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Use of environmental variables to infer gene flow and population structure in the gopher tortoise (*Gopherus polyphemus*) and predict the seroprevalence of an emerging infectious disease

A Dissertation

Submitted to the Graduate Faculty of the  
University of New Orleans  
In partial fulfillment of the  
Requirements for the degree of

Doctor of Philosophy  
In  
Conservation Biology

By

Rachel Wallace Clostio

B.S. Baylor University, 2003

August 2010

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**Abstract:**

Understanding worldwide declines in reptiles due to factors such as habitat loss and emerging infectious disease has become an increasingly important focus in conservation biology. Here, I use novel approaches from the field of landscape genetics to combine spatial genetic data with landscape data at both regional and local spatial scales to explore natural and anthropogenic landscape features that shape population structure and gene flow in a federally threatened reptile, *Gopherus polyphemus*. I also utilize approaches from the field of spatial epidemiology to examine the extent to which environmental variables can be used to predict the seroprevalence of an associated pathogen *Mycoplasma agassizii* in gopher tortoise populations. Using mitochondrial data, I find evidence of a historical barrier to gene flow that appears to coincide with the Apalachicola River. I also discover low genetic diversity and evidence of population bottlenecks in the western portion of the range. My evaluation at the regional scale shows that dispersal is limited by geographic distance, areas of low elevation and major roads ways. A fine-scale study reveals no evidence of spatial genetic structure within a 14 x 35 km area. However, soil type is significantly correlated with pairwise genetic distances between individuals, suggesting that this variable influences fine-scale population structure in the gopher tortoise. In addition to soil, high density canopy cover is an important factor impeding gene flow at the local level for females, while land cover type explains some of the genetic variance between males. Finally, temperature and precipitation appear to be important predictors of the seroprevalence of the pathogen *Mycoplasma agassizii* in gopher tortoises. The probability of an individual testing seropositive for exposure to this disease increased with high temperature and low precipitation values. The methods presented in this dissertation evaluate novel approaches for assessing the influence of environmental variables on population structure, dispersal and disease occurrence and could be applied in future studies of other threatened and endangered taxa.

**Keywords:** Gopher tortoise, *Gopherus polyphemus*, mtDNA, microsatellites, GIS, landscape genetics, causal modeling, *Mycoplasma agassizii*, spatial genetic structure



## CHAPTER ONE: DISSERTATION INTRODUCTION

### INTRODUCTION

#### *Declines in reptiles*

We are currently experiencing the world's sixth mass extinction event. However, this extinction differs from previous events because declines are the result of human expansion and not natural catastrophic events (Ehrlich and Ehrlich 1981; Wilson 1989). Anthropogenic changes in land use that have led to habitat loss, fragmentation and degradation are the major culprits for most species current extinctions and declines. Other causes of species loss include the introduction of invasive species, human exploitation and disease (Ehrlich and Ehrlich 1981; Wilson 1989; Gibbons et al. 2000). The greatest losses have occurred in species rich communities known as biodiversity 'hotspots' such as tropical forests, coral reefs and wetlands (Brooks et al. 2002). Species at the greatest risk of extinction are those that are naturally rare, long-lived, have restricted range distributions, specific habitat requirements, poor dispersal capabilities and low fecundity (Olden et al. 2008; Davidson et al. 2009). In addition to regional species level extinction, populations at the periphery of a species' ranges can also suffer from local extinctions (Brown 1984; Gaston 1990). Furthermore, populations isolated by habitat loss are likely to lose genetic diversity over time and become increasingly vulnerable to extinction (Lande and Barrowclough, 1987). One example of this scenario is the rare dusky gopher frog (*Rana servosa*). This species has limited dispersal ability and is restricted in terms of breeding habitat to open canopy ponds and terrestrial habitat to upland longleaf pine. Habitat loss and fragmentation are both hypothesized to have led to the local extinction of dusky gopher frogs (*Rana servosa*) in Louisiana and much of Mississippi. The remaining populations are highly isolated and exhibit very low genetic variation (Richter and Seigel 2002; Thurgate and Pechman 2007; Richter et al. 2009). Recovery for this species will require not only increasing the quality of habitat and reintroducing individuals into extirpated parts of the range but also restoring connectivity between populations. Protecting and increasing the amount of suitable habitat for a species as well as restoring connectivity for dispersal is necessary to prevent population isolation, loss of genetic variation and ensure long-term population viability (Ruggiero et al. 1994). However, one of the most difficult challenges facing conservationist is preserving and increasing habitat for wildlife in the wake of expanding agriculture and urbanization.

One group of species that is experiencing global declines is reptiles. Although little focus has been placed on reptiles, it appears they are at greater risk of extinction than even amphibians (Gibbons et al. 2000). According to the ICUN, at least twice the number of reptiles as compared to amphibians are extinct, endangered or threatened (IUCN 2000). Furthermore, Brooks et al. (2002) noted that based on amounts of habitat loss in areas of high species diversity, fewer reptile species are listed as threatened or endangered than would be expected. This suggests that many declines in reptiles have still gone unnoticed. Reptiles in general have many life history traits that make them vulnerable to extinction including limited dispersal, delayed sexual maturity and low recruitment (Gibbons et al. 2000). Six common causes of reptile declines include habitat loss and degradation, invasive species, environmental pollution, disease, unsustainable harvesting and global climate change (Gibbons et al. 2000). As with many groups, habitat loss has been the greatest cause of population declines for reptiles. For example, losses in wetland areas in the United States have negatively impacted many semi-aquatic turtle species (Gibbs 1993). Similarly, a 97% loss of longleaf pine forests in the southeastern United States has reduced many reptile species in both population size and distribution (Guyer and Bailey 1993; Gibbons et al. 2000). This link between habitat loss and population declines has been found for many additional reptile species (Hauswaldt and Glenn 2005; Mockford et al. 2007; Brown et al. 2008; Velo-Antón et al. 2008). Populations subject to habitat loss and fragmentation are likely to lose genetic variation and undergo population differentiation due to the effects of genetic drift on small populations (Frankham et al. 2002).

Anthropogenic changes in land use have also led to increased disease transmission in wildlife by inadvertently changing the ecology of the host or pathogen (Daszak et al. 2000; Daszak et al. 2001) and expanding urbanization has promoted the introduction of novel pathogens into wildlife populations through the release of domestic and captive animals (Bradley and Altizer 2006). Many researchers believe that reptile declines due to disease currently rival those of habitat loss (Gibbons et al. 2000). To date, reviews have been published on the influence of emerging infectious diseases on reptiles (Jacobson 1994; Gibbons et al. 2000) but most studies have focused on diseases observed in captive species. Research conducted on wild populations is limited to the description of clinical symptoms and reports of occurrence (Schumacher 2006).

For example, studies concerning herpesvirus in reptiles, which can cause high mortality rates, have described the isolation of novel strains (Johnson et al. 2005), its occurrence in captive individuals (Soares et al. 2004), and identified this virus as the causative agent of observed clinical symptoms (Hughes-Hanks et al. 2010). However, current research has neglected to examine transmission dynamics among natural populations and relate disease occurrence in natural populations to environmental factors. It is possible that changes in environmental factors may increase stress and disease susceptibility in the host, which would lead to an increase in occurrence. Climate change has already been related to an increased susceptibility of amphibian species to disease (Rachowicz et al. 2005; Araujo et al. 2006). Although no studies have addressed the relationship between environmental factors, stress and disease susceptibility in reptiles, it is likely to exist (Gibbons et al. 2000).

Research that evaluates the historical population genetic structure of declining reptile species can be used to improve management strategies in the wake of habitat loss. For example, current studies utilizing molecular markers in reptiles have identified distinct groups of populations for conservation (Ciofi et al. 1999; Mockford et al. 2007; Echelle et al. 2009). Microsatellite data can also be used to focus management efforts on populations that are genetically distinct or have experienced a reduction in population size (Ciofi et al. 1999; Echelle et al. 2009) as well as suggest methods that can be used to increase gene flow and genetic diversity through translocations (Kuo and Janzen 2004). Furthermore, recent contributions from the field of landscape genetics has enabled the use of molecular markers to understand how anthropogenic features, such as roads (Coulo et al. 2006; Gauffre et al. 2008), and recent habitat modification effect contemporary population structure (Moore et al. 2008).

Although molecular markers have allowed us to incorporate genetic data into species recovery plans, more research is needed to understand the direct effects of anthropogenic land use changes and other environmental variables on reptile populations. Fortunately, the newly emerging fields of landscape genetics and spatial epidemiology offer novel means of examining the influence of environmental factors on population structure, species movement and disease prevalence. These methods have the ability to evaluate the influence of habitat fragmentation on gene flow, identify discrete barriers to movement, such as roads, and identify important dispersal

routes (Storfer et al. 2007). Furthermore, these novel approaches can be used to relate spatially explicit environmental data with disease risk, identify landscape features that impede disease transmission and generate maps of predicted disease occurrence.

### *Landscape genetics*

In many cases population genetic structure is not only influenced by the spatial distance separating populations but also by the quality of the landscape between populations (Taylor et al. 1993; Holderegger and Wagner 2006). This connection between population genetic structure and landscape quality can be quantified by statistically evaluating the positive or negative association between genetic differentiation and one or more key landscape variables (Storfer et al. 2007). The field of landscape genetics is a combination of methodological approaches in landscape ecology and population genetics (Mantel et al. 2003). It allows researchers to merge molecular and environmental data collected from multiple landscape layers using geographic information systems (GIS). At its simplest level, landscape genetics can be used to detect genetic discontinuities by identifying spatially explicit changes in allele frequencies between population or individuals. The location of such genetic breaks can then be correlated with environmental variables using GIS and statistical methods and the number of studies utilizing these techniques have vastly increased over the last 15 years (Holderegger and Wagner 2006). Furthermore, the methods used in this field are constantly improving, allowing researchers to generate more realistic landscapes, test competing hypotheses and examine larger datasets at both the individual and population level (Balkenhol et al. 2009; Segelbacher et al. 2010) This has allowed the results from landscape genetic studies to be more applicable to the practical management of threatened and endangered species (Epps et al. 2007; Segelbacher et al 2008).

Common statistical methods used to detect genetic discontinuities include Bayesian clustering algorithms such as STRUCTURE (Prichard et al. 2000), Monmonier's algorithm (Monmonier 1973) and a technique known as Wombling (Manel et al. 2003; Storfer et al. 2006). Bayesian clustering methods, which assign individuals to groups based on the allele frequencies of genotypic data, are the most commonly used methods for delineating population structure. For example, Funk et al. (2005) used STRUCTURE to determine that gene flow was restricted by elevation in the spotted frog (*Rana luteiventris*). Similarly, Guillot et al. (2005) identified

population clusters from wolverine samples and then used this data to associate breaks between populations with known anthropogenic landscape features. Determining the number of population clusters can be difficult in some cases (Evanno et al. 2005) especially when genetic differentiation between populations is low (Latch et al. 2006). However, incorporating spatial coordinates into clustering algorithms and introducing options in these programs that relax previously stringent assumptions that may be unrealistic in natural populations has led to substantial improvements in population delineation in the wild (Falush et al. 2003; Evanno et al. 2005; Chen et al. 2007).

Once genetic breaks are identified, specific landscape features are correlated with genetic data in order to identify features that may promote or impede gene flow. Full and partial Mantel tests as well as other multivariate methods are frequently used to examine the relationship between a genetic distance matrix and distances among populations based on landscape features (Mantel et al. 2003; Storfer et al. 2007). Distance measurements that take into account the presence of landscape features are often generated using a least-cost path (LCP) approach (Storfer et al. 2007). LCP analyses are conducted using GIS software to calculate a single path of least resistance between populations based on a grid of resistance values whose weight reflects the resistance posed by specific landscape types to animal movement. Spear and Storfer (2008) combined partial Mantel tests with a LCP approach to determine that genetic connectivity in the coastal tailed frog (*Ascaphus truei*) was linked to the distribution of forested areas. An LCP approach was also used to determine that contemporary land use rather than historical landscape configuration accounted for genetic variation in the Jerusalem cricket (Vandergast et al. 2007). However, in a recent review of statistical methods used in landscape genetics, it was found that the commonly used Mantel test may have a high type I error rate and several alternatives have been suggested (Balkenhol et al. 2009). It has been suggested that multivariate methods, that use a multiple regression (Legendre et al. 1994) or a general linear modeling approach (Foll and Gaggiotti 2006), perform better and give lower type I errors rates (Balkenhol et al. 2009). However, Mantel and partial Mantel tests are still two of the most widely used approaches in landscape genetics (see Storfer et al. 2007 for review) and when used within a causal modeling framework (cf. Cushman et al. 2006) provide a powerful means of discriminating between competing hypotheses that examine the influence of multiple landscape features on gene flow.

Although landscape genetic approaches have made significant contributions to studies of population structure, there are several considerations and limitations. First, the selection of molecular markers must be appropriate for the temporal scale of the question under investigation. The higher rate of mutation in nuclear microsatellites make them suited for studying more recent population structure, while slower evolving mitochondrial DNA is better for examining historical processes (Balkenhol et al. 2010). Second, an appropriate sampling scheme is important for testing hypothesis at different spatial scales (Storfer et al. 2007; Schwartz and McKelvey 2009). For example, a more uniform sampling approach is necessary to evaluate landscape variables at a fine scale. Third, the most commonly used methods, such as LCP, require the user to assign subjective resistance values to landscape features. It is important that these values are based on research from previous ecological studies or multiple levels of resistance should be tested to determine the relative importance of each variable (Cushman et al. 2006; Balkenhol et al. 2010).

Only a few studies have attempted to test the reliability of current landscape genetic methods by simulating genetic data under realistic landscape hypotheses (Latch et al. 2006; Chen et al. 2006; Balkenhol et al. 2009). These aforementioned simulation studies have found that some approaches are more likely than others to detect the true relationship between landscape and gene flow. Newer approaches need to calculate resistance across the landscape more realistically by accounting for multiple pathways and corridor widths using an isolation-by-resistance (IBR) approach (McRae et al. 2008). Borrowing from electrical circuit theory, McRae and Beier (2007) showed that this approach improved connectivity estimates between populations for both a plant and animal species and outperformed models that considered only geographic distance. Future studies examining the influence of historical and contemporary land use changes on population genetic structure will benefit from the continual advances being made in this field.

### *Spatial Epidemiology*

GIS has more recently been combined with the field of epidemiology to generate a better understanding of environmental variables that correlate with disease distribution and prevalence. Factors such as expanding urbanization, changes in land use and climate change have long been

attributed to disease outbreaks (Daszak et al. 2000; Bradley and Altizer 2006). However, to date only a few studies have used a spatial approach to evaluate the impact of specific environmental variables on disease seroprevalence.

Disease emergence may result from changes in land use or environmental variables that modify the ecology of either the host or pathogen altering abundance, increasing host susceptibility or enhancing disease transmission (Daszak et al. 2000; 2001). Several studies have also shown that human population expansion and associated environmental changes can promote the emergence of infectious diseases in wildlife populations (see Daszak et al. 2000; 2001; Bradley and Altizer 2006 for review). A classic example is that of declines in the black-footed ferret (*Mustela nigripes*) due to canine distemper introduced by domestic dogs (Thorne and Williams 1988). Studies on rabies in raccoons have also shown that transmission slowed through heavily forested areas, indicating that cleared areas associated with human land use aided the spread of this disease (Smith et al. 2005). Anthropogenic effects can also increase stress and disease susceptibility in species. For example, a study in the great tit (*Parus major*) found birds were exhibiting greater stress and reduced plumage in urban environments, suggesting an increased susceptibility to disease under urban environmental conditions (Isaksson et al. 2006). Increased lungworm infections in leopard frogs have also been observed after their immune system was suppressed by exposure to pesticides (Gendron et al. 2003).

Although it can be difficult to determine the link between environmental factors and immune response (Rachowicz et al. 2005), several recent studies have found a correlation between environmental variables and disease occurrence. For example, a recent study of Sin Nombre virus in Canadian deer mice found that disease incidence was significantly related to habitat quality, fragmentation, and temperature (Langlois et al. 2001). Amphibian chytridiomycosis outbreaks have also been linked to high elevation and low temperatures (Daszak et al. 2003). Das et al. (2002) found that the abundance of Lyme disease ticks was negatively related to non-sloping, urban areas without forests. This use of statistical methods to identify environmental variables that are risk factors for occurrence is an important new advance in the study of wildlife disease. These techniques can also be used to understand the relationship

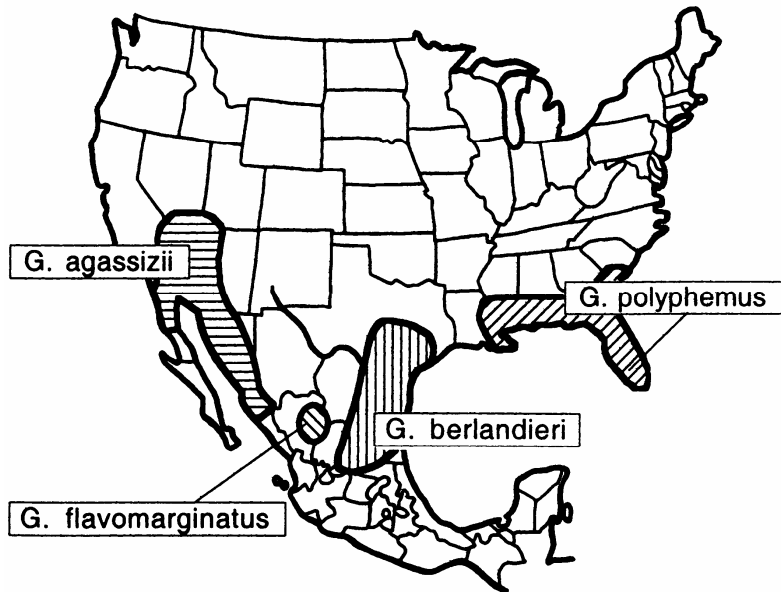
between environmental variables and disease in declining reptile species and in so doing may provide a means of predicting disease outbreaks.

### *Study Species*

In North America, only four species of tortoise are extant and all of these belong to the genus *Gopherus*. All species within this genus inhabit xeric habitats and are characterized by structural specializations for digging and burrowing. This group is believed to have an early Oligocene origin (Bramble 1982). Within the family Testudinidae, all *Gopherus sp.* form a monophyletic group comprised of two clades (Lamb and Lydeard 1994). The first clade is made up of the desert tortoise (*G. agassizii*) and Texas tortoise (*G. berlandieri*), while the second clade contains the gopher tortoise (*G. polyphemus*) and bolson tortoise (*G. flagomarginatus*). These two groups differ not only genetically but also show much structural dissimilarity from each other (Bramble 1982). Based on mitochondrial sequence data, it is predicted that these two lineages diverged during the early Miocene period (Lamb and Lydeard 1994). The four *Gopherus* species have distinct non-overlapping distributions and surprisingly, species within the same lineage do not inhabit adjacent ranges (Figure i). One suggested reason for the disjunct distribution of *G. polyphemus* and *G. flagomarginatus* is the extinction of a giant tortoise species *G. donloli* that allowed *G. berlandieri* to move its range southward (Reynoso and Montellano-Ballesteros 2004). Currently all tortoise species within the *Gopherus* genus are experiencing population declines due to habitat loss and are at risk of extinction (Morafka et al 1994).



Figure 1. Distribution of the four species of *Gopherus* in North America. Figure modified from Reynoso and Montellano-Ballesteros (2004).



Fossil evidence suggests that gopher tortoises once occurred from central Texas northward to the central plains (Bramble 1982; Reynoso and Montellano-Ballesteros 1994). Climate changes during the Pleistocene have since reduced the gopher tortoise range and the species is now distributed from eastern Louisiana to the Florida peninsula and up the Atlantic coast to southern tip of South Carolina (Auffenburg and Franz 1982; Figure ii). Due to population declines, the gopher tortoise is protected throughout its range and populations in Mississippi and South Carolina are designated as endangered at the state level. All populations west of the Mobile and Tombigbee rivers are federally listed as threatened (USFWS 1987).

The greatest threat to the gopher tortoise has been habitat loss, especially within the western portion of the tortoise's range, which has declined by at least 82% (Lohofener and Lohmeier 1984). Habitat loss has been also dramatic in the eastern portion of the gopher tortoise's range, with a 61% reported reduction in suitable habitat between 1952 and 1999 (Conner and Hartsell 2002). Furthermore, the conversion of natural pine forests to pine plantations of slash or loblolly pine has had detrimental effects on tortoise populations (Aresco and Guyer 1999; Hermann et al. 2002), particularly as this type of habitat conversion has been primarily responsible for most of the habitat loss in Louisiana and Mississippi (Lohofener and Lohmeier 1984).

Figure 2. Distribution of *Gopherus polyphemus* throughout the southeastern United States.



<http://www.gophertortoiseCouncil.org>

The gopher tortoise is also threatened by habitat fragmentation, degradation, road mortality, human exploitation, predation and disease (USFWS 1987). Several life history traits make this species particularly susceptible to extinction including delayed sexual maturity, low fecundity, low juvenile survivorship, and specific habitat requirements (Deimer 1986). Age at sexual maturity varies across the range as this is determined by tortoise size and individual growth rate, which is dependent on both habitat quality (Aresco and Guyer 1999) and length of the active season. For example, in Georgia tortoises reach sexual maturity at 19-21 years of age (Landers et al. 1980) while in Florida tortoises are mature at 10-15 years (Iverson 1980). Reproductive success in the gopher tortoise is generally low. Females usually lay a single clutch per year (Iverson 1980) but not all produce a clutch annually (Smith et al. 1997). Clutch sizes vary across populations and among individuals with means ranging from 4.8-7.0 eggs (Iverson 1980; Landers et al. 1980; Diemer 1986; Smith et al. 1997; Epperson and Heise 2003). A positive correlation has also been found between clutch size and female body size with larger females laying up to 12 eggs per season (Iverson 1980; Landers et al. 1980). Hatching success has been shown to be as low as 29% (Epperson and Heise 2003) and hatchling mortality is highest (44-65%) in the first 30 days after hatching (Epperson and Heise 2003; Pike and Seigel 2006). These same studies also found that almost no hatchlings survive past 1-2 years of age.

The low number of individuals emerging from nests has been attributed to many factors but the greatest appears to be nest predation, especially by armadillos (Landers et al. 1980). A

large percentage of hatchling mortality is caused by mammals and the invasive red imported fire (*Solenopsis invictus*), which attacks emerging hatchlings (Landers et al. 1980; Epperson and Heise 2003; Pike and Seigel 2006). Based on this information, it has been estimated that an adult female produces a successful clutch only once every ten years (Landers et al. 1980). Therefore, it appears that recruitment is so low that this species cannot compensate for current population declines due to human causes.

Gopher tortoises have specific habitat requirements and are considered specialists of the longleaf pine ecosystem because of the fact that their range overlaps by 90% with the historic distribution of this habitat type (Guyer and Bailey 1993). This tortoise species is also generally associated with well-drained sandy soils (Auffenburg and Franz 1982; Diemer 1986; USFWS 2005). In fact, populations in some areas are restricted to relict sand ridges left over from the Pleistocene (Auffenburg and Franz 1982; Lohoefener and Lohmeier 1984). Consequently, gopher tortoises have a naturally patchy distribution throughout their range. Gopher tortoises are also thought to be closely associated with longleaf pine forests because they require open canopy habitat that provides abundant grasses for forage, areas for basking and sites for nest laying (Auffenburg and Franz 1982; Lohoefener and Lohmeier 1984; Deimer 1986). The availability of appropriate areas for nest construction may also be extremely important because this species has temperature-dependent sex determination like many other turtles and tortoises.

In addition to habitat loss through land conversion, substandard forest management is the major cause of habitat degradation. In order to maintain fire-dependent longleaf pine habitat, frequent controlled burns are necessary (Noss 1988). However, fire is often excluded from private properties, forests near urban areas and pine plantations, leading to further habitat degradation. Habitats not maintained by fire usually become overgrown within a few years and tortoises are forced to the perimeter or along roadways. Habitat degradation is probably responsible for a large proportion of the road mortality that occurs because tortoises move along road ways and are then at greater risk of being killed crossing roads (Lohoefener and Lohmeier 1984).

A more recently realized threat to gopher tortoise populations is disease. In 1989, an upper respiratory tract disease (URTD) was observed in tortoises from Sanibel Island, Florida. Brown et al. (1994) determined that URTD in the gopher tortoise was caused by *Mycoplasma agassizii* and since then a number of population declines have been attributed to this disease (Gates et al. 2002; Rabatsky and Blihovde 2002; Seigel et al. 2003). Clinical signs of this disease include discharge from the nares and eyes, conjunctivitis and edema of the eyelids (Brown et al. 1999). Chronic infection of gopher tortoises with *M. agassizii* will cause the destruction of the respiratory epithelium and eventually the death of the animal (Brown et al. 1994). Transmission occurs by direct contact most likely during courtship, mating or male-male competitions.

Tortoises displaying clinical signs of URTD and/or testing positive for exposure to *M. agassizii* have been found throughout the natural range (Smith et al. 1998; Berish et al. 2000; Diaz-Figueroa 2005). Currently, the level of decline attributable to this disease is unknown. However, the continued increase in urbanization often leads to the relocation of gopher tortoises and disease transmission is a major concern when translocating individuals. Tortoises that show clinical signs of illness are at the greatest risk of spreading the pathogen but individuals that are infected without showing overt symptoms can be silent carriers (Brown et al. 2002). Furthermore, previous studies have suggested that environmental factors could play a role in disease occurrence (Lederle et al. 1997; McLaughlin et al. 2000; Kahn and Mendonca 2005). More information on the presence of this disease in natural populations is needed to enable wildlife managers to continue recovery efforts without introducing infectious individuals into naive populations.

In light of the current declines, future population viability of this species is a major concern and many studies have focused on learning more about the ecology of this species to improve habitat management. However, the loss of this species from longleaf pine ecosystems is of even greater concern because of its role as a keystone species. The gopher tortoise is a fossorial species that constructs burrows several meters deep (Diemer 1986). These burrows provide refuge for a number of vertebrate and invertebrate species (Diemer 1986; Witz et al. 1991). Some species closely associated with gopher tortoise burrows are also in decline, including the eastern indigo snake (*Drymarchon corais couperi*), eastern diamondback (*Crotalus*

*adamanteus*), dusky gopher frog (*Rana sevosa*) and several species of pine snake (*Pituophis spp.*). The action of maintaining burrows also increases local plant diversity within the surrounding habitat (Kazcor and Hartnett 1990). Therefore, if the gopher tortoise is allowed to go extinct other species and ecosystem processes within its habitat are likely to suffer.

Incorporation of genetic data into ongoing recovery efforts is needed in order to (i) assess population genetic structure, (2) quantify loss of genetic diversity, 3) identify barriers to gene flow that isolate populations and 4) delineate landscape features that promote movement (USFWS 1990). This data can be used to designate management units that allow for more regional and focused recovery goals. It can also be used to generate relocation guidelines that specify maximum distance and landscape features that individuals should not be moved across (USFWS 2009). Another area in need of additional research is the association between gopher tortoise and *M. agassizii*. Data that identifies habitat or climatic variables associated with occurrence could be used to better understand mortality events linked to URTD and assist wildlife agents in managing this disease.

### *Study Sites*

The following dissertation utilizes data from sample sites located throughout the the historical range of longleaf pine. Natural longleaf pine communities are described as a park-like savanna with scattered longleaf pine and a grassy understory (Figure vi.). These communities can be divided into two general types: sandhills that occur in areas of rolling hills away from the coast and flatwoods that occur along the coast (Noss 1988). Currently, less than 4% of the original extent remains and the majority is composed of stands of trees <40 years of age (Outcalt and Sheffield 1996). The remaining areas of old-growth longleaf pine make up only 0.00014% of their pre-settlement extent and currently comprise a total of 4846 ha in area (Varner and Kush 2004). In fact, losses of longleaf pine have been greater than those of wetlands (Figure vii.), although loss of wetlands receives more attention and protection (Noss et al. 1995).

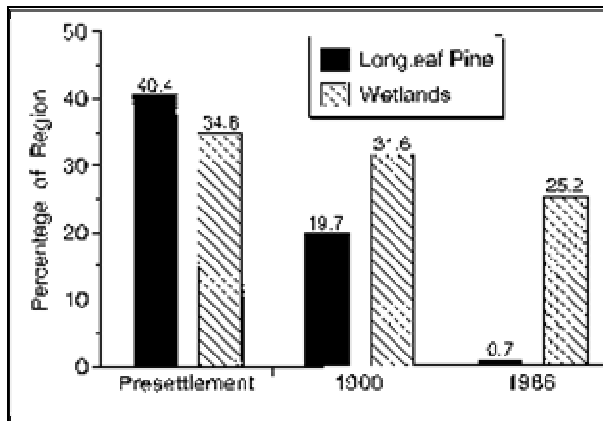
Figure 3. Historical distribution of longleaf pine, adapted from The Longleaf Alliance <http://www.longleafalliance.org>.



Figure 4. Recently burned longleaf pine habitat in Tangipahoa Parish, Louisiana.



Figure 5. Comparison of remaining longleaf pine and wetlands habitat remaining from pre-settlement to 1986, adapted from Noss et al. (1995).



The immense loss of longleaf pine habitat that has occurred led Noss et al. (1995) to classify this community as an endangered ecosystem. Threats to this habitat include conversion of natural pine areas to agriculture or pine plantations, urbanization and exclusion of fire. The species diversity in longleaf pine communities is extremely high (Noss 1988) due to the large number of seeds produced for granivores and the high abundance of insect populations that support numerous insectivores (Van Lear et al. 2005). There is also a fairly high level of endemism: 40% of plants found along the coastal plains are restricted to longleaf pine forests (Van Lear et al. 2005). However, many species associated with this habitat are in decline and already listed for protection (Noss 1988). For example, 14% of mammals closely associated with longleaf pine are listed as species of special concern while many generalist mammals that may also prey on gopher tortoise hatchlings have increased within these habitats (Van Lear et al. 2005). Because much of the remaining longleaf pine forests exist as isolated fragments, reductions in genetic diversity are likely for many species closely associated with this ecosystem.

Sample sites used in this study were located throughout the southeast and spanned a wide range of habitat types varying in gopher tortoise habitat quality. In total, 24 sample sites were used in this study with collection locales spread across all six states that constitute gopher tortoise range. In Louisiana, samples were collected from all three parishes where the gopher tortoise still remains. Within Louisiana, suitable gopher tortoise habitat is lacking with much of it having been converted to pine plantations. Furthermore, in Louisiana the species has been described as functionally extinct (Lohoefer and Lohmeier 1984) and is considered to be in danger of extinction (Auffenburg and Franz 1982). The collection site in Tangipahoa Parish was located within the south section of the Sandy Hollow Wildlife Management Area (WMA), which is owned by the Louisiana Department of Wildlife and Fisheries. Sandy Hollow is an upland longleaf pine savanna with a primarily grassy understory dominated by blue stem (*Schizachyrium tenerum*). This WMA is burned frequently and managed mostly for bobwhite quail (*Colinus virginianus*) hunting. Two sites were sampled in Washington Parish: Ben's Creek WMA and the Florida Gas Transmission corridor. Ben's Creek is owned by the Weyerheuser Paper Company but is leased by the Louisiana Department of Wildlife and Fisheries. The dominant overstory species is loblolly pine (*Pinus taeda*) with only a small tract of longleaf pine maintained on the property. Since the site is managed for commercial pine, the habitat is a mix of

open to closed canopy pine, clear cuts and wildlife food plots. Most tortoises at this site occur within the one area of longleaf, along gas and power line right of ways, adjacent to road sides, or at the perimeter of food plots. The Florida Gas Transmission right of way is a large corridor that is frequently mowed. This same transmission corridor also crosses the Ben's Creek site but these two sites were sampled approximately 5 miles apart. The last site in Louisiana was private land in St. Tammany Parish. The habitat was dense loblolly pine with a thick scrub understory and the tortoises sampled were being excavated from burrows and relocated because of a subdivision development project.

Population densities in Mississippi are low and most of the remaining individuals exist within the Desoto National Forest (Auffenburg and Franz 1982). Two of the nine sites sampled in this state were in wildlife management areas (WMAs) in Marion and Pearl River Counties. Habitat at both sites was composed of a longleaf overstory and an open grassy understory maintained by fire. Four sites were located within the Desoto National Forest (Harrison, Perry, and Forrest Counties) where habitats ranged from open canopy longleaf pine forest to dense loblolly and slash pine (*Pinus elliottii*) stands. One of these sites was also located within the Camp Shelby National Guard Training Facility which overlaps with the northern portion of Desoto National Forest. Longleaf pine forests in this area are managed by The Nature Conservancy and are well maintained using prescribed burning techniques. The three remaining sites were located further North (Greene Co.) and consisted of one well managed site located within the Chickasawhay National Forest and two sites on property owned by The Westervelt Company. Much of the habitat on this last collection site was clear cut. Previous studies have suggested that recruitment in Mississippi is very low (Epperson and Heise 2003) and populations are projected to be functionally extinct in the near future (Lohoefer and Lohmeier 1984).

Three sites were sampled in Alabama, two of which were located west and one of which was located east of the Tombigbee and Mobile rivers. The two sites located in the federally protected portion of the range were located in Mobile County where the gopher tortoise has been described as rare (Auffenburg and Franz 1982). The first site was located on private property and sampled tortoises were being relocated for a development project. Habitat on this site was a mix of open areas and dense stands of young pines with tortoises sampled in both habitat types. The

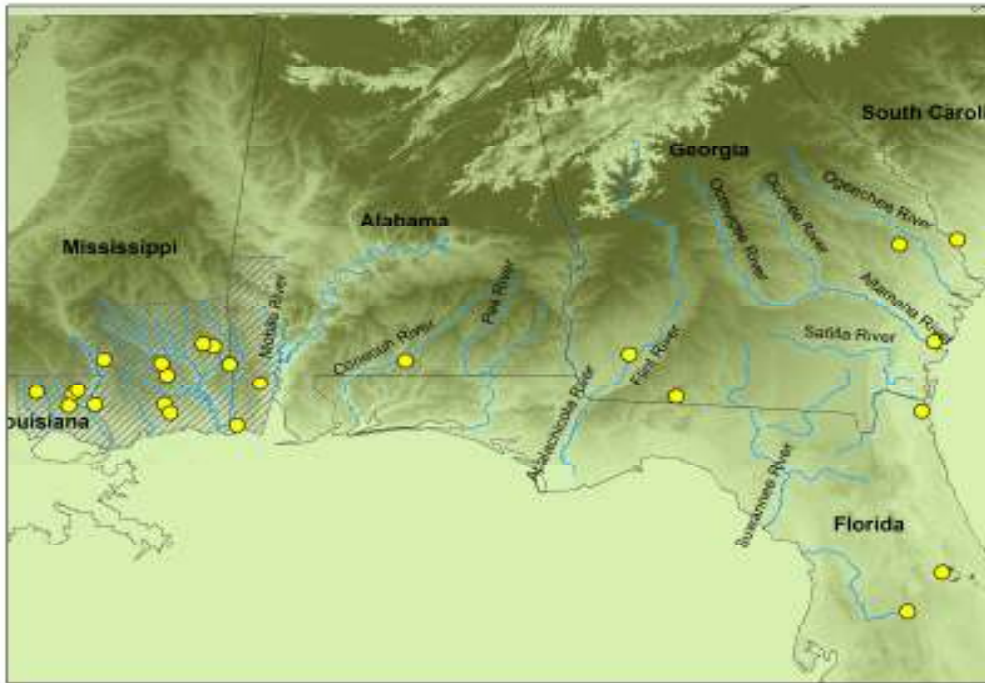


second site was owned by the Mobile Co. school board but leased by Grand Bay National Wildlife Refuge. The understory at this site was over grown with invasive cogon grass (*Imperata cylindrica*), which has a high silica content and is not eaten by tortoises. Most of the tortoises were located along the perimeter of this grass mat. The final site was at the Solon Dixon Forestry Education Center in Covington County. Gopher tortoises at this study site were sampled from stands of longleaf pine that had an open grassy understory. Human population increases across southern Alabama have been modest (USFWS 1987) but have probably occurred mainly in major cities like Mobile. Overall, tortoise populations in Alabama, outside of Mobile County, exist at higher densities than those in either Louisiana or Mississippi (Auffenburg and Franz 1982).

In Georgia samples were obtained from four collection sites. Two sites were located in the southwestern portion of the state and the remaining two were located near the Atlantic coast. Wade Tract Preserve located in Thomas County is one of the few sites with remaining old-growth forests. The Joseph W. Jones Ecological Research Center in Baker County has many habitat types but is mainly composed of longleaf pine-wiregrass uplands. Both of these sites support large gopher tortoise populations. The remaining sites were located in Bulloch and McIntosh Counties. Individuals from these two sites were part of relocation projects and no information on habitat quality at the original sites was available.

The three collection sites in Florida were located within Nassau, Lake and Seminole Counties. These individuals were also excavated and moved to Nokuse Plantation in the Florida panhandle. Information on habitat quality for these collection sites was also not available. The final sites in South Carolina were located in Jasper County but were only a few miles apart so they were combined into one population. The density of remaining populations in South Carolina is very low with the average colony containing only three active burrows (Auffenburg and Franz 1982).

Figure 6. Location of sampling sites throughout the southeastern United States included in this study.



#### *Research approach and questions*

The main goal of this study is to investigate how both anthropogenic land use change and natural environmental variables influence overall population genetic structure in the gopher tortoise. I examined populations of *G. polyphemus* because little research has been done on either their local or range-wide population genetic structure or factors related to the seroprevalence of upper respiratory tract disease. This species has strict habitat requirements that will allow me to investigate the influence of regional landscape features on gene flow and genetic structure between populations. Gopher tortoise are also abundant enough in some areas to allowing sampling of a large number of individuals from a single population for a fine-scale study which aims to examine the local effects of land use change and natural features such as rivers on measures of genetic relatedness between individuals. Lastly, the recent emergence of an infectious disease (URTD) provides the opportunity to assess how environmental variables correlate with seroprevalence. The will allow us to understand how these indirect factors may contribute to the prevalence of a disease that has been linked to population declines. The results of this study should provide information that can be incorporated into management plans to

improve recovery efforts. The questions I wish to address through the broader framework of this dissertation are as follows:

1. How are populations of *G. polyphemus* delineated across the range and does their structure coincide with putative geographic barriers to gene flow?
2. Does the molecular data show evidence of recent or historical population declines?
3. How do landscape features influence regional population structure and gene flow and how can this knowledge be used to inform long-term management?
4. Do these landscape features differ from those that affect fine-scale population structure?
5. How might key environmental variables related to habitat quality and climatic factors influence the seroprevalence of the pathogen *M. agassizii* in gopher tortoises?

I used molecular, ecological and environmental data to explore the impact of natural and anthropogenic landscape features on the population structure and connectivity of gopher tortoise populations. I also utilize environmental data to investigate the influence of habitat quality and climatic variables on seroprevalence of the pathogen *M. agassizii* within gopher tortoise populations. This information can be used to better understand potential factors associated with the decline of this federally threatened reptile and develop management plans for its long-term survival.

This research is presented in four parts. First, I examine the genetic diversity and population genetic structure of the gopher tortoise at sites throughout the natural range using both mitochondrial and nuclear microsatellite markers with the aim of detecting population declines and comparing historical and contemporary population structure. Second, I use novel methods from the field of landscape genetics along with a causal modeling framework to examine the relative importance of landscape features important to gopher tortoise habitat choice and movement on population genetic structure. Third, I used an individual-level approach to determine whether spatial genetic structure existed at the local level and evaluate the influence of landscape features on fine-scale dispersal patterns. Fourth, I determine if specific environmental variables believed to influence tortoise health are associated with an increased occurrence of a pathogen, *M. agassizii*. Finally, I integrate the results from all four studies to discuss the current population structure of the gopher tortoise and the likely future effects of both anthropogenic and natural landscape features on species movement and disease occurrence.

## **CHAPTER TWO: Phylogeography and population genetics of the federally threatened gopher tortoise (*Gopherus polyphemus*) throughout the southeastern United States**

### INTRODUCTION

Genetic data can have significant influence on the management and recovery of declining species. The delineation of subdivided populations can help guide translocation of wildlife populations for the purpose of augmenting small declining populations (Bouzat et al. 2008; Hendrick 1995) or reintroducing extirpated species (Valentine et al. 2007; Frankham et al. 2002). The recognition of deep phylogenetic divergence between populations alerts wildlife managers to geographic areas that have been historically separated and can thus identify geographical regions across which individuals should not be exchanged (Avice 2004). In this way, information on natural patterns of gene flow can be used to better mimic natural species movement, preserve genetic diversity and prevent unwanted admixture and outbreeding (Frankham et al. 2002; Hartl and Clark 1997). Furthermore, molecular data can be used to identify populations with low genetic diversity that are in need of well focused recovery goals (Frankham et al. 2002). Taken together, genetic data and ecological studies can provide wildlife managers with a more complete picture of species demography and population structure and both should therefore be an important part of any species recovery plan

The gopher tortoise (*Gopherus polyphemus*) is one of only four extant tortoise species found in North America and it is the only tortoise species found east of the Mississippi River (Ernst et al. 2009). This species is distributed from eastern Louisiana across the southeast, up to southern South Carolina, and over most of the Florida peninsula (Auffenberg and Franz 1982). Gopher tortoise populations are patchily distributed across their range due to strict habitat requirements that include well-drained sandy soils and an open under-story maintained by fire (Auffenberg and Franz 1982; Baskaran et al. 2006). The fossorial gopher tortoise is an important keystone species of longleaf pine communities (Eisenburg 1983; Guyer and Bailey 1993) and in constructing burrows it increases local plant species richness (Kaczor and Hartnett 1990) and provides refuge for several vertebrate and invertebrate species (Milstrey 1986, Witz et al. 1991). However, during the last 100 years over 80% of populations have experienced declines

throughout their historic range (Auffenburg and Franz 1982). Habitat loss has been greatest in the western portion of the range with populations in Louisiana near extinction (Auffenburg and Franz 1982) and an estimated 5% of the original habitat remains in Mississippi (Lohoefener and Lohmeier 1984). Auffenburg and Franz (1982) projected that all gopher tortoise populations not on protected lands would be extirpated by 2025 if action was not taken to protect the species from further declines. In 1987, the U.S. Fish and Wildlife Service responded to public concern and listed all populations west of the Tombigbee and Mobile Rivers as federally threatened. Populations in the remaining portion of the range have also suffered from significant habitat loss but were originally not included due to a lack of data and are now currently under review (USFWS 1987; USFWS 2009).

The range-wide decline of gopher tortoise populations is due to a number of factors that include habitat loss, fragmentation, human predation and low juvenile recruitment (USFWS 1987). Currently, less than 14% of the historical longleaf pine ecosystem remains, and of this, less than 1% is old-growth forest (Simberloff 1993). Southeastern pine forests began declining as early as the 1630s, with the greatest losses occurring between 1830 and 1910 (Frost 1993; Smith et al. 2001). Natural pine communities have continued to decline in the southeast, being replaced by areas of young planted pine, harboring lower levels of wildlife and plant diversity (Conner and Hartsell 2002). Furthermore, in counties where the tortoise occurs, human populations have increased by greater than 1000% over the last 100 years (U.S. Census Bureau; SOBS 2006), surpassing agriculture as the primary cause of forest loss in the southeast (Connell and Hartsell 2002). Although numerous surveys have shown that tortoise populations are in decline (Auffenburg and Franz 1982; Lohoefener and Lohmeier 1984; SOBS 2006), wildlife representatives are currently unaware of when population declines began or during what period declines were greatest. A better understanding of past population declines will therefore provide crucial information on when changes in historical population size occurred and identify which anthropogenic factor(s) may have been the major cause.

Increasing urbanization has necessitated the relocation of gopher tortoises from sites under development. To date, these relocations have occurred in the absence of any guidelines based on natural population structure or landscape features that might be used to delineate

management units for conservation (USFWS 1990, USFWS 2009). Therefore, current relocation strategies potentially threaten the natural population structure of this species. Management plans created for the recovery of gopher tortoise populations note the need for incorporating genetic data into population management schemes (USFWS 1990; FFWCC 2007). Specifically it has been proposed that molecular data could be used to: 1) develop relocation guidelines based on natural population differentiation, 2) direct the restocking of declining populations on public lands and 3) designate evolutionarily significant units (ESUs) and management units (MUs) for conservation (Moritz 1994) so that wildlife authorities can develop more focused recovery plans (USFWS 1990; FFWCC 2007).

Despite this clear management need for genetic data, only two studies have used molecular markers to examine the distribution of genetic variability in gopher tortoise populations (Osentoski and Lamb 1995; Schwartz and Karl 2005) and no previous studies have attempted to rigorously assess population structure using multiple samples from states across the entire species range. Osentoski and Lamb (1995) used restriction fragment length polymorphism (RFLP) analysis of amplified mitochondrial DNA (mtDNA) fragments to examine historical population structure throughout the southeast. Their studies revealed three geographic assemblages: the “Eastern” and “Western” assemblages on either side of the Apalachicola River drainage, and a third assemblage “Mid-Florida”, which corresponded to a historical upland ridge on the Florida peninsula. However, the majority of samples in this study (57%) were from localities in Florida alone and only one location was from within the federally listed portion of the range. Furthermore, the use of RFLPs to examine genetic variation in only a few loci provides limited resolution of historical population structure. A more recent study carried out by Schwartz and Karl (2005) used nine microsatellite loci to designate 21 sites located in Florida and southern Georgia into eight genetic assemblages based on an analysis of molecular variance (AMOVA), including one group that roughly corresponded to the “Mid-Florida” assemblage identified by Osentoski and Lamb (1995). This study was able to resolve population structure but focused on only a small portion of the range within Georgia and Florida. It is therefore likely that by using two molecular markers evolving at different rates and a more comprehensive sampling strategy will better resolve genetic assemblages across the entire range of this species and provide a framework for recovery plans. Furthermore, combining data from a relatively

slowly evolving mitochondrial marker and a more rapidly evolving nuclear genetic loci will allow us to determine both historical and contemporary population structure, and provide information necessary for the designation of ESUs and MUs (Avice 2004).

In contrast to both previous studies, the present study is the first to adequately sample the western, federally listed portion of the range and examine regional population structure across the gopher tortoise's entire distribution using mitochondrial and nuclear data. The objectives of this study were to: 1) examine historical gene flow and identify reciprocally monophyletic phylogeographic units which could be recognized as ESUs, 2) delineate fine-scale population structure using nuclear microsatellites for the designation of MUs and 3) test for evidence of demographic declines and determine whether historical forest loss or more recent urbanization has been the major driver behind population declines. We then discuss how these results can be used to address current gaps in population management and target key populations in recovery efforts for this threatened species.

## MATERIALS AND METHODS

### *Sample Collection*

Blood samples (n = 452) were collected from 24 sites across six states in the southeastern U.S. that comprise the range of the gopher tortoise, including 15 locations where the tortoise is federally listed (Table 1, Figure 1). Blood samples were collected by drawing 1 to 2 cc from either the brachial venipuncture or subcarapacial venous sinus. Samples were stored in vials with lithium heparin and then placed on ice while in the field. Once in the laboratory, samples were centrifuged and plasma was removed. The pelleted red blood cells were then stored at 4.0°C until DNA extraction. Samples received from other researchers were also provided as concentrated red blood cells.



Table 1. List of sample sites (with the site acronyms used), number of samples obtained from each site, the state where each site was located, the latitude and longitude coordinates for each site, and the sample collector(s).

Site	Sample No.	State	Latitude	Longitude	Collector
Sandy Hollow WMA (SH)	5	Louisiana	30.80	90.37	R.Clostio
Private Site, Blonde (ST)	4	Louisiana	30.65	90.06	R.Clostio
Florida Gas Transmission Line (FGP)	36	Louisiana	30.78	90.00	R.Clostio; T. Zacharia
Ben's Creek WMA (BC)	21	Louisiana	30.83	89.96	R.Clostio
Bogue Chitto WMA (BG)	4	Mississippi	30.67	89.79	R.Clostio
Marian County WMA (MC)	23	Mississippi	31.17	89.70	R.Clostio
Camp Shelby Training Facility (CS)	21	Mississippi	31.14	89.13	TNC Camp Shelby <sup>1</sup> ; R. Birkhead
Desoto National Forest (DNF1)	25	Mississippi	30.67	89.10	R.Clostio
Private Site, Wiggins (DNF2)	12	Mississippi	30.58	89.04	A.Dinsmore; P. Kahn
Desoto National Forest (DNF3)	45	Mississippi	31.00	89.07	R.Clostio
Turkey Fork Recreational Area (TF)	12	Mississippi	31.35	88.72	R.Clostio
Westervelt Property, Sandhill (SAH)	7	Mississippi	31.32	88.61	M. Bailey
Westervelt Property, Leaksville (LK)	14	Mississippi	31.12	88.45	M. Bailey
Grand Bay National Wildlife Refuge (GB)	3	Alabama	30.44	88.38	R.Clostio
Meeker Creek Tract, Saraland (AL)	45	Alabama	30.90	88.15	R.Clostio; J. Ennen
Solon Dixon, Andalusia (SD)	20	Alabama	31.16	86.70	R.Clostio
Jones Ecological Research Center (GG)	26	Georgia	31.23	84.47	R. Birkhead
Wade Tract Preserve, Thomasville (WT)	26	Georgia	30.75	84.00	R. Birkhead
Private Site, Statesboro (SCI)	24	Georgia	32.45	81.78	T. Tuberville
Private Site, Darien (SRS)	22	Georgia	31.37	81.43	T. Tuberville
Tillman Sandhill Reserve, Jasper Co. (SC)	23	S. Carolina	32.50	81.20	T. Tuberville
Private Site, Lake Co. (Lake)	4	Florida	28.38	81.70	R.Clostio
Private Site, Nassau Co. (FL)	19	Florida	30.59	81.56	R.Clostio
Private Site, Seminole Co. (SM)	11	Florida	28.80	81.35	R.Clostio

<sup>1</sup>The Nature Conservancy's Camp Shelby Field Office, Mississippi

### *Molecular methods*

Genomic DNA was isolated from blood using a cell lysis buffer (10mM Tris pH 8.0, 1mM EDTA, 1% SDS) with 60µg proteinase K and incubated for a minimum of three hours at 55°C. DNA was then precipitated by potassium acetate solution (3M potassium acetate, 11.5% glacial acetate acid) and 1 volume of isopropanol (Sambrook and Russell 2001). DNA was resuspended in TE (10 mM Tris pH 8.0, 1 mM EDTA) and diluted to 50 ng/µL prior to polymerase chain reaction (PCR) amplifications. DNA concentrations for each sample were quantified using the Nanodrop1000 (Thermo Fisher Scientific Inc., USA).

**MtDNA Sequencing** – A 836 base-pair (bp) portion of the mitochondrial genome was amplified using primers CytbF2 (5' GGGGTTCTCAGTAGATAACGC 3') and CRR3 (5' GAAATTTTCTCTTCTGGGCGC 3'), designed from a larger mitochondrial fragment amplified using L14724 and H16464 (Meyer et al. 1990). This 836 bp region encompasses a 646 bp fragment of the cytochrome *b* gene (Cyt *b*), threonine transfer RNA, proline transfer RNA, and 90 bp of the control region (CR). PCR was performed in a 20µL volume reaction with 10mM Tris, 3.0 mM MgCl<sub>2</sub>, 2X bovine serum albumin, 0.5 units of *Taq* polymerase (Invitrogen, USA), 0.2 mM of each dNTP and 20 pmol of each primer. The mixture was heated to 94°C for 3 min, followed by 35 cycles of 94°C 30s, 46°C for 30s, and 72°C for 45s, with a final extension step at 72°C for 10 min.

We used single stranded conformational polymorphism (SSCP) to assess sequence variation among PCR products. A subset of 234 samples was analyzed for sequence variation using the SSCP method of Sunnucks et al. (2000). Using this technique, single base pair differences can usually be resolved with 99% efficiency for fragments less than 300 bp and more than 80% efficiency for fragments up to 400 bps (Sunnucks et al. 2000). In order to reduce the size of the fragments under investigation, the 836 bp amplified product was broken into four smaller fragments of 114, 188, 234 and 296 bps by digestion overnight with one unit of restriction enzyme, *Mbo*I (New England Biolabs, USA) at 37°C. Following restriction digest, an equal volume of formamide loading dye (95% formamide, 10mM NaOH, 0.1% bromophenol blue, 0.1% xylene) was added to each PCR product and heat denatured at 95°C for 5 mins prior to snap cooling on ice. A total of 3-4µL of the denatured digested DNA was then loaded on a

non-denaturing 4% polyacrylamide gel made using the SeqGel MD solution (National Diagnostics, USA). SSCP gels were run for 2.5 h at 14 W and were maintained at 4°C using a refrigeration pump (VWR Scientific). Products were visualized using standard silver staining, following the method of Sunnucks et al. (2000). Haploypes that displayed different banding patterns in SSCP analysis were used as a reference samples in all subsequent SSCP gels. Following SSCP analysis, at least two individuals from each sampling site were sequenced to verify that sequence haplotypes conformed to the corresponding SSCP banding pattern. Prior to sequencing, a total of 10µL uncut PCR product was purified for sequencing using 10µL of ExoAP following the protocol of Glenn and Schable (2005). Sequencing was carried out on an automated ABI 3170 DNA sequencer using the BigDye Terminator Cycle Sequencing Kit V1.1 (Applied Biosystems, USA). Sequences were edited using Sequencher 4.0 (Gene Codes Corporation, USA) and subsequently aligned using ClustalW (Thompson et al. 1994).

Nucleotide diversity  $\pi$  (Nei 1987), the average proportion of nucleotide differences between all pairs of sequences, and the proportion of nucleotide polymorphisms observed ( $\theta$ ) (Watterson 1975) were estimated using DNASP 5.0 (Librado and Rozas 2009). Tajima's D (1989) and Fu and Li's D\* (Fu and Li 1993) was also estimated to test for selective neutrality and evidence of population expansion or contraction. The distance between pairs of haplotypes was calculated using the Jukes-Cantor model (Jukes and Cantor 1969), assuming uniform substitution rates among sites and maximum likelihood estimates of the rates of nucleotide substitution in MEGA 4.0 (Tamura et al. 2007). In order to quantify the proportion of the total variance attributable to the east-west divide identified by Ostenoski and Lamb (1995), ARLEQUIN 3.1 (Excoffier et al. 2005) was used to compute a minimum spanning network among haplotypes and examine the geographic distribution of molecular variance using an analysis of molecular variance (AMOVA).

**Microsatellite Sequencing** – A total of 35 microsatellite loci were examined and their utility as genetic markers was assessed on the basis of their degree of polymorphism and ability to reliably amplify. These candidate loci included four loci previously amplified from *Emydoidea blandingii* (Ostenoski et al. 2002) and seven loci previously isolated from *Gopherus agassizii* (Edwards et al. 2003) that cross amplified in gopher tortoises. We also evaluated nine

loci previously published for *G. polyphemus* (Schwartz et al. 2003), and five additional unpublished loci isolated from *G. polyphemus* (Tuberville 2008). An additional 10 dinucleotide repeat loci were then isolated in the present study using the enrichment protocol of Glenn and Schable (2005). Ten of these 35 loci amplified well, proved to be variable, and were selected for use in further genetic analyses (Table 2). These ten loci were amplified in two multiplex assemblies of 5 loci each using the QIAGEN Multiplex PCR Kit (Qiagen, USA). Microsatellites were run on an ABI 3170 (Applied Biosystems) and genotypes were edited using GENEMAPPER 4.0 (Applied Biosystems).

Table 2. Microsatellite loci used in the study, the repeat motif, primer sequences, range in allele size ( $R_A$ ) across all sample sites, number of alleles ( $N_A$ ) across all sample sites, and primer source.

Locus	Repeat No.	Primers Sequences	$R_A$ (bp)	$N_A$	Source
Multiplex A					
Gp15	GA <sub>(15)</sub> GT <sub>(8)</sub>	F: 5HEX-CCTATTTTTCCCCCTCACAGT R: GAAAATAAAAACAGTCCCAACCA	208-275	19	Schwartz et al. 2003
Gp26	GT <sub>(12)</sub>	F: GACAACCATCTTTACCCACA R: 5HEX-TCCCAAGACATAAGTCAGTAGC	357-369	6	Schwartz et al. 2003
Gp30	GT <sub>(13)</sub>	F: 5NED-GAATGCAGCACTGCTTGGA R: CGAAGAGGGAGCACGTTTAG	194-228	11	Schwartz et al. 2003
Gp96	GA <sub>(11)</sub>	F: 56FAM-TCAGTTACCGGATAATGTTTCAGTG R: TGCTGTTACCTCGTGCATGT	138-152	7	Schwartz et al. 2003
Gp102	GT <sub>(5)</sub> CT <sub>(13)</sub> CA <sub>(5)</sub>	F: 56-FAM-AGCTGCCTGACTGCTATGCT R: GCATAATCAGCATCAACAACAAA	297-341	14	Schwartz et al. 2003
Multiplex B					
Gp5	GATA <sub>(19)</sub>	F: 5HEX-TCTGTAATGCCTAGAATC AA R: TGCCATTTCTGTAAAGTTC	300-364	17	Tuberville 2008
Gp12	CAAA <sub>(11)</sub>	F: 56-FAM-CTTTGGAAGCCATTGTAATA R: CATTGACACCAGTTTAACTA	318-362	12	Tuberville 2008
Gp14	ATGG <sub>(8)</sub>	F: GTCCTGGGATTACAATCAAT R: -5HEX-CCAATCTTTTCGTAATGTAT	142-190	11	Tuberville 2008
Gp105	CA <sub>(10)</sub>	F: GGGAGAGGAGACTGGAAAGC R: -5HEX-TTTAAGGAGAGAGGGTTGTTCC	226-272	16	R. Clostio, UNO <sup>2</sup>
Gp201	CA <sub>(16)</sub>	F: -56-FAM-TTACGCCATCCCACAAAGC R: ATGCCAGATCCTTGGCCTG	205-215	5	R. Clostio, UNO <sup>2</sup>

<sup>1</sup>University of New Orleans, New Orleans, LA

Microsatellite alleles for each locus were binned using FLEXIBIN (Amos et al. 2007). The presence of genotyping errors due to null alleles, stutter peaks and large allele dropout was assessed using MICROCHECKER (Oosterhout et al. 2004). The program ARLEQUIN 3.1 (Excoffier et al. 2005) was used to examine data for significant deviations from Hardy-Weinberg equilibrium (HWE) for all loci within each group using a triangular contingency table and a modified version of the Markov-chain random walk algorithm (Guo and Thompson 1992). A likelihood ratio test was used to detect non-random associations between alleles at different loci (linkage disequilibrium). The effect of variation in sample size on genetic diversity was examined by testing for correlations between sample number and the total number of alleles or observed heterozygosity in the R package (R Development Core Team). Population genetic distances were estimated using an  $F_{ST}$  analog,  $\theta_{ST}$  (Excoffier et al. 1992), in ARLEQUIN (Excoffier et al. 2005). The significance threshold for the rejection of the null hypothesis was adjusted using a Bonferroni correction.

To determine how genetic variation was partitioned among populations we used a spatial analysis of molecular variance or SAMOVA (Dupanloup et al. 2002). SAMOVA defines groups of populations that are maximally differentiated by using two constraints. Groups must be genetically homogenous and geographically adjacent. The number of groups of populations (K) that best explain the data was assessed for values of K between 2 and 23 using 100 independent simulated annealing processes. The value of K that maximized the among group variance component ( $F_{CT}$ ) while minimizing the variance among populations within groups ( $F_{SC}$ ), was selected to represent the optimal number of groups.

A Bayesian assignment approach, implemented in the program STRUCTURE 2.2 (Pritchard et al. 2000), was also used to determine the number of K groups that best explain the data. Individuals were assigned to clusters based on the similarity of their multilocus genotypes, while minimizing deviations from HWE and LE (Pritchard et al. 2000). The mean and variance of the log probability of the data for values of K from 1 to 10 were constructed from 20 replicate runs of STRUCTURE. Five replicate runs were also produced for K values ranging from 11 to 20. For all analyses of K, the Markov chain Monte Carlo (MCMC) was run for 1,000,000 iterations with an initial burn-in of 100,000 iterations. As suggested by Falush et al. (2003),

simulations were run under the admixture ancestry model and the assumption of correlated allele frequencies among samples. The best estimate of the number of clusters (K) was determined using the method of Evanno et al. (2005), which identifies the best estimate of K as that which corresponds to the maximum change in the log probability of the data for successive values of K. For the best estimate of individual assignments, membership coefficients were averaged over 20 replicate runs using the “Greedy” algorithm and 100 permutations of the data in the program CLUMPP (Jakobsson and Rosenberg 2007). CLUMPP also addressed problems associated with label switching and multimodality. DISTRUCT 1.1 (Rosenburg 2004) was then used to visualize the estimated membership coefficients outputted from CLUMPP.

The program TESS 2.1 (Chen et al. 2007) was also used to assess Bayesian clustering of individuals into K groups. Unlike STRUCTURE, TESS utilizes both individual spatial data and multilocus genotypes to assign individuals to clusters. The MCMC algorithm was run for a length of 40,000 sweeps and a burn in of 10,000 sweeps. The model was first run without admixture, as suggested by the authors, for values of K ranging from 2 to 23. The value of K at which the number of clusters no longer increased and the change in the deviance information criteria (DIC) began to slow was selected as the optimum K value, for the no-admixture model (Chen et al. 2007). The results from the no-admixture model were then used to set the maximum K value under a model of admixture. The conditional autoregressive Gaussian (CAR) admixture model (Durand et al. 2009) was then used to analyze values of K ranging from 2 to 10 with 20 replicates for each K value. To estimate individual membership coefficients the algorithm was then run an additional 100 times at the optimal K value and the 20 runs with the lowest DIC values were retained. Admixture estimates were then averaged using CLUMPP and final estimated membership coefficients were displayed using DISTRUCT 1.1.

Two programs were used to test for recent reductions in population size at sample sites where 10 or more individuals were sampled. The program BOTTLENECK (Cornuet and Luikart, 1997) was used to assess signatures of past bottleneck events using the heterozygosity excess method of Cornuet and Luikart (1997) and a two-phase model (TPM) of mutation (DiRienzo et al. 1994). The significance of the test was assessed using a Wilcoxon signed-rank test (Luikart 1997). In addition, we used the method of Garza and Williamson (2001) to calculate the M

statistic using ARLEQUIN 3.1 (Excoffier et al 2005). This method divides the mean number of alleles by the range in allele size, to calculate the M ratio. Using M, it is possible to detect reductions in population size of more than 100 generations in the past (Garza and Williamson 2001). Populations that have experienced a marked reduction in size have been shown to exhibit M values  $<0.70$  (Garza and Williamson 2001).

A program developed by Storz and Beaumont (2002) using a hierarchical Bayesian coalescence model was also used to investigate evidence for a historical decline in population size. MSVAR carries out a coalescent analysis of multilocus microsatellite data to estimate time since change in population size (T), current population size ( $N_0$ ), and ancestral population size ( $N_1$ ). Since there is little evidence of population subdivision within populations west of the Mobile River, we combined individuals from all sample sites in the federally listed portion of the range. The prior mean for current population size was taken from a field survey conducted by Lohoefener and Lohmeier (1984). Mean ancestral population size was obtained by estimating the historical range of the gopher tortoise based on the total hectares of soil types classified as priority ( $\sim 4.8 \times 10^5$  ha) or suitable ( $\sim 1.4 \times 10^6$  ha) from habitat suitability studies (USFWS 1990) within the federally listed range and density estimates (0.32 tortoises/ha, priority; 0.148 tortoises/ha, suitable; Lohoefener and Lohmeier 1984). The mean time since population size was hypothesized to have begun changing was estimated to be as early as the 1630s (Frost 1993; Smith et al. 2001) and a generation time of 33 years (Gruver 2002) was used for the model. Eight loci showing a step-wise pattern of mutation were included in the analyses (Gp15, Gp201 excluded). Five independent chains were run for a total of  $4 \times 10^8$  steps, recording parameter values every 20,000 iterations in the chain for a total of 20,000 draws. The output was checked for convergence using the Gelman-Rubin statistic calculated in BOA (Smith 2005) as implemented in R. Plots of the estimated posterior density for each chain were also plotted to check for overall consistency in shape. The last 10,000 draws from each chain were then combined across all five chains for a total of 50,000 points in order to estimate the highest probability density (HPD) limits for each parameter. The log of ( $N_0/N_1$ ) (Beaumont 1999) was calculated to determine whether population decline or growth had occurred.



## RESULTS

### *Mitochondrial DNA results*

A total of 94 individual mitochondrial sequences of 743 base pairs in length were generated. Although 22 polymorphic sites were identified among all sequences analyzed, only six haplotypes were found to be unique (Table 1). The three most divergent haplotypes were easily distinguishable using SSCP. An additional three haplotypes were found through sequencing and were found to be only a single mutational step from the most closely related haplotype. This weak ability of SSCP to discriminate single base pair mutations has been observed in other studies (Pauly et al. 2007).

A minimum spanning network of the data (Figure 1) recovered two primary haplogroups (A and B). Haplogroup A corresponded to sample sites in Louisiana, Mississippi, and Alabama. The most common haplotype (West01) constituted 97% of all individuals sequenced in this region (Appendix IA). The two remaining haplotypes in group A (West02 and West 03) were found in sample sites from Louisiana (SH) and Alabama (AL), respectively, and differed from West01 by only a single base-pair substitution. Haplogroup B corresponded to sample sites in Georgia, South Carolina, and Florida. Haplotype East01 was found in 73% of the individuals sequenced for the region east of the Apalachicola River. A second haplotype (East02) was found in only a single individual from Florida (SM) and was one base-pair from the most common haplotype (East01). A third distinct haplotype (East03) was found exclusively in all individuals sampled from the two sites located in western Georgia (GG and WT) and was three base-pairs from the common haplotype of the region (East01). Haplogroups A and B showed no geographic overlap and appeared to be separated by the Apalachicola River drainage.

The mean absolute pairwise sequence differences between western (haplogroup A) and eastern (haplogroup B) sequences ( $16.314/743 = 2.20\%$ ) was 2.05 to 2.19% higher than within haplogroup differences (haplogroup A:  $0.096/743 = 0.01\%$ ; haplogroup B:  $1.177/743 = 0.15\%$ ). Net sequence divergence between A and B haplogroups was also low (p-distance = 0.022). Overall nucleotide diversity ( $\pi$ ) and polymorphism ( $\theta$ ) across all 94 sequences was 0.00998 and 0.00589, respectively. Within haplogroups A and B, nucleotide diversity and polymorphism was also low (haplogroup A:  $\pi = 0.00013$ ,  $\theta$  per site = 0.00086; haplogroup B:  $\pi = 0.00159$ ,  $\theta$  per site

= 0.00136) and tests of selective neutrality showed inconsistent results. Specifically, Tajima's D was significantly positive ( $D = 2.079$ ,  $p < 0.05$ ), while Fu and Li's  $D^*$  was not ( $D^* = 0.142$ ,  $p > 0.10$ ).

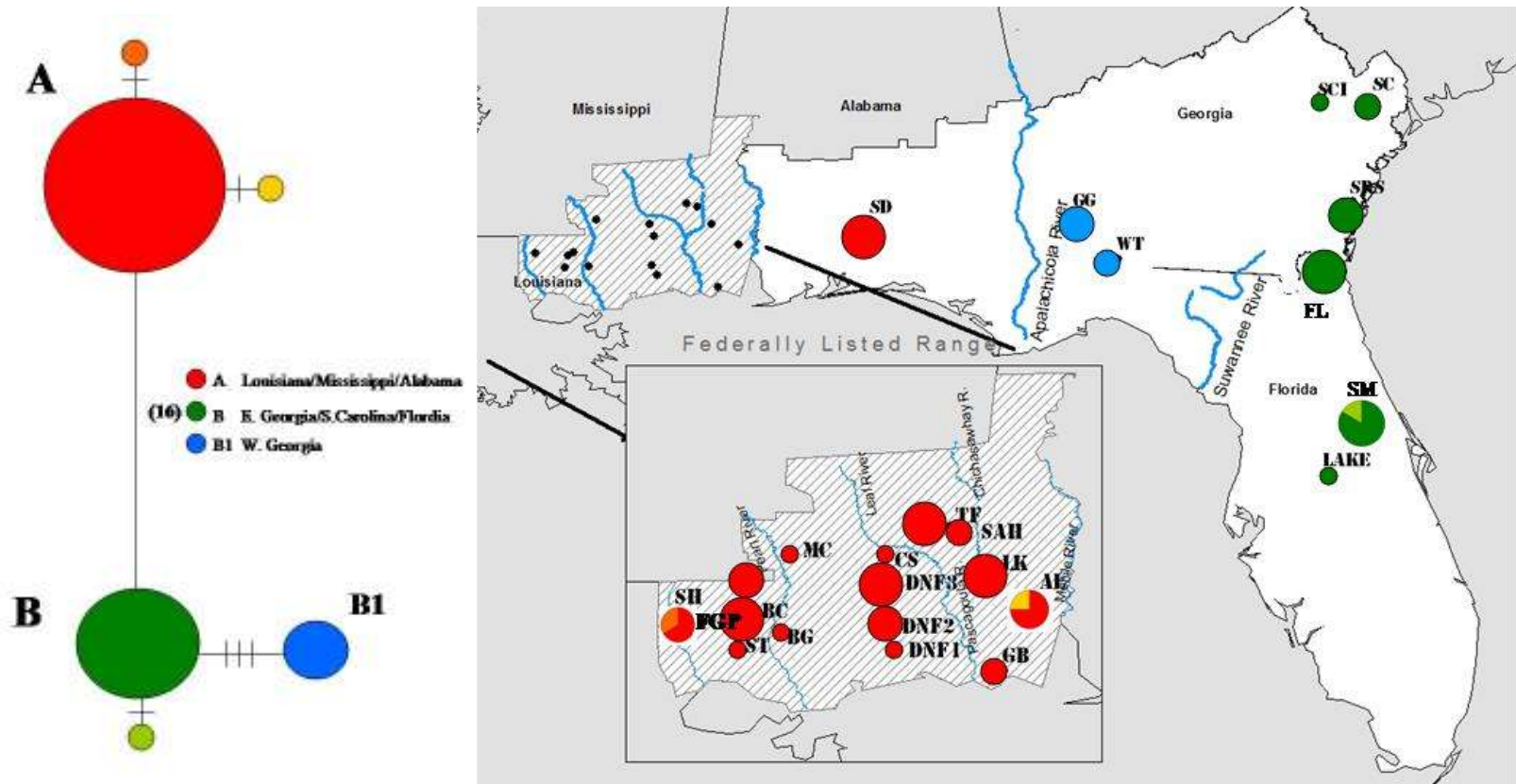
We used an AMOVA to group sample sites east and west of the Apalachicola River. More than 81% of the variation was due to genetic differentiation between haplogroups A and B, while only 0.53% of variation was within groups. A second AMOVA was then conducted where sample sites from western Georgia (GG and WT) were placed into a third group. This population configuration led to an even higher proportion of variance among groups (99.6%).

Table 3. Distribution of six mitochondrial haplotypes found in 24 gopher tortoise sampling sites.

		Haplotypes					
		A			B		B1
Population	Number	West01	West02	West03	East01	East02	East03
SH	3	2	1				
ST	2	2					
FGP	6	6					
BC	5	5					
BG	2	2					
MC	2	2					
DNF1	2	2					
DNF2	5	5					
DNF3	6	6					
CS	2	2					
TF	6	6					
SAH	4	4					
LK	6	6					
GB	3	3					
AL	4	3		1			
SD	6	6					
GG	4						4
WT	3						3
SRS	4				4		
SCI	2				2		
SC	3				3		
FL	6				6		
SM	6				5	1	
Lake	2				2		
<b>Total</b>	<b>94</b>	<b>62</b>	<b>1</b>	<b>1</b>	<b>22</b>	<b>1</b>	<b>7</b>

Figure 1. Geographical distribution of gopher tortoise haplotypes across the southeastern United States sampled from 24 sites. (a) Minimum spanning network of absolute pairwise differences between gopher tortoise mitochondrial DNA haplotypes. The area of each circle is proportional to the number of sequences analysed at each site and the number of mutational steps separating haplotypes is indicated by the hash marks. Note that 16 mutational steps separate haplogroups A and B. (b) Location of haplotypes by sample site. The sample site abbreviations correspond to Table 1. The shaded area of the map indicates the federally listed portion of the gopher tortoise range.

(a)(b)



### *Nuclear microsatellite DNA results*

Only one locus Gp201 showed deviations from HWE across multiple populations after Bonferroni correction. An analysis performed using MICROCHECKER (Oosterhout et al. 2004) confirmed that this was most likely due to the presence of null alleles. Tests for linkage disequilibrium indicated that 70 of the 1080 pairwise comparisons were significantly linked after correction for multiple tests. However, physical linkage of loci is unlikely since there was little evidence of consistent pairwise associations across sample sites. However, two sites (DNF2 and SCI) exhibited significant linkage disequilibrium at 20 (44%) and 11 (24%) of the possible pairwise comparisons, respectively.

A linear regression of sample size and total number of alleles demonstrated a significant correlation ( $r^2=0.40$ ,  $p = 0.0005$ ). However, no relationship was found for sample size and observed heterozygosity (Table 4). A further examination of the data found that populations with extremely small sample sizes ( $\leq 5$ ) were driving the majority of the correlation between sample size and total number of alleles. Therefore, populations with less than 10 samples were removed from tests of recent reduction in population size.

Table 4. Total number of alleles ( $A_N$ ), observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) by sample site.

Sample Site	N	$A_N$	$H_o$	$H_e$	Sample Site	N	$A_N$	$H_o$	$H_e$
SH	5	2.1	0.33	0.34	LK	14	3.1	0.4	0.44
FGP	36	3.9	0.37	0.41	GB	3	2.2	0.4	0.41
BC	21	3.0	0.36	0.4	AL	45	4.7	0.47	0.5
ST	4	2.1	0.23	0.38	SD	20	5.9	0.67	0.66
BG	4	2.3	0.45	0.4	GG	26	4.9	0.57	0.59
MC	23	3.2	0.36	0.39	WT	26	5.0	0.59	0.64
DNF1	12	2.8	0.46	0.45	SCI	24	4.2	0.41	0.55
DNF2	25	4.5	0.38	0.45	SRS	22	4.2	0.38	0.46
DNF3	45	4.0	0.38	0.44	SC	23	4.2	0.39	0.46
CS	21	3.4	0.39	0.44	FL	19	4.7	0.38	0.5
TF	12	3.3	0.44	0.45	Lake	4	2.8	0.42	0.57
SAH	7	2.7	0.33	0.44	SM	11	4.2	0.42	0.56

\* Sample site abbreviations are as in Table 1

Estimates of  $\theta_{ST}$  were calculated using all loci with and without the locus Gp201. As  $\theta_{ST}$  values and tests of significance for the two estimates were very similar, only values based on all

loci are reported. A total of 201 out of 276 pairwise comparisons were statistically significant following Bonferroni correction (Table 5). The highest estimates of  $\theta_{ST}$  were between western and eastern sites either side of Apalachicola River. Estimates of  $\theta_{ST}$  between sample sites in Louisiana and Mississippi were low and most did not differ significantly from zero. Two sample sites in western Alabama (GB and AL), showed moderate to high levels of differentiation ( $\theta_{ST}$  0.107-0.285) and moderate levels of genetic divergence ( $\theta_{ST}$  0.034-0.170) from other western sites, respectively. There was also a large amount of genetic differentiation between one site in central-southern Alabama (SD) and all of the other 23 sample sites ( $\theta_{ST}$  0.123-0.362). Differentiation between sample sites in the eastern portion of the range (Georgia, South Carolina, and Florida) was also surprisingly high ( $\theta_{ST}$  0.151-0.372) with the exception of sites that were less than 70km apart, such as SM and Lake in Florida ( $\theta_{ST}$  0.025), WT and GG in western Georgia ( $\theta_{ST}$  0.036) and SC in South Carolina and SCI in eastern Georgia ( $\theta_{ST}$  0.079).

The mean log likelihood from replicate runs in STRUCTURE was used to determine the best value of K using the method of Evanno et al. (2005). By means of this method, evidence of hierarchical population structure was evident, with K = 2 producing the highest value of  $\Delta K$ , with a smaller peak at K = 4 (Figure 3). When individual membership coefficients were aligned for 20 replicate runs for K = 2, all individuals sampled west of the Mobile and Tombigbee Rivers fell into one cluster and all individuals sampled east of the Apalachicola River fell into a second cluster. The population located in central-southern Alabama (SD) showed mixed ancestry from both clusters. When K = 4, individuals from Louisiana and Mississippi formed the majority of samples within the first cluster (Figure 4a). The second cluster was composed of 57% of the individuals from one site in Mississippi (LK) east of the Chickasawhay River and individuals from two populations in eastern Alabama (GB and AL) with many individuals sampled from other sites in Mississippi showing low levels of membership within this cluster. A third cluster was made up of tortoises sampled from western Georgia (GG and WT) and one site from Alabama (SD) east of the Mobile River, which also showed mixed ancestry with individuals from cluster two. Individuals sampled from South Carolina, Florida, and eastern Georgia all formed a fourth discernable cluster with the exception of one individual from South Carolina that assigned with high membership to the third cluster.

Analysis of multilocus genotype data in TESS (Chen et al. 2007) produced a similar pattern of population substructure as that observed for STRUCTURE with a few exceptions. First, in this spatially explicit analysis, five clusters were supported rather than four (Figure 4b). One cluster consisted of individuals sampled from all sites in Louisiana, Mississippi and two sites in Alabama (GB and AL) west of the Mobile River. In contrast, individuals from the site SD in Alabama formed a separate, second distinct cluster. Similar to STRUCTURE, the fourth cluster was made up of the two sample sites in western Georgia (GG and WT). However, TESS assigned individuals from the remaining six sites located near the Atlantic coast into two distinct clusters. The first group contained the two sites in eastern Georgia (SRS, SCI) and the site in South Carolina (SC). The final cluster encompassed all three sites in Florida. Several individuals from the SRS site in eastern Georgia showed moderate levels of membership with the Florida cluster. As observed with the STRUCTURE analysis, one individual from South Carolina was assigned to the cluster containing sample site SD and four individuals from one population in eastern Georgia (SCI) showed mixed ancestry with the western cluster.

Table 5. Pairwise  $\theta_{ST}$  values for 24 populations of gopher tortoises. Bold values are statistically significant following Bonferroni corrections,  $\alpha \leq 0.0001$ .

	SH	ST	FGP	BC	BG	MC	DNF1	DNF2	DNF3	CS	TF	SAH	LK	GB	AL	SD	GG	WT	SCI	SRS	SCI	FL	LAKE	SM	
SH	0.00																								
ST	0.04	0.00																							
FGP	0.01	0.04	0.00																						
BC	0.00	0.01	0.00	0.00																					
BG	0.06	0.00	0.04	0.01	0.00																				
MC	0.00	0.05	0.01	0.00	0.09	0.00																			
DNF1	0.01	0.07	0.05	0.03	0.02	0.04	0.00																		
DNF2	0.02	0.04	<b>0.05</b>	0.01	0.00	<b>0.05</b>	0.00	0.00																	
DNF3	0.00	0.08	<b>0.07</b>	<b>0.03</b>	0.07	<b>0.04</b>	0.04	<b>0.03</b>	0.00																
CS	0.00	0.04	<b>0.04</b>	0.00	0.02	0.02	0.01	0.01	0.01	0.00															
TF	0.02	0.03	<b>0.09</b>	<b>0.03</b>	0.05	<b>0.06</b>	0.04	<b>0.04</b>	0.03	0.02	0.00														
SAH	0.06	0.04	<b>0.09</b>	0.04	0.01	0.11	0.05	0.04	0.07	0.05	0.01	0.00													
LK	0.01	0.08	<b>0.05</b>	0.02	0.05	0.03	0.04	0.03	0.02	0.00	0.02	0.06	0.00												
GB	<b>0.12</b>	<b>0.10</b>	<b>0.15</b>	<b>0.10</b>	0.08	<b>0.13</b>	<b>0.11</b>	<b>0.08</b>	<b>0.07</b>	<b>0.05</b>	<b>0.03</b>	<b>0.08</b>	0.05	0.00											
AL	<b>0.27</b>	0.25	<b>0.29</b>	<b>0.25</b>	0.18	<b>0.27</b>	<b>0.17</b>	<b>0.13</b>	<b>0.18</b>	<b>0.17</b>	<b>0.12</b>	0.17	0.18	0.11	0.00										
SD	<b>0.17</b>	<b>0.21</b>	<b>0.23</b>	<b>0.18</b>	<b>0.19</b>	<b>0.21</b>	<b>0.18</b>	<b>0.19</b>	<b>0.18</b>	<b>0.17</b>	<b>0.14</b>	<b>0.18</b>	0.16	<b>0.17</b>	<b>0.16</b>	0.00									
GG	<b>0.29</b>	<b>0.33</b>	<b>0.35</b>	<b>0.30</b>	<b>0.31</b>	<b>0.34</b>	<b>0.31</b>	<b>0.31</b>	<b>0.30</b>	<b>0.30</b>	<b>0.27</b>	<b>0.28</b>	0.27	<b>0.28</b>	<b>0.31</b>	<b>0.19</b>	0.00								
WT	<b>0.26</b>	<b>0.28</b>	<b>0.31</b>	<b>0.26</b>	<b>0.26</b>	<b>0.31</b>	<b>0.27</b>	<b>0.27</b>	<b>0.27</b>	<b>0.25</b>	<b>0.24</b>	<b>0.24</b>	0.24	<b>0.24</b>	<b>0.27</b>	<b>0.15</b>	<b>0.04</b>	0.00							
SCI	<b>0.42</b>	<b>0.43</b>	<b>0.46</b>	<b>0.42</b>	<b>0.43</b>	<b>0.45</b>	<b>0.41</b>	<b>0.41</b>	<b>0.41</b>	<b>0.40</b>	<b>0.36</b>	<b>0.39</b>	0.39	<b>0.36</b>	<b>0.39</b>	<b>0.28</b>	<b>0.30</b>	<b>0.24</b>	0.00						
SRS	<b>0.53</b>	<b>0.54</b>	<b>0.53</b>	<b>0.51</b>	<b>0.53</b>	<b>0.53</b>	<b>0.50</b>	<b>0.49</b>	<b>0.47</b>	<b>0.48</b>	<b>0.45</b>	<b>0.49</b>	0.48	<b>0.41</b>	<b>0.50</b>	<b>0.35</b>	<b>0.37</b>	<b>0.32</b>	<b>0.12</b>	0.00					
SCI	<b>0.51</b>	<b>0.53</b>	<b>0.51</b>	<b>0.49</b>	<b>0.52</b>	<b>0.51</b>	<b>0.50</b>	<b>0.49</b>	<b>0.48</b>	<b>0.47</b>	<b>0.45</b>	<b>0.49</b>	0.47	<b>0.44</b>	<b>0.50</b>	<b>0.33</b>	<b>0.35</b>	<b>0.29</b>	<b>0.05</b>	<b>0.21</b>	0.00				
FL	<b>0.50</b>	<b>0.49</b>	<b>0.52</b>	<b>0.50</b>	<b>0.48</b>	<b>0.51</b>	<b>0.48</b>	<b>0.48</b>	<b>0.47</b>	<b>0.47</b>	<b>0.43</b>	<b>0.45</b>	0.47	<b>0.41</b>	<b>0.45</b>	<b>0.35</b>	<b>0.34</b>	<b>0.32</b>	<b>0.27</b>	<b>0.20</b>	<b>0.37</b>	0.00			
LAKE	0.48	0.45	<b>0.49</b>	<b>0.49</b>	0.43	<b>0.50</b>	<b>0.43</b>	<b>0.43</b>	<b>0.46</b>	<b>0.45</b>	<b>0.39</b>	0.40	0.44	<b>0.37</b>	0.41	<b>0.28</b>	<b>0.32</b>	<b>0.25</b>	<b>0.24</b>	<b>0.21</b>	<b>0.35</b>	<b>0.22</b>	0.00		
SM	<b>0.48</b>	<b>0.46</b>	<b>0.48</b>	<b>0.47</b>	<b>0.44</b>	<b>0.49</b>	<b>0.43</b>	<b>0.42</b>	<b>0.44</b>	<b>0.43</b>	<b>0.39</b>	<b>0.41</b>	0.42	<b>0.35</b>	0.42	<b>0.28</b>	<b>0.30</b>	<b>0.24</b>	<b>0.23</b>	<b>0.24</b>	<b>0.33</b>	<b>0.25</b>	0.03	0.00	

Figure 2. The seven groupings supported by a spatial analysis of molecular variance (SAMOVA) for sample sites distributed across the southeastern United States. The shaded area indicates the federally threatened portion of the gopher tortoise's range located west of the Mobile and Tombigbee Rivers.

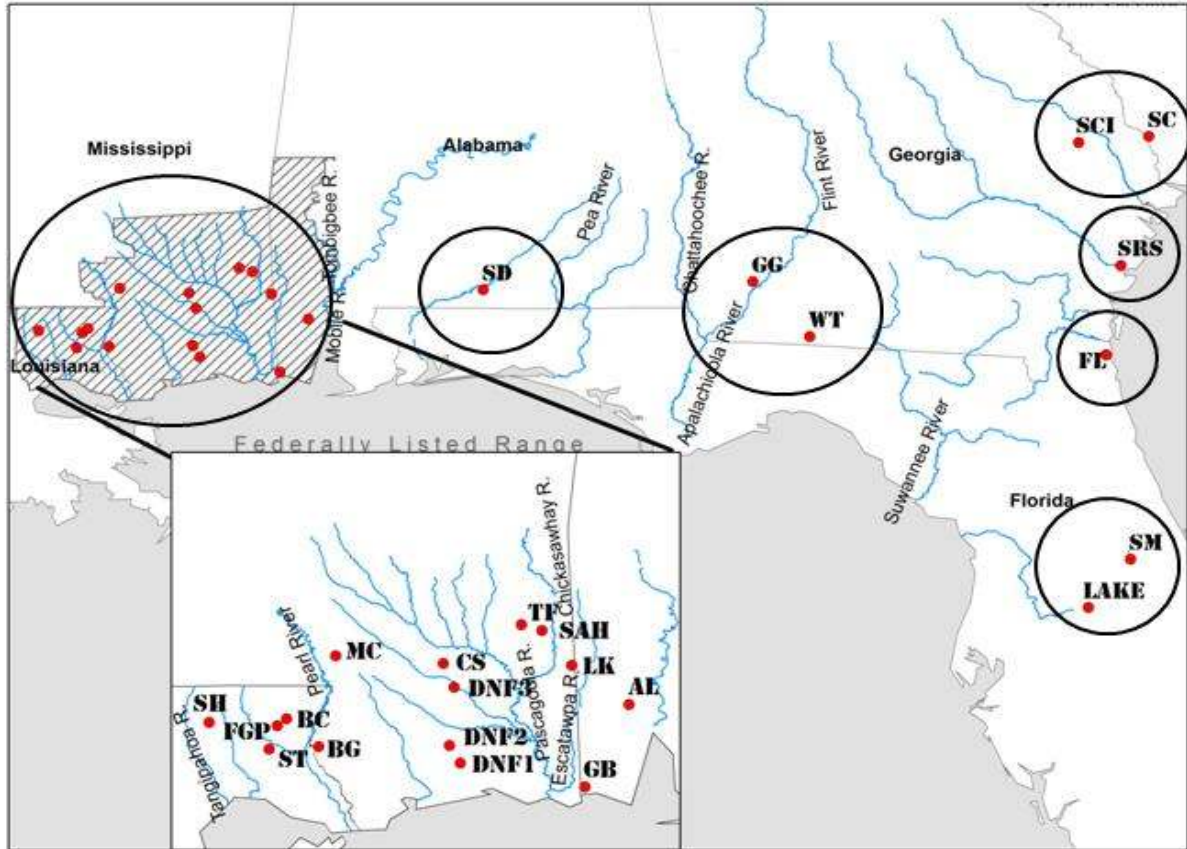




Figure 3. The rate of change in the mean log probability of the data between successive K values for K groups ranging from 2 to 20 based on the method of Evanno et al. (2005). Data indicates that four is the best estimate of the number of clusters.

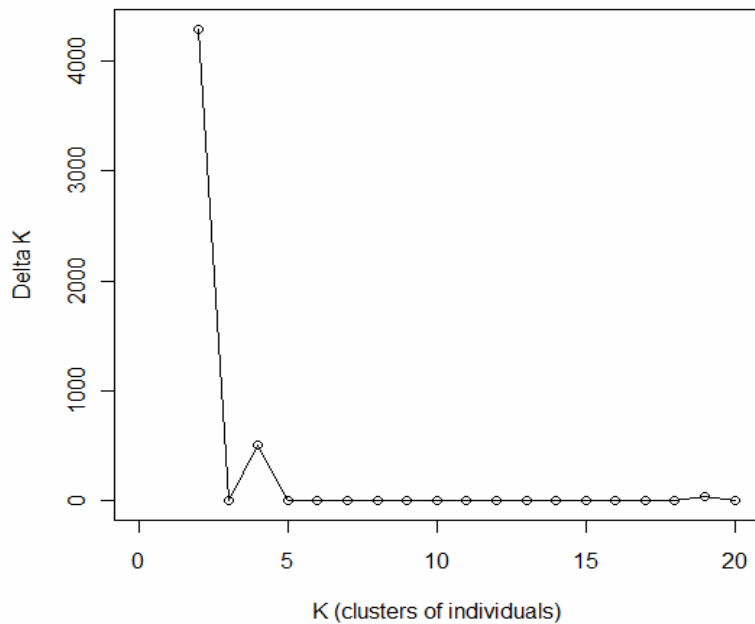
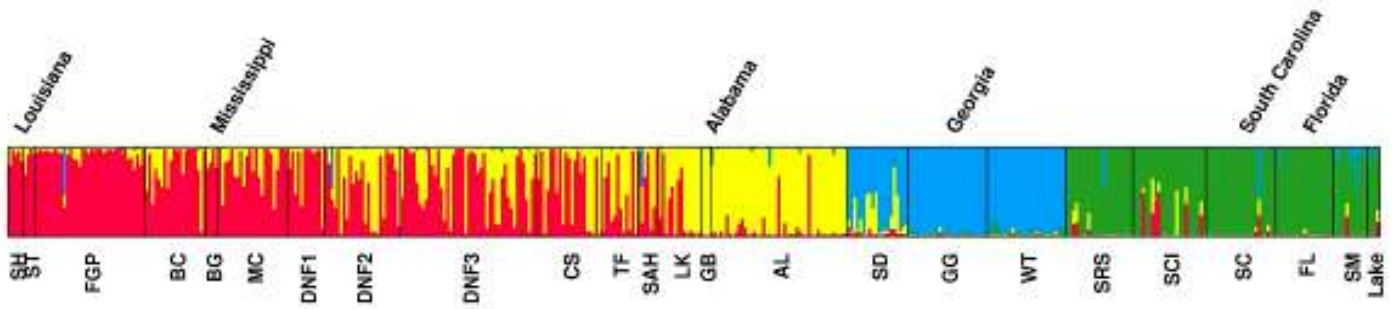
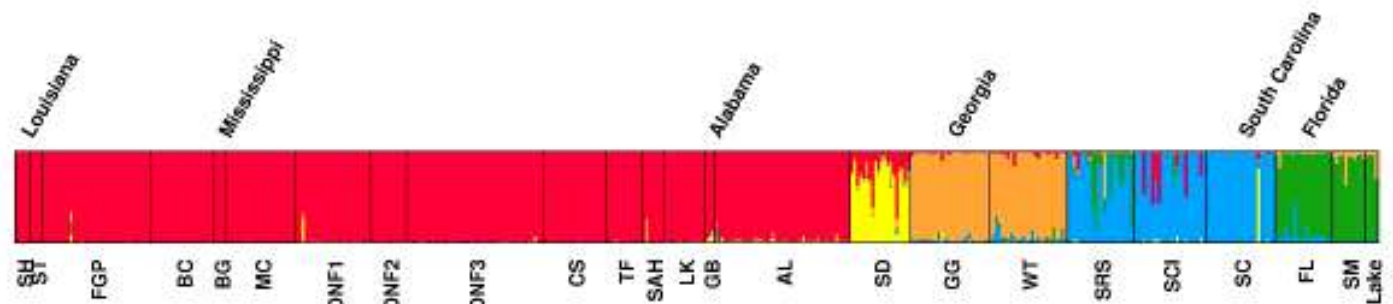


Figure 4. (a) Membership coefficients for 452 individuals sampled from 24 sites using a STRUCTURE 2.2 showing K =4 clusters and (b) from TESS 2.1 for K = 5 clusters. Labels below each plot indicate the site individuals were sampled from and label above plots display the state of origin.

(a)

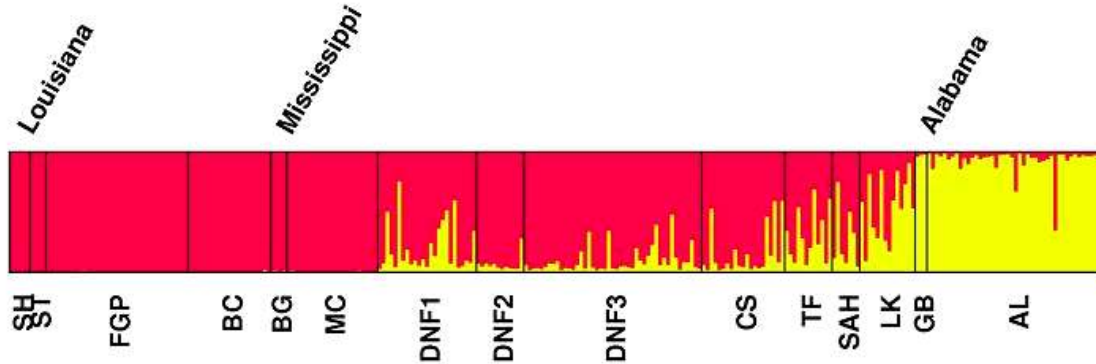


(b)



High levels of genetic divergence between sample sites east of the Mobile River ( $\theta_{ST}$  0.151-0.372) most likely made it difficult to delineate finer population structure evident within the western portion of the range where the majority of  $\theta_{ST}$  values were lower than 0.05. Therefore, an additional analysis was carried out in STRUCTURE and TESS to uncover potential signatures of population structure west of the Mobile River. The analysis was confined to 15 western sample sites showed that a maximum of two clusters was most likely (Figure 2). These corresponded to the same clusters identified by STRUCTURE using the entire data set (Figure 4a).

Figure 5. Membership coefficient results from TESS for 277 individuals sampled from 15 sites located west of the Mobile and Tombigbee Rivers showing  $K = 2$  clusters.



Results from BOTTLENECK found one sample site out of 18 (DNF1,  $p = 0.027$ ) that displayed evidence of significant heterozygote excess using the Wilcoxon signed-rank test and three additional populations showed evidence of significant heterozygote deficiency (DNF2, GG, SC). Garza and Williamson's  $M$  value showed strong evidence for bottleneck events in four populations (LA, MC, SCI, SM), where values ranging from 0.56-0.59 are well below those typical of bottlenecked populations ( $<0.70$ ) (Garza and Williamson 2001). Another 12 populations (DNF1, DNF2, DNF 3, AL, BC, FL, Lake, MC, SC, SRS, TF, WT) also showed evidence of a population decline with  $M$  values ranging from 0.61- 0.69. The remaining four populations (LK, CS, SAH, SD) produced values between 0.71- 0.74. These values were still below those normally observed in stable populations, which typically produce  $M$  values  $> 0.82$  (Garza and Williamson 2001).

The Bayesian coalescence model implemented in MSVAR indicated a significant decrease in population size for populations across the federally listed range. Reasonable convergence among chains was observed; the Gelman Rubin statistic ranged from values of 1.00-1.28 (Gelman et al. 1995). The log ratio of the mean current ( $N_0$ ) versus mean ancestral ( $N_1$ ) population size was -5.6, providing strong evidence for population decline and no support for either population growth ( $\log(N_0/N_1) > 1$ ) or a stable population size ( $\log(N_0/N_1) = 0$ ). Current effective population size was estimated to be between 100-3,400, while ancestral population size was estimated to range from 39,000-760,000 (Table 4). The estimated time that the population in the federally listed portion of the range began to decline was between 10,000 and 50,000 years.

## DISCUSSION

The present study provides the first detailed range-wide analysis of population genetic structure for gopher tortoise populations distributed throughout the southeastern United States. A deep phylogenetic divergence between eastern and western populations across the Apalachicola River drainage is consistent with the phylogenetic break observed in a previous study conducted by Osentoski and Lamb (1995). This pattern is also observed in other co-distributed species of the southeastern United States (Avice et al. 1979) including other species that also utilize upland longleaf pine-sandhill habitat (Avice 2000; Ellsworth et al. 1994; Pauly et al. 2007) and other chelonian species (Avice 2000). Estimates of net sequence divergence for gopher tortoise haplogroups east and west of this historical biogeographic divide were also similar to those found in other turtle species (Walker and Avice 1998). It has been postulated that this drainage was inundated by rising sea levels several times during the Pliocene and Pleistocene interglacial periods, creating a barrier for most terrestrial species such as the gopher tortoise (Avice 2000) and confining most freshwater aquatic taxa to the more northern, upper reaches of rivers, preventing gene flow among drainages (Avice 2004). In our study, the northern east-west divide for gopher tortoises appears to correspond to the upper western tributary of the Apalachicola (the Chattahoochee River). Two other turtle species, *Sternotherus minor* and *S. odoratus*, also display this same east-west divergence pattern (Walker and Avice 1998). However, another upland longleaf pine specialist (the flatwood salamander *Ambystoma cingulatum*) shows a deep phylogenetic break around the Flint River, the eastern tributary to the Apalachicola (Pauly et al

2007). For other southeastern species the exact geographic break is difficult to discern due to limited sampling. Records show that in the last four years the Flint River has been below three feet and tends to be low during summer and fall months when tortoises are most active (USGS 2009). The gopher tortoise populations may therefore be able to migrate west of the Flint River when water levels were low. It may also be possible that individuals were translocated to sites west of the Flint River during flood events (pers. comm. R. Birkhead).

Although a distinctive phylogeographic break was revealed among eastern and western groups, little genetic variation was evident within these two major haplogroups. Our data did show a distinct haplotype present in individuals from western Georgia, which was not found in the previous study (Osentoski and Lamb 1995). However, the present study did not find any evidence for haplotypes associated with the Ocala uplift in west-central Florida, which was cut off from the mainland during the Miocene and Pliocene (Osentoski and Lamb 1995). This can probably be attributed to a lack of sampling in this region of Florida. Low levels of genetic diversity at the mitochondrial level have also been found in other chelonian species (Edwards 2003; Velo-Antón et al. 2008). Previous research by Avise et al. (1992) proposed that the micro-evolutionary rate for the mitochondrial genome in the order Testudines is significantly slower than the conventional mtDNA clock, explaining the low levels of mtDNA polymorphism found in the gopher tortoise.

In contrast to the mitochondrial data, our microsatellite dataset revealed high genetic differentiation between numerous sample sites and evidence of regional population substructuring. As observed in the mtDNA analysis, the greatest levels of nuclear genetic divergence were found between populations east and west of the Apalachicola River, further supporting this region as a strong historical barrier to gene flow. Finer population genetic structure across the entire southeastern U.S. varied slightly with respect to the assignment method used. However, the best number of clusters appears to be either five or six. All clustering algorithms grouped the sample sites west of the Mobile River completely separate from sample sites located further east. Also, no individuals from the federally listed range showed any evidence of shared ancestry with the eastern populations according to Bayesian assignment methods. The exact number of clusters within the western portion of the range was less clear.

STRUCTURE revealed a maximum of two populations in the western portion of the range, while TESS recovered only one, except when the analysis was limited to sites west of the Mobile River. Membership of individuals from the SD site in Alabama, located between the Mobile and Apalachicola drainages, was also ambiguous. STRUCTURE grouped these individuals with tortoises sampled from western Georgia, while TESS recognized this sample site as an independent cluster. All methods used to discern subpopulations identified sample sites in western Georgia as a distinct cluster separate from other sites in the eastern portion of the range. These results are in agreement with the mitochondrial data, which revealed a distinct haplotype in the same geographic area of Georgia. Lastly, the Bayesian algorithm utilized in TESS, which incorporates spatial information, placed individuals from the final six sampling sites along the Atlantic coast into two groups while STRUCTURE recognized these as a single cluster.

Population structure was ambiguous for 1) the federally listed region located west of the Mobile River and 2) the population located between the Mobile and Apalachicola River. The SD sample site located in Alabama between the Mobile and Apalachicola rivers showed high genetic differentiation from all other sites. This site was grouped separately from other populations in both TESS and SAMOVA analyses. Furthermore, this site is separated from other sampling locations by major river drainages to both the east and west. However, when  $K$  was set to 2 in STRUCTURE analyses, individuals from the SD sample site appeared to be an admixture of eastern and western genotypes indicating that this portion of the gopher tortoise range may possibly represent a secondary contact zone. Nevertheless, mitochondrial DNA haplotypes for individuals sampled from SD are phylogenetically distinct from individuals in western Georgia, indicating that this population has undergone a period of historical isolation from tortoise populations in western Georgia. One explanation for the discrepancy between nuclear and mitochondrial patterns of population structure might be male-mediated nuclear gene flow. Male gopher tortoises are known to disperse long distances for mating opportunities (Eubanks et al. 2003; McRae et al. 1981). Therefore, it is possible that male but not female tortoises have dispersed west across the Chattahoochee River. Further sampling in this area is warranted in the future to better understand the patterns found in this area.

The 15 sites sampled west of the Mobile and Tombigbee rivers, in the federally listed portion of the range, also showed unclear patterns of population structure, as well as little genetic differentiation. Both SAMOVA and TESS grouped all sites into one cluster, suggesting that individuals in this region may have formed a panmictic population in the recent past. These sites did show significant differentiation from all sites further east of the Mobile and Tombigbee rivers, in the unlisted portion of the range. This suggests that the Mobile and Tombigbee River drainage is likely acting as a barrier to gene flow for this terrestrial species, as it does for other southeastern species (Burbrink et al. 2008; Soltis et al. 2006). When only sites west of the Mobile River were analyzed, data supported the existence of two groups east and west of the Chickasawhay River, with the exception of one site in Mississippi located immediately east of the Chickasawhay River (LK) that showed equal amounts of membership to both clusters. This provides evidence that the Chickasawhay River acts as a barrier to dispersal and limits gene flow in the western portion of the range.

The Bayesian assignment tests displayed little evidence for human-mediated translocations of tortoises between regions. Most individuals were assigned to the groups from which they were sampled. However, four individuals from SCI in eastern Georgia showed mixed ancestry with the individuals from the western portion of the range. SCI also showed high levels of linkage disequilibrium, which might be taken as an indication of possible genetic admixture resulting from recent introductions. One way that the parents of these admixed individuals might have been introduced to the SCI population is through the collection of tortoise for races, which were popular up until the 1980s. Tortoises were also often brought to the coast from other regions for sale due to the fact that tortoise meat was believed to have medicinal value (Lohofener and Lohmeier 1984). Only one individual from South Carolina appeared to have been recently translocated from the SD site in south-central Alabama. The extremely long distance over which it appears these translocations occurred makes it more likely that these were human-mediated movements and not natural dispersal events. More translocations would probably have been observed if sampling in the eastern portion of the range had been greater since this is where the majority of human-mediated movements occur (FFWCC 2007).

Eastern populations demonstrated a higher total number of alleles and higher observed heterozygosity. This is most likely due to the fact that the number of tortoises in the eastern portion of the range were historically larger than those in the west (Lohoefener and Lohmeier 1984). The total amount of longleaf pine habitat and suitable soils available to tortoises decreases and becomes more patchily distributed from east to west (Conner and Hartsell 1999; USDA NRCS), naturally supporting smaller populations in the western portion of the range (Auffenburg and Franz 1982). Interestingly, the sample site located in south-central Alabama (SD) had the highest heterozygosity. This result could be attributed to any one or a combination of the following three factors: 1) gopher tortoise populations in Alabama tend to be larger and denser than those in Louisiana and Mississippi (Auffenburg and Franz 1982; SOBS 2008); 2) human populations in Alabama have increased at a slower rate (SOBS 2008, USFWS 1987) leading to less severe tortoise population declines in this predominantly rural area and 3) the SD site could be a secondary contact zone, as suggested by the microsatellite data.

The program BOTTLENECK failed to detect signatures of a genetic bottleneck for the majority of populations. This could be due to the small sample sizes used in this study as well as the fact that this test may only be able to detect bottlenecks that occurred within the last few generations (Garza and Williamson 2001; Williamson-Natesan 2005). The M statistic can detect much more historical bottlenecks and is most effective when population declines have lasted several generations (Garza and Williamson 2001). M values obtained in the present study were much lower than would be expected in a stable population. Gopher tortoises experienced substantial reductions in habitat from 1850 to 1930 when logging began in the South (Van Lear et al. 2005) and since this initial decline the area of natural longleaf pine stands has continued to decrease over the last 50 years (Conner and Hartsell 2002). Interestingly, our coalescent analysis provided evidence of a 1000 fold decline beginning ~ 10,000-50,000 years ago and continuing to present, suggesting that population declines for the gopher tortoise began sometime during the late Pleistocene. Fossil evidence for *G. polyphemus* provides support for a historical range that extended into West Texas during the middle Pleistocene (Reynoso and Ballesteros 2004). Reynoso and Ballesteros (2004) also suggested that starting during the late Pleistocene all *Gopherus* species began to experience range reductions that have continued to the present day.

## *Conclusions*

Understanding patterns of gene flow in the gopher tortoise is important for the future management of the remaining populations, which are geographically isolated due to habitat loss and increasing urbanization. As human populations continue to expand, populations of gopher tortoises are increasingly relocated for development. It is important now more than ever to understand the genetic population structure of this species. Populations east and west of the Apalachicola Drainage displayed deep levels of phylogenetic divergence and should be considered separate ESUs. These two divergent lineages are reciprocally monophyletic and show significant genetic differentiation at nuclear loci, meeting Moritz's criteria for designation as ESUs (1994). Therefore, the movement of individuals across this drainage should be avoided since the translocation of individuals across a historical barrier could lead to outbreeding depression through the disruption of coadapted gene complexes and reduce evolutionary potential (Moritz 1994; Avise 2004).

Defining MUs requires the delineation of current population structure to address short-term management goals (Moritz 1994). Populations in western Georgia should be considered a distinct management unit based on both mitochondrial and nuclear data. Further sampling throughout Georgia is needed to determine the geographic extent of this western Georgia subpopulation. Gopher tortoise populations in the coastal strip encompassing Florida, eastern Georgia, and South Carolina could be managed as a single unit although relocation should focus on translocating individuals less than 7 km based on studies of natural movements in gopher tortoises (Eubanks et al. 2006; McRae et al. 1981). Gopher tortoises between the Mobile and Apalachicola rivers should also be managed as a distinct population but further sampling is needed in this region as it may be a contact zone for eastern and western populations. Federally listed populations west of the Mobile and Tombigbee River appear to have historically been a distinct panmictic population but habitat loss has likely isolated many populations and is currently impeding gene flow. Relocations in the federally listed portion of the range should avoid translocating tortoises across the Chickasawhay River. Further studies will examine more explicitly the role of rivers and other landscape features on gene flow in gopher tortoises. This knowledge can be used to better understand population structure and develop strategies that allow for natural movement between populations. Finally, care should be taken to avoid further



loss of genetic variation, which may lead to a loss of evolutionary potential and an inability to deal with novel pathogens or climate change. Gopher tortoise recovery plans should focus on expanding areas of open longleaf pine habitat in order to promote population growth and natural population connectivity thus securing the future viability of this species.

## **CHAPTER THREE: The Influence of Landscape on Gopher Tortoise Population Structure at the Regional Level**

### INTRODUCTION

Although the delineation of population structure has been a major focus of conservation genetics, recent advances in spatially explicit landscape genetic tools have led to greater insights into the landscape features and habitat variables that influence gene flow and population differentiation (Mantel et al. 2003; Storfer et al. 2007). It is well known that regional population dynamics are shaped by landscape complexity, which is a product of both natural environmental heterogeneity and anthropogenic land use change (Meffe and Carroll 1997; Storfer et al. 2007). The amount of genetic differentiation among populations is therefore determined by both natural landscape factors that either promote or inhibit gene flow such as connectivity of suitable habitat or presence of major rivers and the effect of artificial barriers to species movement such as roads or areas of high intensity development (Vos and Chardon 1998; Vignieri 2005; Cushman et al. 2006; Vandergast et al. 2007; Clark et al. 2008; Spear and Storfer 2008). Measures of genetic distance between populations can be used to correlate population structure with the presence of specific landscape features in order to gain a better understanding of natural and anthropogenic constraints to species movement. Knowledge gained from the delineation of landscape features that contribute to regional population structure can then be used to improve the management of threatened and endangered species by identifying dispersal corridors in need of protection or artificial barriers that need to be modified to maintain natural patterns of gene flow between populations.

The gopher tortoise (*Gopherus polyphemus*) constitutes a model species for landscape genetic analysis due to its limited dispersal capabilities, specific habitat requirements and vulnerability to habitat fragmentation (Lohoefer and Lohmeier 1981; Auffenburg and Franz 1982; Gibbons et al. 2000). The distribution of this species throughout the southeastern U.S. is naturally patchy and most populations are restricted to habitats of longleaf pine-oak uplands, xeric hammock, sand pine-oak ridges, or ruderal successional types (Auffenburg and Franz 1982). Tortoise densities are highest in open canopy habitats with well-drained sandy soils and lowest or absent in areas of mesic hardwoods or dense pine plantations of loblolly (*Pinus taeda*)

and slash pine (*Pinus elliottii*) (Lohoefener and Lohmeier 1984; Aresco and Guyer 1999; Baskaran et al. 2006). The gopher tortoise has experienced range-wide population declines due to the dramatic loss and fragmentation of longleaf pine forests over the past 100 years (Auffenburg and Franz 1982; Lohoefener and Lohmeier 1984; USFWS 1987; Noss 1988; SOBS 2006). Some of the most drastic declines have occurred west of the Mobile and Tombigbee Rivers within the federally listed portion of the range that encompasses southeastern Louisiana, southern Mississippi and southern Alabama (Lohoefener and Lohmeier 1984; USFWS 1987). Tortoises in Louisiana are at the greatest risk of extinction due to poorer habitat quality (Auffenburg and Franz 1982) and conversion of suitable habitat to agriculture and urbanization (Lohoefener and Lohmeier 1984). In southern Mississippi, gopher tortoises tend to occur at low densities (Lohoefener and Lohmeier 1981; Auffenburg and Franz 1982) and with less than 5% of suitable habitat remaining, most populations are now restricted to the Desoto National Forest (Lohoefener and Lohmeier 1981 Auffenburg and Franz 1982). Alabama contains the highest density of populations in the western range, having been less affected by human population growth and changes in land use than southeastern Louisiana and Mississippi (Lohoefener and Lohmeier 1981; Auffenburg and Franz 1982).

Several factors make gopher tortoises particularly susceptible to local extinction. In the western portion of their range, Lohoehener and Lohmeier (1984) noted that the extreme isolation of many populations might have led to loss of genetic diversity and further population declines in the region. As observed in other chelonian species (Gibbons 1986), gopher tortoises also show limited dispersal capabilities with the majority of seasonal movements being <500 m in distance (McRae et al. 1981; Diemer 1992; Eubanks et al. 2003). Long-distance dispersal events for the purposes of mating, nesting, seasonal migrations, or departure from unsuitable habitat are usually < 1 km (McRae et al. 1981; Gibbons 1986; Eubanks et al. 2003). The limited dispersal ability of gopher tortoises combined with their specialized habitat preferences and the extensive habitat fragmentation and urbanization within the region is therefore likely to isolate populations and make them more vulnerable to extinction. This habitat selectivity and limited dispersal might also lead to significant genetic differentiation, making the gopher tortoise a good model for examining the effects of both natural and anthropogenic features on population structure.

An understanding of species-specific natural history is essential to predict what features might drive patterns of genetic differentiation among populations. Data on the ecology of the gopher tortoise suggest that rivers, elevation, roads, soil and land cover type may all affect gene flow at the regional scale (Auffenburg and Franz 1982; Eubanks et al. 2003; Baskaran et al. 2006). In fact, rivers have long been suspected to constitute barriers to gene flow since tortoises are strictly terrestrial (Ernst et al. 2009) and strongly associated with upland longleaf pine habitat (Auffenburg and Franz 1982; Guyer and Bailey 1993). A recent habitat suitability study found that the density of gopher tortoise burrows significantly decreased closer to rivers (Baskaran et al. 2006) indicating that this species may generally avoid riparian areas. Molecular markers have also provided evidence that rivers may have influenced both historical and contemporary population genetic structure in the gopher tortoise (Clostio et al. *in prep*). In the western federally protected portion of their range, the U.S. Fish and Wildlife Service used major rivers to delineate four “service areas” (USFWS 2009) across which tortoises cannot be translocated. However, a recent examination of population structure throughout the southeast found evidence to suggest that only the Chickasawhay Rivers acted as an important barrier to gene flow in the federally listed portion of the range (Chapter 2).

Elevation may also influence movement in the gopher tortoise. Previous studies have suggested that gopher tortoises may prefer to use upland ridges as dispersal corridors because these ridges typically provide more sandy soils and appropriate habitat for constructing burrows and foraging (Auffenburg and Franz 1982; Baskaran et al. 2006). Population structure between ridges has been observed in other species that occupy similar habitat (Clark et al. 1999; McDonald et al. 1999) suggesting that upland areas may provide important conduits for dispersal. Roads have also been shown to limit gene flow for many species (Vos and Chardon 1998; Blanchong et al. 2007; Balkenhol and Waits 2009) including turtles (Beaudry et al. 2008; Shepard et al. 2008). Although road mortality has contributed to the decline of gopher tortoises (USFWS 1987), the extent to which roads impact tortoise gene flow is poorly understood yet might have important management implications.

The effect of different cover types on dispersal has also not been directly investigated. Gopher tortoises are most commonly associated with open canopy longleaf pine habitats

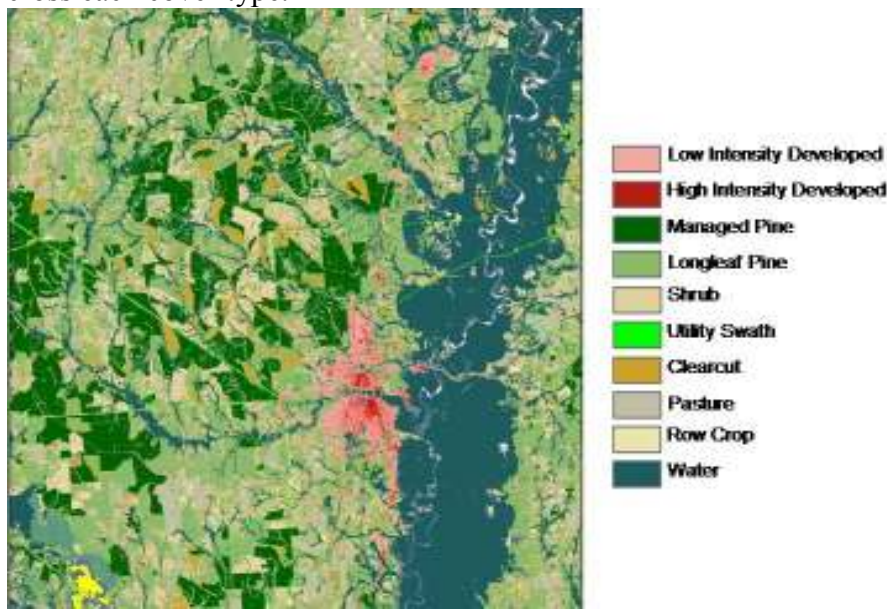
maintained by fire (Auffenburg and Franz 1982; Guyer and Bailey 1993). However, they are also found in man-made open habitats such as pastures and utility right of ways (Baskaran et al. 2006). Areas dominated by wetlands, dense canopy forests or human dwellings are expected to decrease gene flow because tortoises do not generally occupy these types of habitat at high densities (Auffenburg and Franz 1982; Baskaran et al. 2006). In contrast, open canopy land cover types should facilitate movement and increase gene flow among populations as the amount of suitable habitat directly linking sites increases.

Finally, soil type is one of the most significant factors in predicting the location of tortoise burrows (Baskaran et al. 2006). Studies carried out by state and federal agencies based on the location and density of tortoises have led to a classification of soil types into ‘priority’, ‘suitable’, ‘marginal’, and ‘unsuitable’ (USFWS 2005). The presence of gopher tortoise burrows is strongly associated with soils that have high sand and low clay content. These soils are associated with the presence of certain herbaceous plants eaten by tortoises, such as legumes (USFWS 2005). Therefore, soil types classified as priority or suitable should facilitate tortoise dispersal due to the availability of forage material and increased probability of locating burrows for refuge.

The effects of these key landscape features on gopher tortoise population structure and potential for dispersal can be investigated by measuring the physical distance between populations while accounting for resistance to movement across one or more landscape variables. One of the most popular methods of examining species gene flow while incorporating landscape heterogeneity is least-cost path (LCP) analysis (Wang et al. 2009; Cushman et al. 2009; Spear and Storfer 2008; Epps et al. 2007; Stevens et al. 2006; Spear et al. 2005). LCP analysis finds the shortest single path from a source site to a destination site based on amount of resistance a particular landscape feature poses to species movement. This method utilizes raster data layers composed of equally sized cells (usually 30 m<sup>2</sup>) that represent various landscape attributes (Figure 1). These cells are reassigned cost values reflecting the magnitude of resistance a given feature is understood to have on movement. This allows landscapes to be modeled as gradients of resistance to dispersal rather than discrete areas of suitable and unsuitable habitat or simple absolute linear barriers to gene flow (Cushman et al. 2006). These landscape resistance

surfaces can then be used to compute a least cost path (LCP) connecting two points in the landscape. Multiple landscape features can then be simultaneously assessed by using geographic information systems (GIS) to combine the multiple raster data layers. The relative importance of landscape features is then assessed by correlating LCP distances with levels of genetic differentiation between populations using Mantel tests (Mantel 1967) or other distance matrix approaches (Legendre et al. 1994). The significance of these matrix correlations is then assessed through permutation tests.

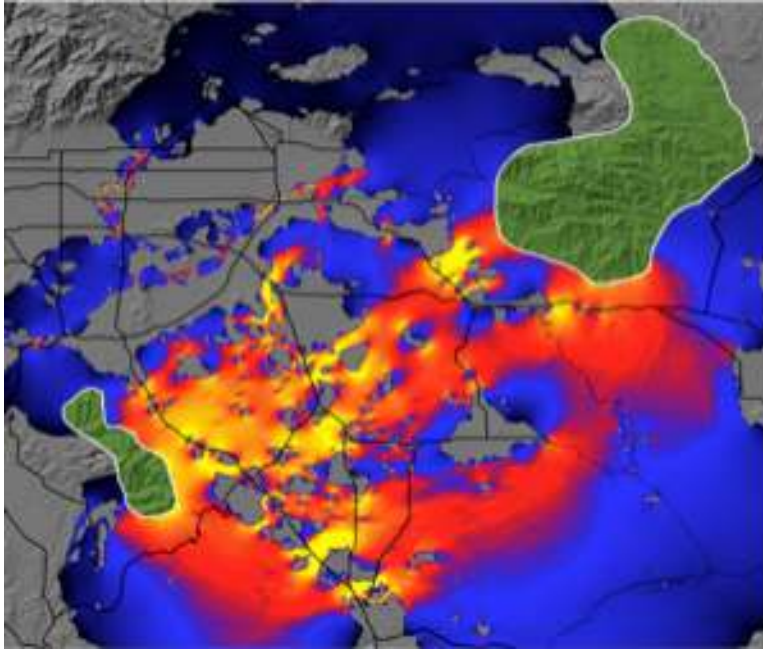
Figure 1 Example raster depicting ten land cover classes. Values can be associated with each class to reflect the amount of resistance a gopher tortoise is expected to experience in order to cross each cover type.



As an alternative to LCP, a more robust method for examining the influence of landscape features on gene flow is an approach based on electrical circuit theory known as isolation by resistance (IBR) (McRae 2006; McRae and Beier 2007; McRae et al. 2008). Although utilizing raster layers in the same way as LCP analysis, IBR analysis differs in that it uses distance metrics based on circuit theory (McRae 2006) to determine the level of conductivity or effective migration between sites or habitat patches, and then computes a resistance distance from these conductance values (Figure 2). This method identifies multiple dispersal pathways and assumes that gene flow between two populations increases as the number and width of connecting pathways increases or when migration through intervening populations is possible (McRae 2006). IBR offers a more robust and realistic option for modeling dispersal across a continuous

landscape than the LCP method which detects only a single optimal dispersal route connecting two points and does not consider other plausible routes. Resistance distances based on this method have been found to explain genetic distance better than straight-line distance and LCPs (McRae and Beier 2007).

Figure 2 A current map from McRae and Shah (2009) constructed in CIRCUITSCAPE 3.5 used to measure resistance distance based on electrical circuit theory. Warmer colors represent areas of high conductance (low resistance) linking one habitat patch to another.



Given our current understanding of gopher tortoise biology and the threats that this species now faces across its range, the goal of the present study was to use a causal modeling approach to assess the relative importance of natural and anthropogenic landscape features in driving gopher tortoise population structure. Three resistance levels for each landscape variable of interest (elevation, roads, soils, and land cover) were used to construct alternative cost surfaces. Partial mantel tests were then used to investigate the relative importance of Euclidean distance, a riverine barrier and the four landscape variables on genetic structure. Findings from these analyses were then evaluated through seven competing models of causality in order to determine which combination of features influenced population genetic structure. A comparison of isolation by resistance (IBR; McRae 2006) and least-cost path (LCP) analyses was also carried out in order to determine which method best explained the observed relationships between genetic differentiation and the hypothesized key landscape features. In order to assess whether

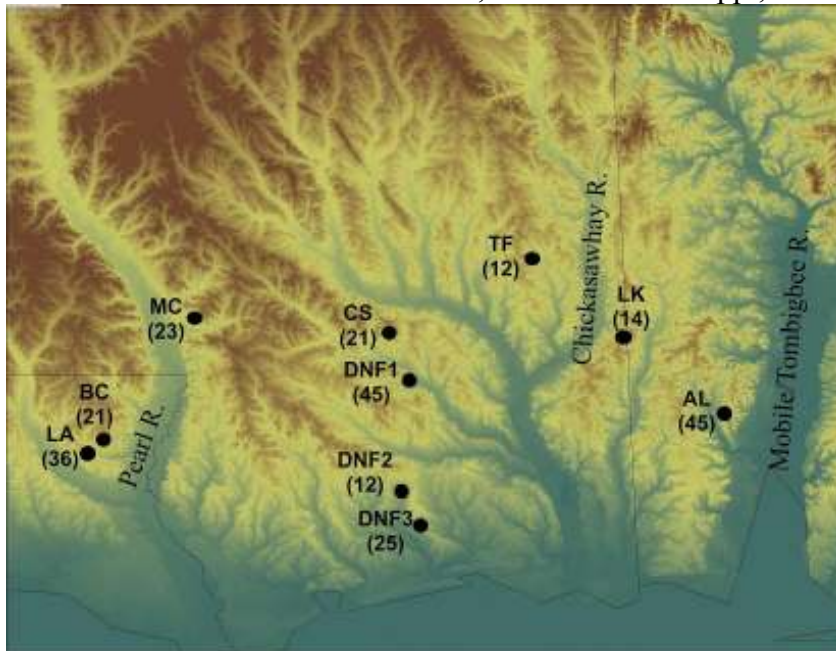
results were model dependent, the effect of multiple landscape variables on genetic structure was also assessed using a multiple regression approach on landscape and genetic distance matrices (Lichstein 2007).

## MATERIALS AND METHODS

### *Genetic analysis*

A total of 254 individuals were collected from 10 sites (Figure 3), located in the federally listed portion of the range, west of the Mobile and Tombigbee rivers. Sites containing less than ten individuals were excluded from analyses because it has been previously shown (Chapter 2) that small sample sizes probably underestimate the true amount of genetic diversity present at a site. DNA samples were genotyped at ten microsatellite loci (Gp5, Gp12, Gp14, Gp15, Gp26, Gp30, Gp96, Gp102, Gp105, Gp201) described in Chapter 2. Pairwise  $\theta_{ST}$  values (Excoffier et al. 1992) were calculated among sample sites using ARLEQUIN 3.1 (Excoffier et al. 2005).

Figure 3 Topographic map derived from a National Elevation Dataset (USGS) of sample sites, located west of the Mobile and Tombigbee rivers, used in this study with sample sizes. Map extent covers southeastern Louisiana, southern Mississippi, and southern Alabama



\* Site abbreviations define in Chapter 2 (Table 1)

### *Spatial analysis*



Euclidean (straight-line) distances between sampling sites were measured in ArcGIS 9.1 (ESRI). A Mantel test (Mantel 1967) was then used to test for an association between genetic and logarithmic Euclidean distance matrices (isolation-by-distance) using the statistical computing software R (R Core Development Team 2009). The effect of the Chickasawhay River on genetic population structure was then assessed using a partial Mantel test (Smouse et al. 1986) where a binary indicator matrix was used to indicate population pairs on the same (0) or opposite side (1) of the river. The Chickasawhay was chosen as a potential barrier to dispersal based on the observation of previous results from Bayesian assignment tests (Clostio et al. *in prep*) indicating that the geographic location of this river coincided with a genetic break between populations in the federally listed range.

Coverage data for landscape features was obtained from multiple sources and all polygon layers were converted to raster datasets before resistance values were assigned to landscape features using ArcGIS 9.2. One criticism of LCP and IBR methods is that the assignment of resistance values is subjective, even when knowledge of species ecology is utilized (Storfer et al. 2007; Cushman et al. 2006). To account for this pitfall, each landscape feature was modeled at three levels of resistance (null, low and high; Table 1), thus permitting comparison of the relative importance of each variable rather than its absolute numerical value (Cushman et al. 2006; Balkenhol et al. 2010). The null model assumed that a particular coverage type had no influence on the movement of tortoises across the landscape and all cells were assigned the same value of one giving essentially Euclidean distances between populations. The low resistance model assumed that resistance due to unfavorable feature types was relatively modest. The high resistance model assigned higher magnitude cost values to unfavorable landscape features. A maximum resistance value of 10 was used for all four landscape features in order to standardize their effects.

Table 1 Resistance values for the three landscape features that were modeled as categorical variables.

Classes	Resistance Model		
	Null	Low	High
<b>Soils</b>			
Priority	1	1	1
Suitable	1	2	2
Marginal	1	3	6
Unsuitable	1	5	10
<b>Roads</b>			
State Highways	1	2	5
Interstates	1	5	10
<b>Land cover</b>			
Water	1	10	10
Wetlands/riparian	1	10	10
Urban	1	10	10
Open understory wet forest	1	8	9
Closed canopy-hardwood forest	1	6	8
Mixed forest	1	5	7
Timber plantation	1	5	7
Agriculture (crops)	1	4	6
Shoreline	1	3	5
Shrub understory	1	3	5
Clearcut	1	4	6
Pasture	1	2	2
Utility swaths	1	2	2
Open canopy forest	1	1	1
Long leaf pine	1	1	1

Resistance to tortoise movement based on elevation was modeled as an inverse linear function over two cost levels representing low and high resistance. Resistance decreased as elevation increased to simulate increasing movement of tortoises along upland ridges. In contrast, resistance values for soils, roads and land cover types were modeled as categorical functions (Table 1). For soils, a map depicting different soil types was acquired from the U.S. Department of Agriculture, Natural Resource Conservation Service (<http://soildatamart.nrcs.usda.gov>) and soil classes were identified as priority, suitable, marginal and unsuitable based on previously published data (USFWS 2005). A cost value of one was assigned to the most suitable soil type and resistance values increased as soil types became less suitable (Table 1). For roads, data was obtained from the U.S. Census Bureau's Census 2000 TIGER line data. Two types of road ways were considered: interstates and major highways. Interstates were assumed to be more difficult for a tortoise to traverse than state highways due to wider corridors and higher traffic volume and were therefore given the highest cost values. All other cells in the data layer not containing roadways of interest were assigned a value of one. In

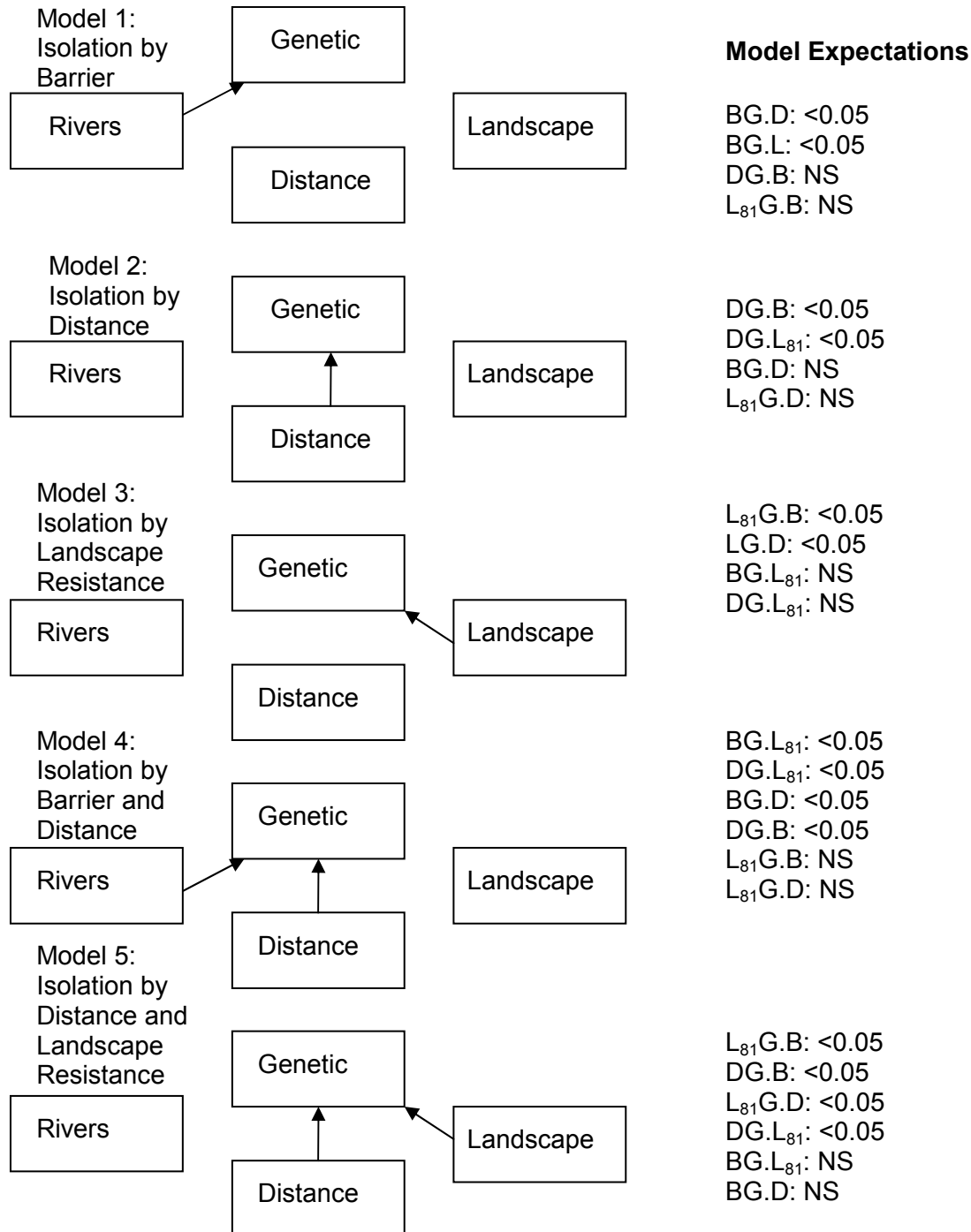
the case of land cover, data was acquired from the Southeast Region Gap Analysis Program ([http://www.basic.ncsu.edu/segap/datazip/region/lc\\_segap.zip](http://www.basic.ncsu.edu/segap/datazip/region/lc_segap.zip)). The original dataset containing 253 land cover classes was collapsed into 16 broader land cover classes to better reflect gopher tortoise ecology and habitat suitability (Table 1). For the low resistance model, the highest resistance values were assigned to habitat types tortoises generally avoid such as urban areas and wetlands. All other unsuitable land cover classes were assigned more moderate values of resistance as tortoises might move through these habitats occasionally. In contrast, the high resistance level modeled all unsuitable habitat types at high values of resistance to movement to reflect a strong avoidance of habitats types that were not open canopy. For both low and high resistance models, preferred habitat (open canopy) received the lowest value of one.

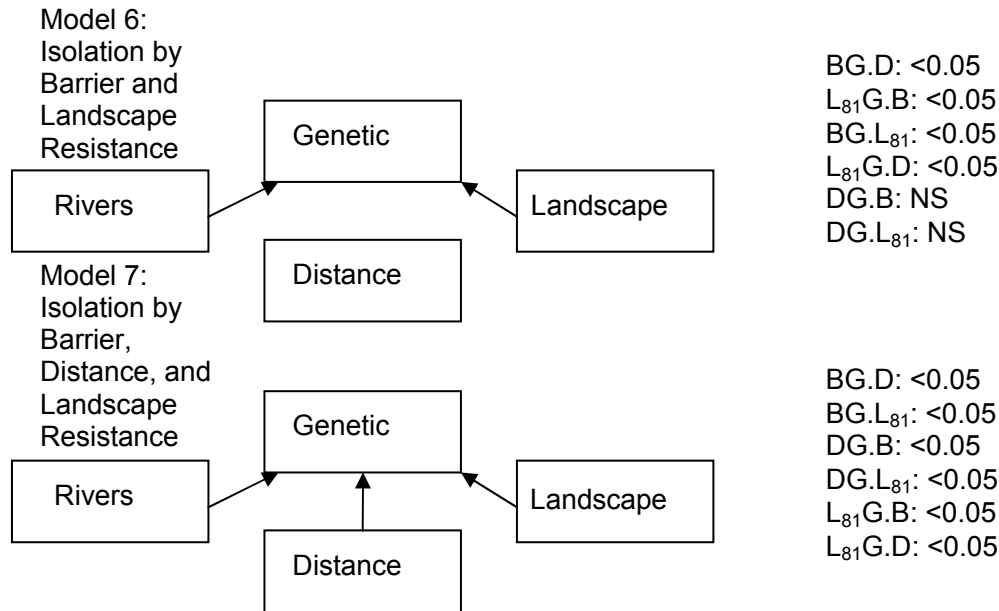
Since it is unlikely that gopher tortoises will experience resistance to movement from a single landscape feature independent of all other landscape variables (Cushman et al. 2006), composite cost surfaces made up of all 4 landscape variables were created. Landscape resistance surfaces were modeled by summing all four variables across all possible combinations of the three resistance levels (null, low and high) using the ‘raster calculator’ feature in ArcGIS 9.1, resulting in a total of 81 cumulative landscape resistance surfaces. Pairwise resistance distances between sample sites were then calculated using two approaches. For IBR, CIRCUITSCAPE version 3.5 (McRae and Shah 2009) was used to determine an average resistance distance that included all possible paths among sites for the 81 resistance surfaces examined. For LCP, a single lowest cost path between each pair of sample sites was calculated in kilometers using the ‘shortest path’ feature in the Spatial Analyst extension of ArcGIS 9.1. As LCP is a computationally intensive method precluding the analysis of all 81 landscape surfaces, LCP distances were only calculated for the landscapes surfaces found to be significant using cost distances based on IBR.

Following Cushman et al. (2006) seven competing models of causality (Legendre and Legendre 1995) were generated *a priori* (Figure 4) to test whether isolation by distance, isolation by barriers (Chickasawhay River), isolation by one or more landscape-resistance gradients (81 resistance surfaces), or some combination of these three factors best explained the variation in genetic distance among sample sites. Each model was defined by a set of statistical predictions

regarding the expected significance of the relationship between a given landscape feature(s) and pairwise genetic distance (Figure 4). These predictions were then tested using a series of partial Mantel tests in order to determine which of the three factors (landscape resistance surfaces, straight-line distance, or the riverine barrier) best correlated with genetic distance, while controlling for one of the other two factors independently. All partial Mantel tests were carried out using the R package and the significance of each test was determined using  $10^4$  permutations of the genetic distance matrix. A model was fully supported if all statistical expectations were met, as determined by the significance of the p-value from the corresponding partial Mantel test.

Figure 4. The seven models of causality used to examine the relationship between genetic distance (G), straight-line distance (D), the Chickasawhay River (B) and landscape resistance distances (L) calculated using both IBR and LCP. The partial Mantel tests used to evaluate each model are listed to the right, under model expectations. A period separates the two matrices tested from the covariate matrix that has been partialled out. In order for a model to be fully supported all model expectations must be met. NS = non significant.





In model 1, the Chickasawhay River was hypothesized to be the dominant driver of genetic distance among sample sites, while neither straight-line distance nor landscape resistance distances based on the 81 cost surfaces are predicted to explain significant patterns of genetic differentiation between sites. The dominant factor for model 2 was straight-line distance while the Chickasawhay River and landscape resistance distances are predicted to be non-significant. In model 3, landscape features were hypothesized to be the single main driver. Models 4 through 6 predicted that two out of the three landscape factors (straight-line distance, Chickasawhay river barrier and landscape resistances) were correlated with genetic distance whereas model 7 predicted that all of these three factors would significantly influence genetic differentiation. To determine the landscape features that best explained genetic distance between sites, landscape surfaces were ranked by their associated correlation coefficients.

Recent studies suggest that partial Mantel tests may exhibit high type I error when they are used to examine pairwise distance measures based on landscape data (Balkenhol et al. 2009). Therefore, results from partial Mantel tests were also compared to those based on multiple regression method of genetic and landscape distance matrices (Lichstein 2007). The significance of the relationship between genetic distance and candidate landscape variables was assessed using 1000 permutations of the genetic distance data in PERMUTE version 3.4 (Legendre et al. 1994). Resistance distances under either a low or high cost model (Table 2) were calculated

independently for each of the four landscape variables using IBR and LCP methods, giving a total of eight landscape resistance distance matrices. A distance matrix made up of straight-line distances and a binary matrix describing the riverine effect was also included as candidate variables in the model. Both forward and backward regression was then used to evaluate which variables should be retained using  $p < 0.05$  for inclusion and  $p > 0.05$  for removal. Significance of the final model was determined by the Bonferroni corrected  $\alpha$ -value ( $p < 0.005$ ).

## RESULTS

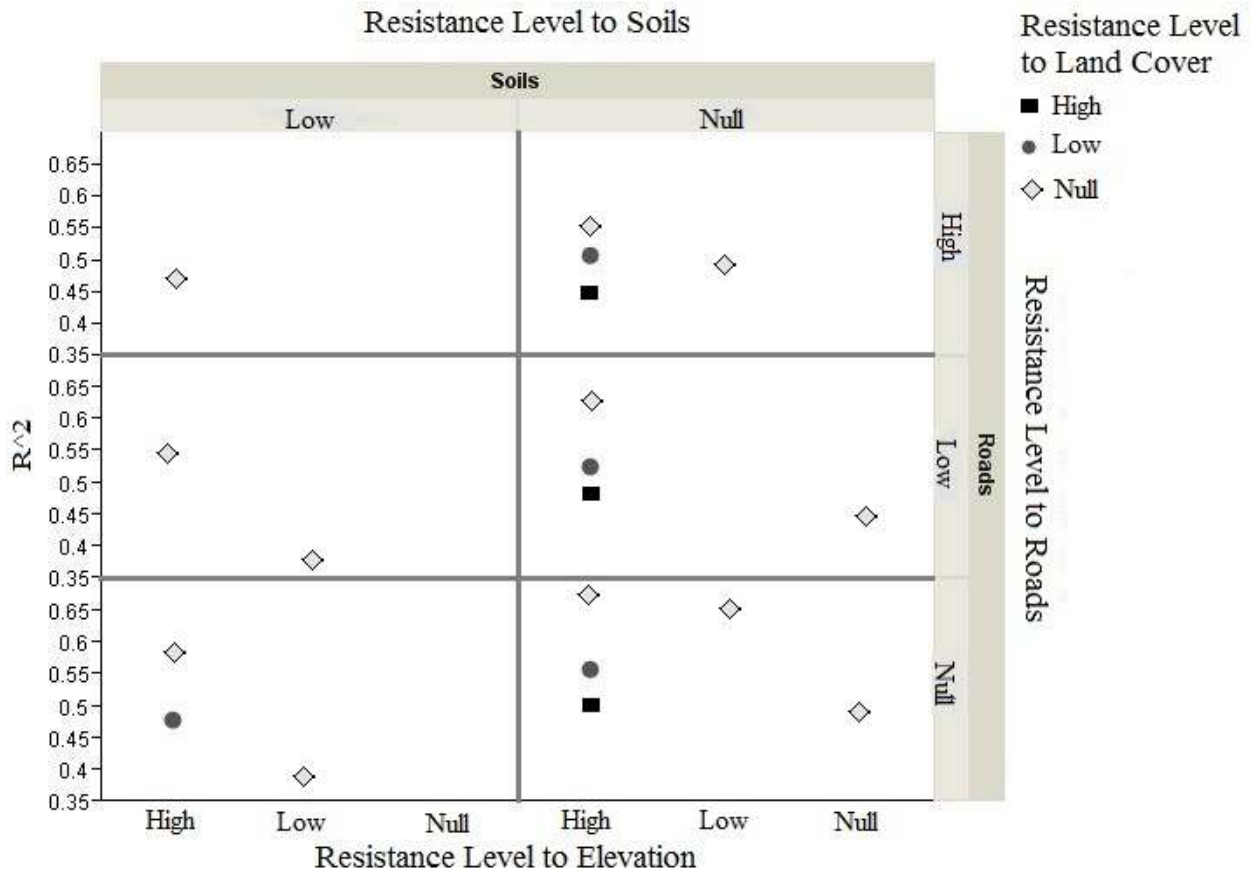
A simple Mantel test revealed a significant effect of geographic isolation on genetic distance ( $R^2 = 0.58$ ,  $p = 0.0001$ ). In contrast, there was no significant association between the Chickasawhay River as a barrier and pairwise genetic structure after controlling for straight-line distance between sites ( $R^2 = 0.23$ ,  $p = 0.20$ ). Seventy nine out of 81 landscape resistance surfaces estimated using IBR were correlated with genetic distance, when controlling for the effects of the Chickasawhay river. In contrast, only 19 landscape surfaces were significantly related with genetic distance after controlling for the effects of straight-line distance ( $R^2 = 0.39-0.69$ ,  $p < 0.05$ , Figure 5). These results supported the statistical expectations of only one of the seven causal models, model 5 (Table 2). This model predicted that pairwise genetic distances are influenced by both straight-line distance and landscape resistance distances with no significant effect of the Chickasawhay River. The top 19 significant landscape surfaces were ranked by the value of their respective correlation coefficients. The highest supported landscapes were found to be associated with high resistance to low elevation and either high or low resistance to major road ways (Figure 5). There was also some support for low resistance to unsuitable soils and resistance to land cover types, although support for the latter was spread across all three cost levels.

Table 2 Results of the partial Mantel test using IBR to evaluate the seven models of causality. An asterisk indicates the only model fully supported. Terms in bold are where a significant association was detected. The null hypothesis was rejected for a statistical expectation if any of the partial Mantel tests that evaluated the 81 landscape resistance surfaces were significant.

<b>Model 1</b>	<b>Expected</b>	<b>Observed</b>		<b>Model 5*</b>	<b>Expected</b>	<b>Observed</b>
BG.D	Sig	p>0.05		<b>L<sub>81</sub>G.B</b>	<b>Sig</b>	<b>79 out of 81 p&lt;0.05</b>
BG.L <sub>81</sub>	Sig	p>0.05		<b>DG.B</b>	<b>Sig</b>	<b>p&lt;0.0001</b>
DG.B	NS	p<0.0001		<b>L<sub>81</sub>G.D</b>	<b>Sig</b>	<b>19 out of 81 p&lt;0.05</b>
L <sub>81</sub> G.B	NS	79 out of 80 p<0.05		<b>DG.L<sub>81</sub></b>	<b>Sig</b>	<b>17 out of 81 p&lt;0.05</b>
<b>Model 2</b>				<b>BG.L<sub>81</sub></b>	<b>NS</b>	<b>p&gt;0.05</b>
<b>DG.B</b>	<b>Sig</b>	<b>p&lt;0.0001</b>		<b>BG.D</b>	<b>NS</b>	<b>p&gt;0.05</b>
<b>DG.L<sub>81</sub></b>	<b>Sig</b>	<b>17 out of 81 p&lt;0.05</b>		<b>Model 6</b>		
<b>BG.D</b>	<b>NS</b>	<b>p&gt;0.05</b>		BG.D	Sig	p>0.05
L <sub>81</sub> G.D	NS	19 out of 80 p<0.05		<b>L<sub>81</sub>G.B</b>	<b>Sig</b>	<b>79 out of 81 p&lt;0.05</b>
<b>Model 3</b>				BG.L <sub>81</sub>	Sig	p>0.05
<b>L<sub>81</sub>G.D</b>	<b>Sig</b>	<b>19 out of 81 p&lt;0.05</b>		<b>L<sub>81</sub>G.D</b>	<b>Sig</b>	<b>19 out of 81 p&lt;0.05</b>
<b>L<sub>81</sub>G.B</b>	<b>Sig</b>	<b>79 out of 81 p&lt;0.05</b>		DG.B	NS	p<0.0001
<b>BG.D</b>	<b>NS</b>	<b>p&gt;0.05</b>		DG.L <sub>81</sub>	NS	17 out of 81 p<0.05
DG.L <sub>81</sub>	NS	17 out of 81 p<0.05		<b>Model 7</b>		
<b>Model 4</b>				BG.D	Sig	p>0.05
BG.L <sub>81</sub>	Sig	p>0.05		BG.L <sub>81</sub>	Sig	p>0.05
<b>DG.L<sub>81</sub></b>	<b>Sig</b>	<b>17 out of 81 p&lt;0.05</b>		<b>DG.B</b>	<b>Sig</b>	<b>p&lt;0.0001</b>
BG.D	Sig	p>0.05		<b>DG.L<sub>81</sub></b>	<b>Sig</b>	<b>17 out of 81 p&lt;0.05</b>
<b>DG.B</b>	<b>Sig</b>	<b>p&lt;0.0001</b>		<b>L<sub>81</sub>G.B</b>	<b>Sig</b>	<b>79 out of 81 p&lt;0.05</b>
L <sub>81</sub> G.B	NS	79 out of 80 p<0.05		<b>L<sub>81</sub>G.D</b>	<b>Sig</b>	<b>19 out of 81 p&lt;0.05</b>
L <sub>81</sub> G.D	NS	19 out of 80 p<0.05				



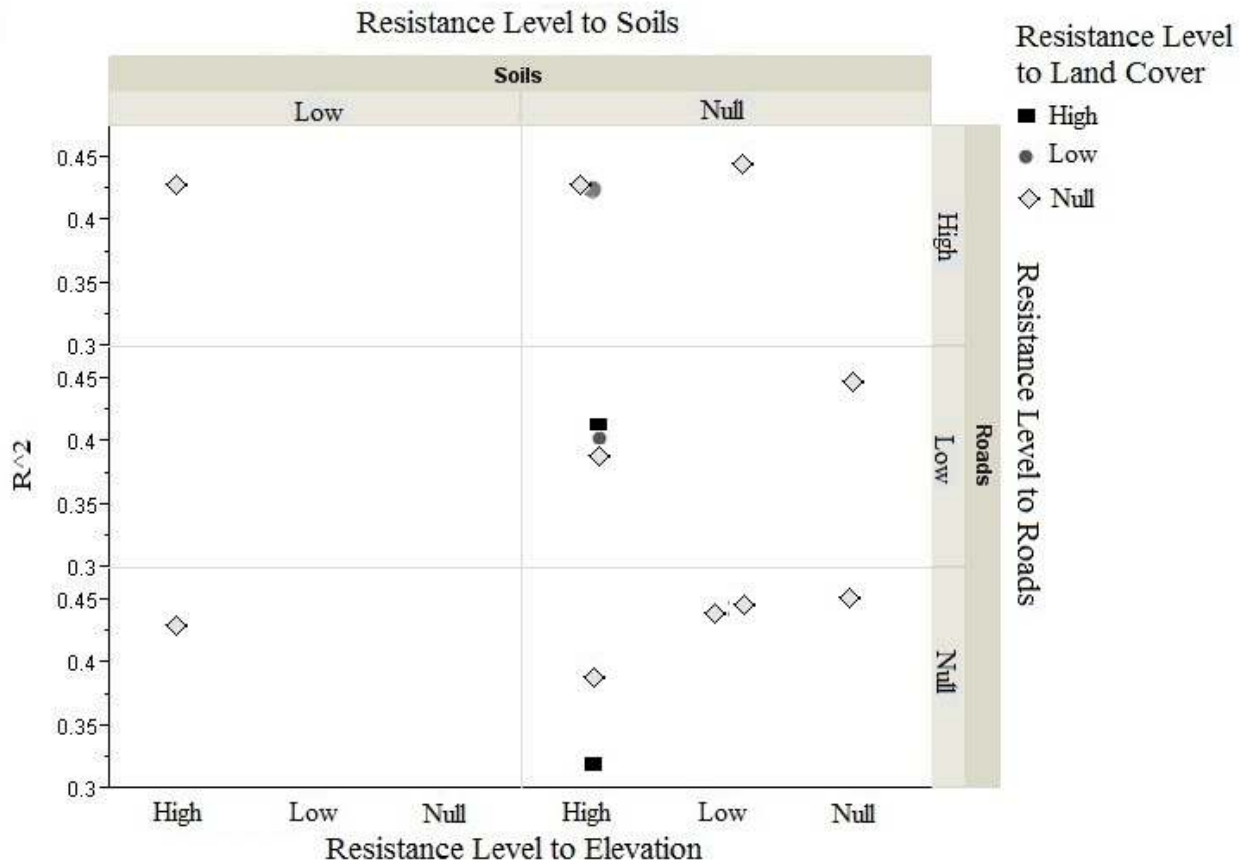
Figure 5 A plot of the 19 landscape resistance surfaces found to be significant after the influence of straight-line distance was removed. Each point represents the coefficient of determination ( $R^2$ ) for a single landscape resistance surface plotted by the four landscape features examined (elevation, roads, soils, and land cover), as well as the three resistance levels: high, low, and null. Only two levels are shown for soils because high resistance to unsuitable soils was not found to be a significant factor in any model. The landscape resistance surface with the highest  $R^2$  value is located in the lower right plot displaying high resistance to elevation and no resistance to soil, roads or land cover.



Only 14 out of the top 19 IBR landscape resistance surface models were significant using LCP distances ( $R^2 = 0.32-0.45$ ,  $p < 0.05$ ; Figure 6). In this case, the highest supported landscape surface was composed of null models for all four landscape variables and resulted in essentially Euclidean distances between populations. The remaining significant landscape surfaces showed support for the same variables as the IBR models: high resistance to low elevation, resistance to roads at both the low and high level and some support for low resistance to soils. Overall correlation coefficients were much lower for models based on LCP distances compared to those based on IBR measures. Furthermore, a simple linear regression showed that the LCP distances

between sample sites were highly similar to straight-line distances ( $R^2 = 0.97$ ;  $p < 0.0001$ ), while IBR distances were less correlated with Euclidean measures ( $R^2 = 0.85$ ;  $p < 0.0001$ ).

Figure 6 A plot of the 14 landscape resistance surfaces found to be significant for LCP distances after the influence of straight-line distance was removed. Each point represents the correlation coefficient for a single resistance model plotted by the four landscape features examined (elevation, roads, soils, and land cover), as well as the three resistance levels: high (H), low (L), and null (N).



Results from the backwards step-wise MRDM analysis using IBR distances between sites indicated that low resistance to low elevation and high resistance to roads best explained the observed genetic distance ( $R^2 = 0.72$ ,  $p = 0.001$ ). The forward regression supported the same two landscape variables, roads and elevation, but with support for the high rather than low resistance elevation model ( $R^2 = 0.76$ ,  $p = 0.001$ ). A backwards step-wise MRDM analysis of landscape variables based on LCP distances found only straight-line distance to be significant ( $R^2 = 0.34$ ,  $p = 0.001$ ). In contrast, a forward regression approach supported only high resistance to low elevation ( $R^2 = 0.34$ ,  $p = 0.001$ ) but not roads.

## DISCUSSION

This study is the first to use a causal modeling approach to examine the influence of landscape resistance surfaces on gene flow using electrical circuit theory. By constructing models of causality *a priori*, this study was able to evaluate the relative importance of straight-line distance, a putative riverine barrier (the Chickasawhay River), and four major landscape features on levels of genetic differentiation between populations across the federally listed range of the gopher tortoise. There was strong support for the effects of both straight-line distance and landscape resistance gradients on genetic distance between sample sites. In contrast to previous observations, the Chickasawhay River did not appear to constitute a substantial barrier to gene flow. It appears that the low elevation of the riparian area rather than the rivers itself was responsible for the observed genetic differentiation in the western portion of the range. Landscape resistance matrices examined using both partial Mantel and multiple regression analysis also indicated that low elevations and roads present significant barriers to gene flow. While the effects of elevation were best supported by the high resistance model, the effects of roads were significant regardless of the magnitude of resistance. There was also a small amount of support for low resistance to unsuitable soils but the majority of support for unsuitable land cover types was at the null resistance level. This suggests that the present distribution of land cover types may not affect contemporary genetic structure in this species as much as other landscape features.

Resistance distances measured between sampling sites using IBR outperformed landscape resistance models based on LCP measures. When compared to a simple model of IBD, the coefficient of determination improved by 11% for the highest supported IBR model after controlling for the effects of straight-line distance and by 20% compared to the highest supported LCP model. LCP distances were very similar to straight-line distances between sample sites and generally failed to explain genetic distance better than a simple model of IBD. In contrast, distance measures based on IBR were less correlated with Euclidean distances and explained a greater portion of the genetic differentiation between sample sites. Similar results were noted by McRae and Beier (2007) who found that distance measures based on electrical circuit theory outperformed IBD and LCP models. Furthermore, partial Mantel tests and MRDM provided

support for the same landscape variables as determinants of population structure, providing further support for low elevation and roads as barriers to gopher tortoise movement. The correlation between genetic and straight-line geographic distances was also high, suggesting that the limited dispersal capability of the gopher tortoise is a major factor contributing to overall population structure. However, even in the best model at least 30% of the genetic variance between sample sites still remains unexplained after accounting for geographic distance and landscape features. This unexplained variance maybe due to important variables that were overlooked in the present study or simply due to the high variance in genetic distance estimates due to the limited number of loci used in the present study . It is also important to point out that these models do not take into account the demographic history of tortoise populations in the region, some of which may have undergone demographic bottlenecks and genetic drift, which might distort estimates of gene flow and population differentiation.

The landscape features identified as important to gopher tortoise population structure generally agreed with knowledge of species ecology (Auffenburg and Franz 1982; Diemer 1986; Baskaran et al. 2006). Behavioral avoidance of roads has been observed in other chelonian species (Shepard et al. 2008) and road mortality is reportedly high (Diemer 1986; USFWS 1987) supporting the hypothesis that major roads may present barriers to tortoise movement. Gopher tortoise populations are also known to prefer high elevation ridges rather than low elevation riparian habitat, which they tend to avoid (Baskaran et al. 2006).

Interestingly, some landscape features thought to strongly influence gopher tortoise movement did not seem to have any significant effect in the present study. Previous studies have suggested that habitat quality influences tortoise movement (Diemer 1986). Much higher population densities are commonly observed in open canopy habitat as compared to agricultural areas, hardwoods and pine plantations (Hermann et al. 2002; Baskaran et al. 2006). However, this study was unable to determine any effect of land cover on tortoise movement. Human populations in this portion of the range have increased rapidly over the last 100 years (USFWS 1987) causing major changes in land cover. This failure to detect any effect of present land cover on genetic differentiation might be due to the long generation time of tortoises (40-60 years) and the lag time expected for molecular markers to track this disturbance. It could also be that the

spatial resolution used here for land cover (30 m<sup>2</sup>) was too fine a grain for effects of land cover to be detected at a regional scale. Lastly, it might be that the distribution of historical land cover rather than contemporary landscape patterns may better explain current population genetic structure this species, as has been observed in other studies (Poissant et al. 2005; Spear and Storfer 2008) and should be the focus of future work.

Sandy, well drained soils are another factor thought to be important to gopher tortoises (Hermann et al. 2002; Baskaran et al. 2006). This study found some support for low resistance to unsuitable soils but it was not a major driver of differentiation. It could be that soil type is not an important factor for tortoises when dispersing over long distances because they do not construct burrows during this time. In fact, Diemer (1992) noted that tortoises often use shallow depressions instead of burrows for shelter while making long-distance movements. It would therefore appear that some factors important to habitat selection and long-term viability of populations such as soil and land cover are not as important to dispersal. Similar discrepancies between habitat types that predict species distribution and landscape features influencing dispersal have also been observed in the literature (Lee-Yaw et al. 2009) and point to the importance of examining candidate landscape variables at both regional and fine spatial scales.

### *Conservation and Management*

This study has important implications for the conservation and management of gopher tortoises in the federally listed portion of the range. Dispersal is critical to maintaining gene flow between populations and attenuating the loss of genetic diversity in fragmented populations. Males travel long distances to find mating opportunities and sub-adults tend to emigrate away from dense populations to reduce competition (Eubanks et al. 2003). Identifying landscape features critical to regional dispersal could therefore provide significant information to conservation managers on the importance of major upland corridors to population connectivity through seasonal migrations, male dispersal, and by females seeking nest sites.

The methods employed in this paper identified the influence of a major anthropogenic feature (roads) on dispersal. It is no surprise that major roads are an impediment to gopher tortoise dispersal since they affect movement in many other species (Cushman et al. 2006;

Blanchong et al. 2007; Shepard et al. 2008) and are likely to have broad-scale regional effects due to the very large spatial distances they cover (Balkenhol and Waits 2009). As habitat loss and fragmentation continue throughout the southeast, preserving potential routes of dispersal between populations will be important to the long-term preservation of the species. Similarly, an understanding of the influence of natural landscape features on population structure will also be important when selecting candidate populations for translocation efforts.

While this study identified landscape features important to movement at a regional level, these factors may not be the same at the local level. In fact, Lee-Yaw et al. (2009) found that for wood frogs (*Rana sylvatica* or *Lithobates sylvaticus*) the landscape variables that explained fine scale dispersal were not the same variables that influenced regional patterns of genetic structure. Features like roads may also vary in their degree of permeability. Although interstate highways appear to constitute barriers to gene flow, smaller, rural roads may actually provide good habitat and promote movement (McRae et al. 1981; Baskaran et al. 2006). Evaluating the influence of more subtle features such as minor roads, local habitat connectivity and gender-specific biases in gene flow is better addressed at a smaller scale. Future work should therefore employ landscape genetic approaches to explore features that influence tortoise movement at finer spatial scales and in so doing potentially identify management practices at the individual level that may impact local dispersal and connectivity of adjacent populations.

## CHAPTER FOUR: Fine-scale spatial genetic structure and landscape connectivity in the gopher tortoise (*Gopherus polyphemus*)

### INTRODUCTION

Habitat fragmentation is one of the greatest threats to wildlife populations (Myers 1997). Fragmentation creates unnatural landscape heterogeneity and creates unsuitable habitat types that constitute barriers to movement (Noss and Csuti 1997). Negative effects of habitat fragmentation may also disrupt fine-scale ecological processes such as local dispersal. This is because individuals must move among suitable patches of habitat to acquire the resources they need. However, dispersal ability over the landscape is not only determined by the distance between suitable habitat patches but also by their level of connectivity to one another (Taylor et al. 1993). Over time, barriers that reduce connectivity between populations or individuals will cause a reduction in gene flow that may ultimately lead to genetic divergence, inbreeding and loss of genetic diversity within remaining fragments. Methodologies from the field of landscape genetics can be used to evaluate landscape connectivity and identify landscape features that impede or facilitate gene flow. This data can then be incorporated into conservation planning for species that have undergone extensive fragmentation (Taylor et al. 1993).

In many cases landscape features are examined only at the regional level using data collected from pre-defined populations. This approach has the potential to bias results because groupings are somewhat arbitrary and populations may in fact be more continuously distributed across the landscape (Schwartz and McKelvey 2009). One issue with this type of discrete sampling is that it can influence  $F_{ST}$  values and lead to inappropriate conclusions concerning the presence of putative barriers (Schwartz and McKelvey 2009). Conversely, several factors make individual-based genetic studies particularly amenable to resolving landscape factors impacting local dispersal. Firstly, the dense, uniform sampling required for individual-based studies makes no *a priori* assumptions about local relatedness structure (Storfer et al. 2006). This allows for a more unbiased analysis of fine-scale genetic structure that can then be correlated to the presence of specific landscape features. Secondly, genetic analyses of fine-scale movement can also reveal patterns that are difficult to observe using traditional field methods, which are often constrained by small sample sizes, extensive field work, equipment costs, and small study areas. Ecological

studies of species movement also tend to underestimate lifetime dispersal distance because long-distance movements are often missed and for long-lived species the time span of studies is too limited (Koenig et al. 1996; Segelbacher et al. 2010). Also, direct observations of dispersal rarely attempt to correlate potentially important landscape features with species movement and thus can only give limited insight into the effects of environmental heterogeneity on dispersal. Lastly, individual-based genetic studies can be used to statistically test for evidence of sex-biased dispersal (Favre et al. 1997; Mossman & Waser 1999) and in so doing provide important information on differences in gene flow between sexes.

Individual-based genetic approaches have traditionally been used to assess local patterns of dispersal and spatial genetic structure (Rousset 2000; Peakall et al. 2003; Vekemans and Hardy 2004; Double et al. 2005). However, this approach is also increasingly being used to detect landscape features that promote or inhibit local movements (Coulon et al. 2004; Broquet et al. 2006; Gauffre et al. 2008; Moore et al. 2008). Since anthropogenic land use changes have been shown to negatively influence species movement (Scribner et al. 2005), understanding how gene flow is related to landscape cover can be critically important to the management of threatened and endangered species. For example, using this approach Broquet et al. (2006) found that logged landscapes altered dispersal in the American marten (*Martes americana*) and disrupted the natural isolation-by-distance pattern found in unlogged landscapes. Similarly, an individual-based approach was also able to show that the distribution of wooded patches significantly explained pairwise genetic distance among roe deer (*Capreolus capreolus*) (Coulon et al. 2004). Studies such as these not only highlight the effectiveness of individual-based data at delineating landscape features important to gene flow but also illustrate how human mediated habitat alterations may impact local genetic structure.

Although the number of landscape genetic studies continues to increase (Holderegger and Wagner 2006) very few studies have evaluated landscape influences on dispersal patterns for reptiles using molecular data (Moore et al. 2008). Reptiles make good models for examining the influence of landscape features on individual gene flow because of their limited dispersal capabilities and often specialized habitat requirements (Gibbons et al. 2000). These same attributes also make reptiles particularly vulnerable to habitat loss and fragmentation, which has



been the cause of numerous population declines within this taxonomic group (Gibbons et al. 2000). These aforementioned factors indicate it should be possible to evaluate the effect of landscape variables on gene flow for reptiles and improve fine scale management.

The gopher tortoise has experienced immense loss in habitat throughout its range due to expanding urbanization and a continuing increase in pine plantations (USFWS 1987). However, in some areas this species is still locally numerous and continuously distributed, permitting an individual-based sampling approach. Furthermore, this species possesses many of the previously discussed attributes that make reptiles especially vulnerable to local habitat fragmentation and extinction, including limited dispersal, low fecundity and specific habitat requirements (Diemer 1986). Previous studies that examined empirical movement have shown that dispersal is limited (< 1 km) and that home range sizes differ among sexes, with males typically utilizing larger areas and dispersing greater distance overall (McRae et al. 1981; Diemer 1992; Eubanks et al. 2003). Furthermore, a recent habitat suitability analysis revealed that many land cover types associated with anthropogenic changes in land use are negatively related to gopher tortoise presence (Baskaran et al. 2006). Therefore, I predict that genetic data can be used to evaluate the influence of landscape features on fine scale population structure for this species. A recent analysis of regional population structure in the gopher tortoise found a significant pattern of isolation by distance among populations and evidence that areas of low elevation and major roadways impede gene flow (Clostio et al. *in prep*). However, Lee-Yaw et al. (2009) found that landscape variables influencing genetic variation at the regional level were not the same as those explaining population structure at a finer scale for wood frogs, indicating that this might also be true for other species such as the gopher tortoise.

Currently little is known about determinants of local spatial genetic structure and how fine-scale fragmentation may impact species ecology and movements for the gopher tortoise. The objectives of the following study were therefore to use an individual-based approach to: 1) investigate fine scale population structure and detect genetic discontinuities using a Bayesian assignment method (Pritchard et al. 2000); 2) Test for male-biased patterns of gene flow; 3) Examine data for evidence of fine-scale spatial genetic structure and spatial autocorrelation; 4) Determine if an isolation-by-distance pattern of gene flow exists at the individual level and 5)

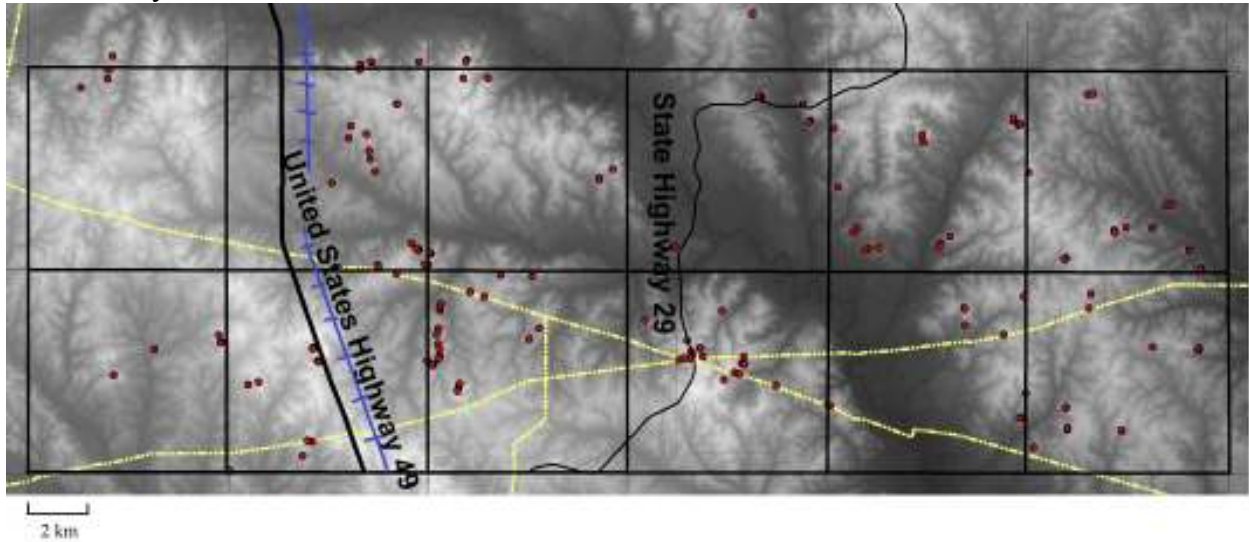
evaluate the influence of anthropogenic and natural landscape features on local dispersal patterns using the recently introduced method of isolation by resistance (McRae 2006). Specifically, I wanted to test the hypotheses that fine scale dispersal is facilitated by suitable soils and open canopy habitat but impeded by areas of low elevation, rapid changes in slope, a riverine barrier, major roads and land cover types related to anthropogenic habitat alteration. The influence of these landscape features on habitat suitability and potentially movement for gopher tortoises was previously described in Chapter 3.

## **METHODS**

### *Study Area*

The study area covered a 14 x 35 km area within the Forrest and Perry Counties of the Desoto National Forest (DNF), located in southern Mississippi (Figure 1). Although the majority of this area is composed of national forest land, the forest is fragmented by pasture, clear cut areas, pine plantations and developed lots. The topology of the area consists of rolling hills with southern pine ridges scattered throughout. The elevation in the study area ranged from 18 to 101 meters and the area is bisected by the Black Creek National Scenic River which has a base discharge of 3,500 cubic feet per second and a mean gauge height of 4.95 feet from 2007 to 2008 (max: 14.28 ft). Two major roadways, U.S. highway 49 and State Highway 29, also pass through the study area. U.S. 49 is a major six lane roadway built in 1928 with a wide median between the north and south bound lanes and wildlife fencing along some corridors.. Mississippi highway 29 is a smaller, two lane roadway that receives a considerable amount of traffic but is flanked by a grassy buffer zone. Two gas transmission right-of-ways that are frequently mowed also cross the study area and may facilitate tortoise movement (Baskeran 2009).

Figure 1 Topographical map of the study area within the Desoto National Forest, Mississippi. Major roads (black lines), gas transmission right-of-ways (yellow dashed lines), railroads (blue crosshatched line) and the Black Creek River drainage (dark area indicating low elevation) are depicted. The location of the 12 sampling quadrants and the 169 individuals sampled (red dots) for the study are also shown.



#### *Genetic sampling and analysis*

Gopher tortoise samples were collected in the fall of 2008 and the summer of 2009 by placing Tomahawk® live traps at the entrance of tortoise burrows and then drawing 1 to 2cc of blood from the subcarapacial venous sinus or the brachial. Data on the location of tortoise burrows within the study area were obtained from surveys conducted in 2005 and 2007 by the Federal Forest Service. The sex of individuals was determined by measuring the concavity of the plastron and the length of the carapace (McRae et al. 1981; Aresco & Guyer 1999). Trapping locations for each individual were recorded using a handheld GPS (Garmin). To ensure uniform sampling throughout the study region, the total area was divided into 12 quadrats measuring 7 x 7 km each with the goal of collecting samples from 15-20 individuals within each quadrat. DNA extraction was carried out as previously described in Chapter 2. A set of 10 microsatellite loci, also described in Chapter 2, were used to examine the spatial distribution of genetic variability. Loci were tested for deviations from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE) using ARLEQUIN 3.1 (Excoffier 2005).

### *Statistical analysis*

Population structure within the study area was assessed using a Bayesian clustering method implemented in the program STRUCTURE (Pritchard et al. 2000). Values of K ranging from 1 to 5 were examined to identify the most likely number of population clusters given the data. A burn-in period of 100,000 was used, followed by 1,000,000 MCMC iterations. A total of 20 runs were conducted for each value of K. An ancestry model allowing for admixture and correlated allele frequencies between populations was used because it better allows for the detection of subtle population substructure (Falush et al. 2003).

To examine genetic data for evidence of sex-biased dispersal, an assignment test was conducted in GENEALEX 6 (Peakall and Smouse 2006) using the test procedure developed by Favre et al. (1997). In this method, a log likelihood assignment index (AI) is used to calculate the probability that an individual's genotype originated from a given sampled population given the allele frequencies of that population (Paetkau et al. 1995; Mossman & Waser 1999). These log transformed AI values were then adjusted to control for population effects by subtracting them from the population means to calculate the assignment correction index (AIC) for each individual. Using this test, it is possible to distinguish immigrants from residents because individuals with a negative assignment index are potential migrants from other areas. If male gopher tortoises disperse greater distances than females then it is predicted that they will have a lower mean assignment index and higher variance in this index compared to females. Cases where gender could not be assigned to adult tortoises or tortoises that had not reached maturity were excluded from sex based analyses.

An analysis of spatial genetic structure was carried out in GENEALEX 6 using the spatial autocorrelation method of Smouse and Peakall (1999) developed for multilocus genotype data. Spatial autocorrelation analyses assess genetic similarity between pairs of individuals for different distance classes to determine the degree of autocorrelation present in the data and the distance to which it extends. To test for statistical significance of the observed autocorrelation coefficient ( $r$ ), 999 permutations of the individual genotypes among the geographic locations were carried out. The resulting autocorrelation coefficients generated through permutation ( $r_p$  values) were then used to construct 95% confidence intervals for each distance class. In order to

do this, a total of 1000 bootstrap replicates of the data were conducted within each distance class to define a 95% confidence interval around  $r$ . If the confidence interval for each distance class contains zero the null hypothesis of no spatial genetic structure cannot be rejected. If positive spatial autocorrelation is observed the first x-intercept can be used to determine the maximal extent of genetic structure. A one-tailed test was also performed by estimating the probability of obtaining a permuted  $r_p$  value greater than or equal to the observed  $r$  value. In this case, if  $p < 0.05$  then the hypothesis of positive spatial structure is accepted. As the ability to detect nonrandom spatial genetic structure is affected by the size of the distance class used and the number of samples per distance,  $r$  was calculated for a range of distance class sizes from 1 km to 6 km at increasing increments of 1 km, as suggested by Peakall et al. (2003). To determine if variation in spatial genetic structure existed between sexes, data for males and females were also analyzed separately.

The genetic distance between individuals ( $a_r$ ), as defined by Rousset (2000) was computed using SPAGEDi 1.3 (Hardy and Vekemans 2002). Because dispersal patterns differ between male and female gopher tortoises (McRae et al. 1981; Eubanks et al. 2003), three pairwise matrices of inter-individual genetic distances were calculated: a dataset including all individuals, a dataset with only adult males, and a dataset including only adult females. To determine if the average genetic distance between males and females was significantly different, a t-test was carried out in the R package (R Development Core Team 2009).

Euclidean distance (in meters) between pairs of individuals was calculated in SPAGEDi using Universal Transverse Mercator (UTM) coordinates of the original capture site for each individual. The effects of isolation by distance were assessed by examining the relationship between pairwise genetic distance ( $a_r$ ) and Euclidean distance using a Mantel test (1967) in R package. The effect of a riverine barrier, Black Creek River, on inter-individual genetic distance was examined using a partial Mantel test (Smouse 1986). The indicator matrix was constructed in a binary form where the value (0) was given to individuals not separated by the river and the value (1) to individuals either side of the Black Creek River.

Based on habitat suitability data (Baskaran et al. 2006) and previous studies of individual movement (McRae et al. 1981; Eubanks et al. 2003), six landscape features were identified that might influence fine-scale dispersal: elevation, slope, roads, percent canopy cover, land cover and soils. Landscape resistance surfaces for each feature were generated in ArcEditor 9.1 (ERSI). Based on ecological knowledge of this species, the highest cost values were given to cells containing areas of: 1) low elevation, 2) large changes in slope, 3) major roadways, 4) dense canopy cover, 5) unsuitable soils that are poorly drained and flood frequently and 6) unsuitable cover types comprising pine plantations, clear cuts, hardwood forests, developed areas, and wetlands. As the level of difficulty tortoises face with each landscape feature is unknown, each of these six feature types were modeled across six levels of maximum resistance 5, 10, 20, 50, 75 and 100.

The landscape resistance surfaces constructed for elevation and slope were derived from a 1-arc second (~30 m) resolution national elevation dataset (NED) acquired from the U.S. Geological Service (<http://seamless.usgs.gov>). Elevation resistance values were standardized using an inverse linear function with the areas of highest elevation receiving the lowest resistance value of one and resistance values subsequently increased with decreasing elevation. Slope data was derived from elevation using the 'slope' function in the Spatial Analyst extension of ArcEditor 9.1 and the appropriate Z factor of 0.00003 was used to calculate slope degree from the NED layer. Areas with the lowest degree of slope were assigned a resistance value of one and resistance increased linearly with increasing slope up to the maximum resistance value.

The GIS data layer depicting roadways was obtained from the U.S. Census Bureau TIGER 2000 line data (<http://www.census.gov/geo/www/tiger/>). Interstate 49 bisects the study site and was presumed to be the greatest barrier to dispersal and was given the highest resistance value, followed by state highways, paved roads and then unpaved roads, which had the least resistance. As minor roads have been shown to promote gopher tortoise movement (Eubanks et al. 2003; Baskaran et al. 2006), all unpaved forest service roads within the study area were assigned the lowest value of one while the surrounding habitat was given a value of two. A single railway which crossed the study area parallel to the interstate was assigned an intermediate

resistance value. In contrast, two utility swaths bisecting the study area are likely to promote dispersal (Baskaran et al. 2006) and were assigned a value of one.

Canopy cover gradients were generated using a 30 m resolution National Land Cover Database 2001 tree canopy layer from the U.S. Geological Service (<http://www.mrlc.gov>). In this layer, tree canopy refers to the layers of foliage at the tops of trees and density was measured using the method of Huang et al. et al. (2001). Landscape resistance surfaces for percent canopy density were developed using a linear function, where areas with density values of zero were assigned a resistance value of one with resistance values increasing with canopy cover up to the maximal resistance value.

A 30 m resolution land cover layer was obtained from the Southeast Region Gap Analysis Program ([http://www.basic.ncsu.edu/segap/datazip/region/lc\\_segap.zip](http://www.basic.ncsu.edu/segap/datazip/region/lc_segap.zip)). The study area contained 18 land cover types including sections of high intensity development, mesic hardwood forest, managed pine, clear cut, pasture, row crops, utility swaths and upland longleaf pine. Areas of longleaf pine as well as other open canopy land cover types such as utility swaths, pastures, and grasslands receiving low resistance values. Developed areas and sections of mesic forest received the highest resistance values while managed pine, clear cut and row crops were given more moderate costs.

Lastly with respect to soils, data was obtained from the U.S. Department of Agriculture, Natural Resources Conservation Service (<http://SoilDataMart.nrcs.usda.gov>). A resistance value of one was assigned to soil types classified as 'priority' by the U.S. Fish and Wildlife Service (2005) and a resistance value of two was assigned to 'suitable' soil types. Resistance values then increased for marginal, foraging only and unsuitable soil types, respectively, up to the maximum resistance value. For categorical landscape variables, the magnitude of the resistance values assigned to favorable versus unfavorable features varied in proportion to the maximal resistance value for that series. For example, for maximal resistance values of 5, resistance levels used for soil type were 1, 2, 3, 4 and 5, while for a maximal resistance level of 10, the values 1, 2, 6, 8, and 10 were used.

The average resistance distance between individuals for each of the six landscape variables examined across each of the six resistance levels was calculated in CIRCUITSCAPE version 3.5 (McRae and Shah 2009). The effect of resistance distance on genetic distance ( $a_r$ ) while controlling for geographic distance was then assessed for each distance matrix using partial Mantel tests implemented in R package.

## RESULTS

Samples were collected from 169 tortoises throughout the study area, including 60 females and 97 males. The remaining individuals could not be sexed due to age (subadults). An average of 15 individuals was sampled across all 12 quadrants. Quadrants that contained sample sizes below this average often contained large amounts of riparian habitat not suitable for gopher tortoises or private lands that were not easily accessible for sampling. A few individuals were sampled adjacent to the study quadrant (<500m) and one individual was opportunistically encountered 2km outside the sampling area.

A total of 158 individuals were successfully genotyped with less than 0.3% missing data for the entire dataset. Two loci were found to be monomorphic (Gp26 and Gp14) and were subsequently removed from the dataset. Of the remaining eight loci, only one (Gp201) was found to deviate from HWE after Bonferroni correction. Three locus pairs out of twenty-eight showed evidence of linkage disequilibrium after Bonferroni correction. Previous studies have shown that null alleles were the cause of HWE deviations for one locus (Gp201) so this locus was removed from further analysis. The mean observed heterozygosity across all remaining loci (0.50) was slightly lower than the expected heterozygosity (0.54) and the number of alleles per locus ranged from 2 to 13 with a mean of 4.5 alleles. Results from STRUCTURE showed that there was no evidence of population differentiation within the study area and the highest likelihood given the data was found for  $K=1$ .



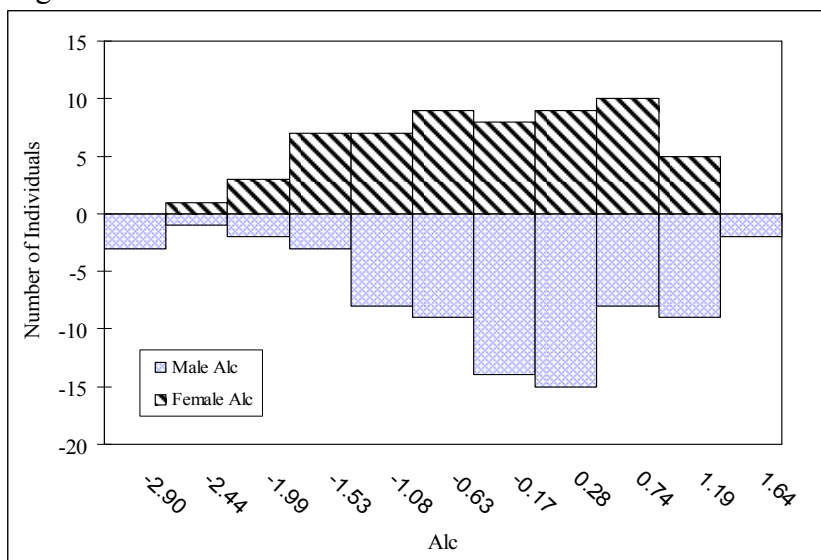
Table 1 The 10 microsatellite loci amplified for the study including the repeat motif, the number of alleles ( $N_A$ ), observed and expected heterozygosity

Locus	Repeat motif	$N_A$	$H_{obs}$	$H_{exp}$
Gp5	4	13	0.79	0.8
Gp12	4	4	0.71	0.63
Gp14	4	1	n/a	n/a
Gp15	2	7	0.69	0.71
Gp26	2	1	n/a	n/a
Gp30	2	4	0.14	0.15
Gp96*	2	2	0.47	0.48
Gp102	2	5	0.47	0.51
Gp105	2	5	0.67	0.68
Gp201*	2	3	0.08	0.35
Mean		4.5	0.50	0.54

\* Indicates loci that significantly deviated from HWE

Males did not have significantly lower AIC values ( $0.06 \pm 0.13$  SE) than females ( $-0.07 \pm 0.12$  SE) (Mann Whitney U-test,  $U = 1984.5$ ,  $p > 0.05$ ). Although the variance in AIC values was higher in males (1.14) than that for females (0.98) this difference was not significant (F ratio test,  $F = 0.87$ ,  $p > 0.05$ ). Only 38% of males were in the negative portion of the assignment distribution whereas 52% of females had negative AIC values (Figure 2).

Figure 2 Frequency distribution of corrected assignment indices (AIC) for male (bottom) and female (top) gopher tortoises. Negative assignment indices indicate individuals that are possible migrants.



The results of the spatial autocorrelation analysis for distance classes ranging from 1 km to 6 km are shown in Table 2. The one-tailed test indicated no evidence of positive spatial genetic structure. Spatial correlograms for distance classes of 2km and 6km are illustrated in Figure 3a and b, respectively. The correlogram for the larger distance class sizes (Figure 3b) shows a positive relationship between distance (km) and  $r$  over short distances although these effects were not significant. All correlograms showed oscillations of high and low autocorrelation, indicating that gopher tortoises occur in high density clusters with intervening areas of lower density (Peakall et al. 2003).

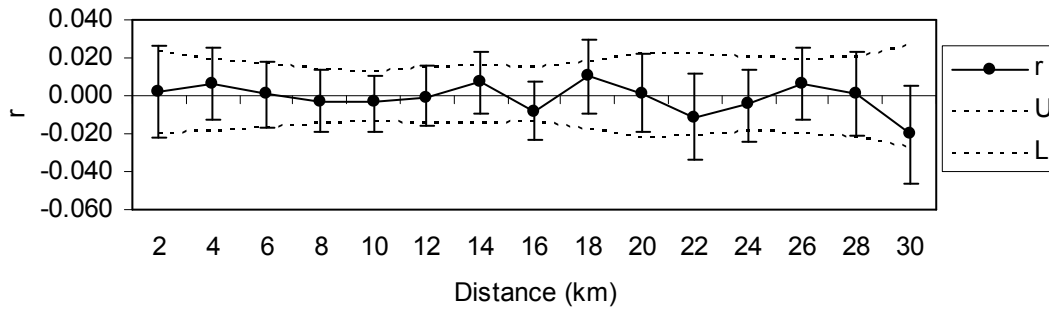
Table 2 Spatial autocorrelation results for the total gopher tortoise dataset for distance classes of 1 to 6 km. The autocorrelation coefficient  $r$  is shown only for the first distance class in each analysis.

Distance Class Size	1km	2km	3km	4km	5km	6km
n	262	563	841	1264	1674	2092
r	0.014	0.002	0.003	0.004	0.002	0.003
U	0.035	0.023	0.016	0.014	0.012	0.021
L	-0.030	-0.020	-0.015	-0.013	-0.011	-0.023
P	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Ur	0.049	0.026	0.022	0.019	0.015	0.013
Lr	-0.021	-0.023	-0.016	-0.010	-0.010	-0.008
Intercept	1.643	6.169	8.156	6.926	7.263	9.507

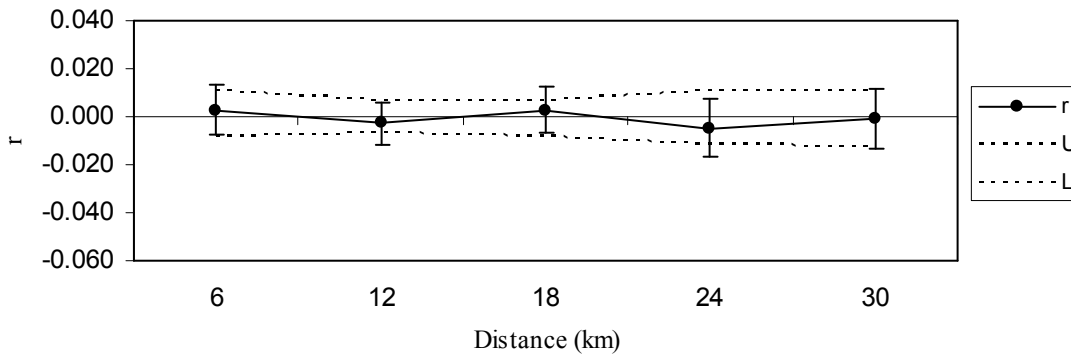
\*  $n$  the number of pairwise comparisons for  $r$ , the upper ( $U$ ) and lower ( $L$ ) bound for the 95% confidence intervals as determined by permutation, the p-value ( $P$ ) for the one-tailed test of positive genetic structure, the upper ( $Ur$ ) and lower ( $Lr$ ) bound of the 95% confidence interval constructed using the bootstrapping method, and the x-intercept.

Figure 3 Correlograms constructed for the total dataset displaying spatial correlation  $r$  for (a) 2 km and (b) 6 km distance classes including 95% confidence intervals (dotted lines) about the null hypothesis of random spatial genetic structure and the 95% error bars about  $r$  determined by bootstrapping.

a)



b)



Spatial autocorrelation analyses for separate sexes also showed no significant pattern of fine-scale spatial genetic structure (Table 3). The one-tailed tests also showed no evidence of positive spatial structure at shorter distances; although, a positive trend between distance and  $r$  values was observed for separate male and female datasets over short distances.

Table 3 Spatial autocorrelation results for the male and female gopher tortoise dataset for analysis with distance classes of 1 to 6 km. The correlation  $r$  is shown only for the first distance class in each analysis.

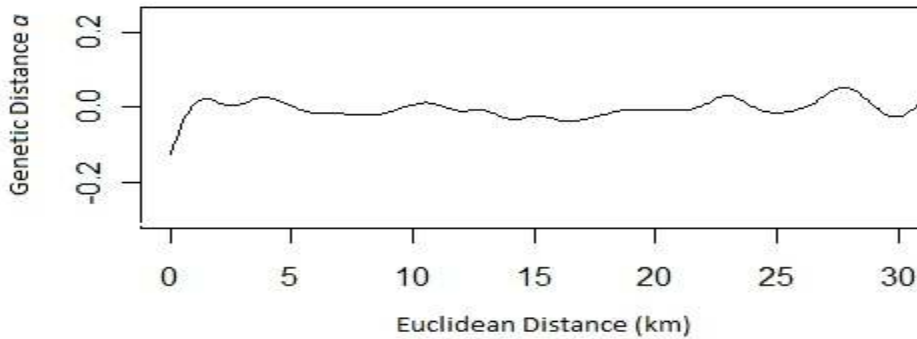
Distance Class						
Size	1km	2km	3km	4km	5km	6km
<b>Males</b>						
n	67	157	237	367	449	549
r	0.034	0.020	0.005	0.005	0.004	0.006
U	0.066	0.041	0.034	0.025	0.021	0.021
L	-0.058	-0.035	-0.028	-0.022	-0.019	-0.015
P	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Ur	0.105	0.069	0.040	0.033	0.030	0.030
Lr	-0.038	-0.029	-0.034	-0.023	-0.020	-0.015
Intercept	2.234	3.625	10.283	10.471	12.704	10.979
<b>Females</b>						
n	38	83	112	169	232	288
r	0.030	0.030	0.014	-0.007	-0.017	-0.022
U	0.057	0.057	0.046	0.038	0.034	0.028
L	-0.050	-0.050	-0.044	-0.033	-0.030	-0.024
P	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Ur	0.155	0.090	0.068	0.034	0.015	0.011
Lr	0.002	-0.024	-0.037	-0.047	-0.052	-0.041
Intercept	1.899	2.906	3.770	22.170	13.788	17.028

\*  $n$  the number of pairwise comparisons for  $r$ , the upper ( $U$ ) and lower ( $L$ ) bound for the 95% confidence intervals as determined by permutation, the p-value ( $P$ ) for the one-tailed test of positive genetic structure, the upper ( $Ur$ ) and lower ( $Lr$ ) bound of the 95% confidence interval constructed using the bootstrapping method, and the x-intercept.

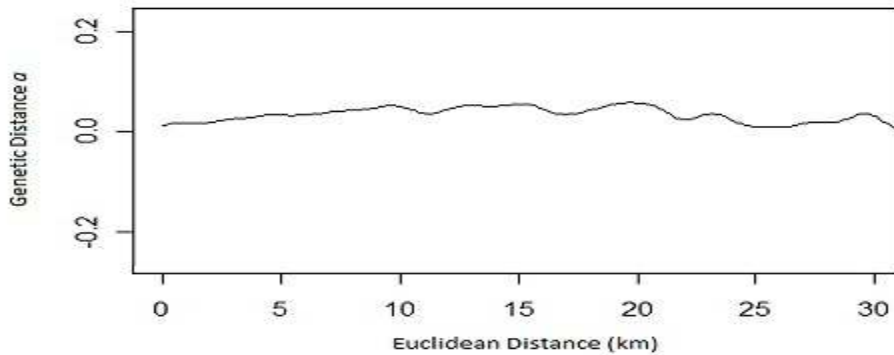
The mean genetic distance ( $a_r$ ) between females (-0.003) was significantly lower than that of males (0.04) as determined by a t-test ( $t = -7.1281$ ,  $p < 0.0001$ ). No pattern of isolation by distance was evident for individuals within the study area ( $R^2 = 0.005$ ,  $p = 0.42$ ) or for either males ( $R^2 = -0.01$ ,  $p = 0.60$ ) or females ( $R^2 = 0.03$ ,  $p = 0.26$ ) (Figure 4). However, a positive trend was observed for females up to ~3km (Figure 5a). A partial Mantel test used to determine the effect of Black Creek River on genetic structure while controlling for geographic distance was similarly non-significant for all three datasets.

Figure 4 Local polynomial regression constructed in R package to visualize the relationship between genetic ( $a_r$ ) and Euclidean distances among individuals for the female (a) and male (b) datasets using a bandwidth of 0.75 to smooth the curve.

a)

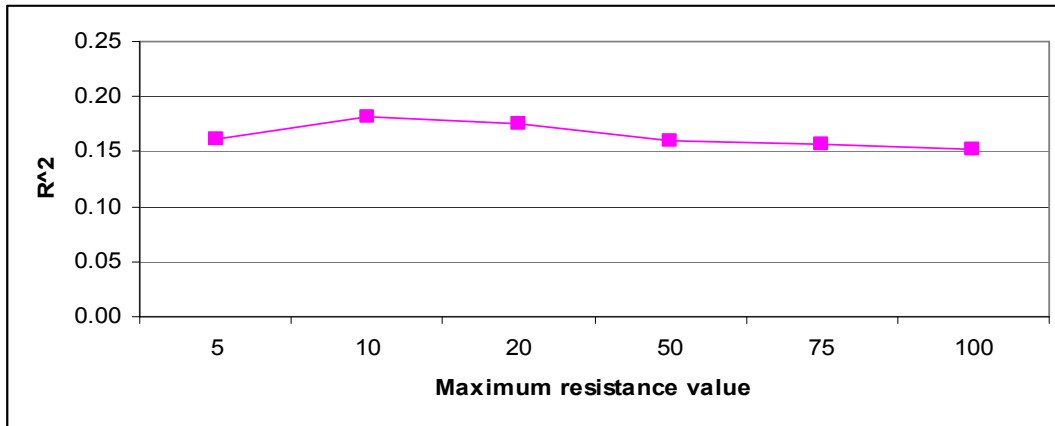


b)



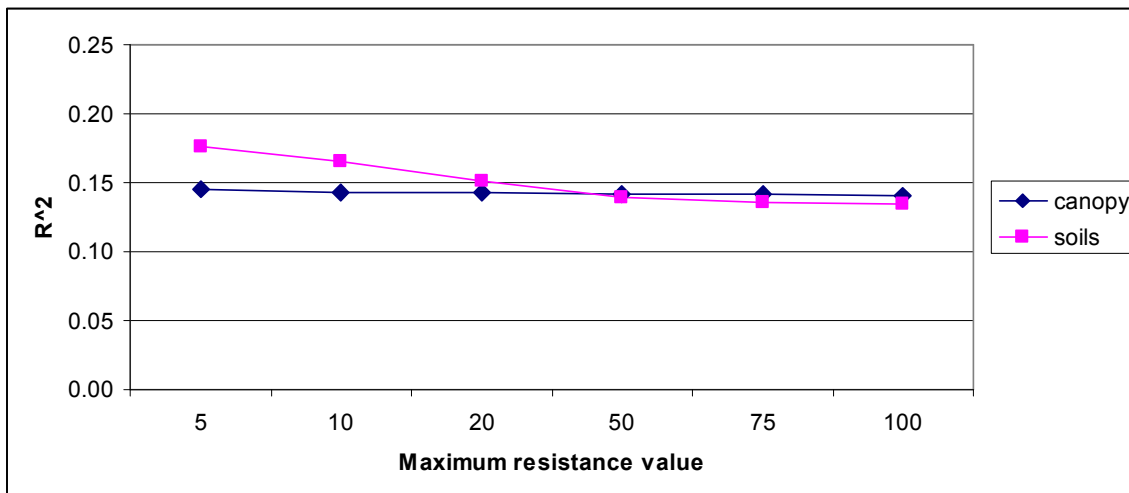
Soil type was the only landscape feature that had a significant effect on inter-individual genetic distances for the combined male and female datasets. The correlation between genetic distance and landscape resistance based on soils increased as the maximum resistance value increased, reaching a maximum for the highest soil resistance value of 100 (Figure 5). A partial Mantel test also found that soil was the only landscape variable that correlated significantly with genetic distance after controlling for straight line distance. Results from the partial Mantel, controlling for the effects of straight line distance, showed a stronger correlation between soil and genetic distance than the simple Mantel. The strongest correlation between genetic distance and soils was in this case observed for a maximum soil resistance value of 10 ( $R^2 = 0.18$ ,  $p = 0.0006$ ; Figure 5).

Figure 5 Coefficients of determination ( $R^2$ ) for partial Mantel tests of genetic distance and landscape resistance distance calculated for soil type at six maximum resistance levels, excluding the effects of Euclidean distance.



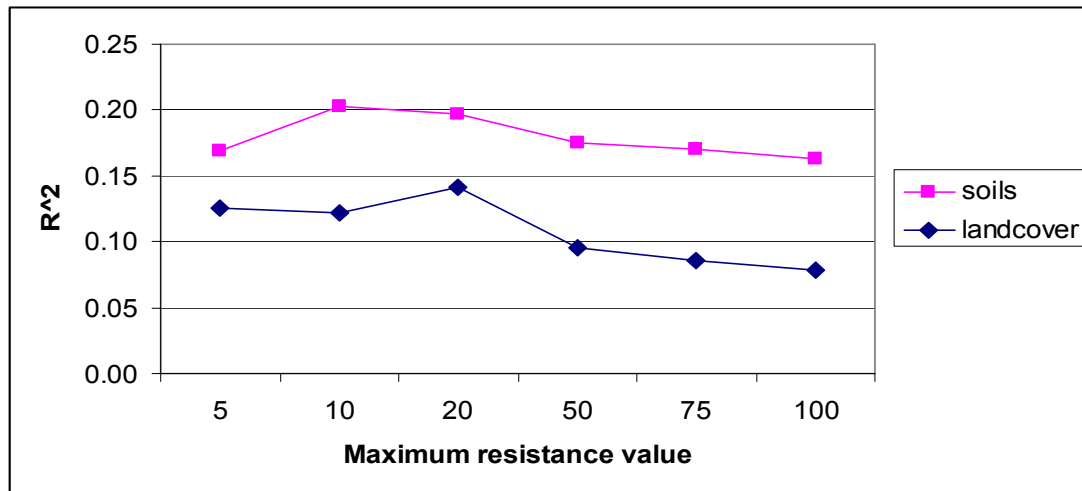
For the female dataset, Mantel tests indicated that both soils and canopy density were significantly correlated with inter-individual genetic differentiation. When a partial Mantel test was used to control for influence of Euclidean distance, the strongest correlation between genetic distance and either soil type or canopy was observed when the maximum resistance value was set to five (Figure 6). As observed for the total dataset combining sexes, soils showed the strongest relationship with genetic distance over any other landscape resistance factor ( $R^2 = 0.18$ ,  $p = 0.03$ ).

Figure 6 Coefficients of determination ( $R^2$ ) for partial Mantel tests of genetic distance between females and landscape resistance distance across six maximum resistance levels calculated for soil type and canopy density, excluding the effects of Euclidean distance.



With respect to males, soil resistance was the only landscape feature that explained genetic distances between individuals using Mantel tests. This association was only significant for maximum resistance levels of 10 or more. Partial Mantel tests revealed significant correlation between genetic distance and both soil and land cover after controlling for straight-line distances. For land cover, only maximum resistance levels of 5, 10, and 20 were significant. In contrast, soils explained the greatest amount of variation in the genetic distance between males at a maximum resistance value of 10 ( $R^2 = 0.20$ ,  $p = 0.006$ ; Figure 7) and this relationship remained significant across all maximum resistance values.

Figure 7 Coefficients of determination ( $R^2$ ) for partial Mantel tests of genetic distance between males and landscape resistance distance across six maximum resistance levels calculated for soil type and land cover, excluding the effects of Euclidian distance.



## DISCUSSION

### *Population genetic structure*

Although spatial heterogeneity and possible barriers to gene flow existed within the study area, STRUCTURE provided no evidence of population structure. Consistent with this result, tests of Hardy-Weinberg equilibrium showed no homozygosity excess, indicating an absence of Wahlund effects. Furthermore, the population showed no sign of widespread admixture as linkage disequilibrium was observed for only three pairs of loci.

### *Sex-biased dispersal*

Previous movement studies that have investigated home range sizes and long-distance dispersal for gopher tortoises have found that females tend to have significantly smaller home

ranges and shorter dispersal distances than males (McRae al. 1981; Deimer 1992; Eubanks et al. 2003). In the present study, the average genetic distance between individuals was significantly lower for females compared to males, implying that females display lower levels of genetic structuring. This suggests that females tortoises are dispersing greater distances than male gopher tortoises. While this could have been an effect of sampling it is not likely since the average geographic distance as well as the variance between sampled males and females was the same. Spatial autocorrelation analyses showed no evidence of positive spatial genetic structure for males or females and an assignment test used to identify individuals as possible migrants found no evidence of sex-biased dispersal. However, several males had assignment indices that were much lower than any of the values calculated for females, implying that they may have emigrated from another area. Overall, the results from this study examining sex-biased dispersal in the gopher tortoise did not support previous movement studies that have suggested male-biased dispersal. Many recent studies have found that estimates of movement using genetic data far exceed those from ecological studies (Segelbacher et al. 2010). If this is the case, it could also be that the failure to detect significant male-biased dispersal might be due to the fact that the spatial scale used in the present study was inappropriate. Results from the regional study in Chapter 2 comparing population structure found using mtDNA, which is maternally inherited, and biparentally inherited microsatellite loci suggested that male-mediated dispersal may be occurring between some populations. Further work should examine dispersal differences between sexes at both regional and local spatial scales.

#### *Landscape features influencing movement*

The effects of geographic distance, a discrete riverine barrier (the Chickasawhay River) and a suite of landscape features (elevation, slope, roads, land cover, canopy density and soils) on genetic distance were assessed using a suite of microsatellite markers. As part of this analysis, each landscape variable was assessed at six maximum resistance levels to determine if results were sensitive to cost assignments. The majority of landscape variables found to have an effect on genetic distances showed a significant relationship across the range of resistance values tested. This is important because examining a range of values ensures that the observed relationship between a landscape feature and genetic structure is not the product of assigned resistance values (Richards-Zawacki 2009).



There was no support for an isolation-by-distance pattern of gene flow at the local level, although a significant IBD pattern was observed at the regional level. It has been suggested that habitat fragmentation might alter natural dispersal patterns and explain the lack of IBD in studies conducted at finer spatial scales. In fact, studies by Broquet et al. (2006) found that distances incorporating movement along suitable habitat patches explained genetic distance among individuals better than straight-line geographic distance in logged forests while individuals in undisturbed forests retained an IBD pattern of gene flow.

Soil type was the only landscape feature that significantly explained genetic distance for the total dataset, although the correlation was very low. Canopy density also explained a small portion of the genetic structure in females whereas male genetic structure was influenced by land cover types. Since the location of open canopy areas is important to females for nesting and (Diemer 1986) it therefore seems likely that female movement would be more positively influenced by suitable soils and habitat with a low percent canopy cover. Male movements are influenced greatly by mating opportunities as they typically mate with several females in a single breeding season (Moon et al. 2006) and have been shown to seek out geographically isolated females for mating events (Boglioli et al. 2003). For these reasons, land cover maybe a more important determinant of movement for males and unsuitable land cover types may be one of the few landscape features that impede gene flow for this sex. For example, through the course of this study, many males were observed traveling along unpaved roads, which may be easier to navigate than areas of dense hardwoods or planted pine when seeking out mating opportunities.

Some of the landscape features previously shown to influence habitat suitability for gopher tortoises failed to explain fine-scale genetic structure. The level of change in topographic features such as elevation and slope may have been so subtle in the study area that they had no effect on local dispersal patterns. For elevation, 68% of the area ranged from only 45 to 80 meters. Areas of high elevation appear to restrict gene flow at the regional level (Chapter 3) but the same effect was not observed at the fine scale. The lack of topology at the local level most likely accounts for this difference.

Slope for the entire area was also low with the majority of the area ranging between only 3° and 14° gradient. Auffenburg and Franz (1982) stated that slopes over 15° may be difficult for tortoises to cross so slopes were probably not steep enough within the study area to impede local movement. Low traffic roads have also been shown to influence the shape and size of home ranges as well as aid in short distance dispersal (Diemer 1992) and major roads were found to be a barrier to dispersal in the regional study. However, neither unpaved roads nor major roadways influenced the genetic structure of gopher tortoises at the local level. One possible explanation for the lack of effect of roadways might be human-mediated movement. Another possible explanation might be that the roads in the present study were only recently constructed. Another individual-based study conducted on a small rodent species also found that a major road had no effect on gene flow or population structure despite a high rate of road mortality (Gauffre et al. 2008). The authors speculated that the road may have been too recent for significant genetic differentiation to have developed.

In conclusion, the present study shows that although geographical distance does not limit gopher tortoise gene flow at the local level, soil type, canopy density and land cover do influence fine-scale genetic structure. The limited effect of landscape features may be a result of the high variance noted in individual measures of genetic distance (Coulon et al. 2004; Broquet et al. 2006), or the limited number of microsatellite loci used in this study. It may also be man-made features examined in this study were too recent to influence molecular markers or the ability of gopher tortoises to disperse greater distances over their long life span than traditional movement studies have been able to detect. Results from the local polynomial regression and the correlogram show that tortoises, especially males, may be capable of dispersing several kilometers. This disparity between direct movement studies and genetic studies has been noted in other species and in most cases dispersal is found to be much greater than expected using molecular markers (Segelbacher et al. 2010).

These results also supported a previous study's finding that landscape features influencing regional population structure are not the same as those that explain fine scale genetic differentiation (Lee-Yaw et al. 2009). Furthermore, the results suggest that changes in management practices at the local level may improve gene flow for this federally threatened

species. For example, females are most likely to benefit from management practices such as prescribed burning and thinning that opens the forest canopy. Males may also benefit from these management actions as dispersal is influenced by unfavorable habitat types such as dense pine plantations and hardwood forests. Overall, this study shows that landscape genetic approaches applied at the individual level have the potential to reveal fine scale landscape effects and improve the management of other species impacted by habitat loss, fragmentation and degradation.

## **CHAPTER FIVE: The influence of environmental factors on the seroprevalence of *Mycoplasma agassizii* in the gopher tortoise (*Gopherus polyphemus*)**

### INTRODUCTION

For threatened and endangered species, emerging infectious diseases are a major area of concern (Daszak et al. 2000), as well as a possible cause of population decline (Smith et al. 2006). To further complicate management efforts, it is often unclear whether wildlife declines due to disease are the result of a recently introduced pathogen or the re-emergence of an endemic pathogen due to some environmental changes (Rachowicz et al. 2005). Environmental changes that are anthropogenic in origin have no doubt influenced the observed increase in wildlife pathogens by reducing and fragmenting habitat and exposing species to novel pathogens (Bradley and Altizer 2006). Fragmentation increases disease risk for wildlife populations through the introduction of domestic animals, invasive species, or previously captive individuals that may harbor novel pathogens (Daszak et al. 2000). The loss of habitat can also cause overcrowding, which facilitates disease transmission (Daszak et al. 2000). Furthermore, anthropogenic changes in land use that negatively affect habitat quality have the potential to compromise the fitness of wildlife populations, making them more susceptible to pathogens (Carey 1993; Rachowicz et al 2005). Environmental variability can also influence disease incidence by stressing animals and reducing their ability to mount an immune response. For example, changes in seasonal weather patterns due to El Nino events or flood events have increased disease prevalence in other species (Grenfell et al. 1998; Baylis et al. 1999). A combination of both anthropogenic and natural factors can also jointly contribute to disease incidence within a specific population. For example, pesticides, introduced species, ultraviolet radiation, changes in temperature and precipitation have all been associated with the emergence of chytrid fungus in amphibian populations (Daszak et al. 1999). A better understanding of how both natural and anthropogenic factors impact wildlife disease is now becoming possible through the use of tools from landscape ecology and geographic information systems (GIS) (Ostfeld et al. 2005). By examining the spatial and temporal variation of environmental variables thought to be involved in disease expression it

might also possible to identify biotic and abiotic factors that may contribute to the emergence and spread of wildlife pathogens (Ostfeld et al. 2005).

Reptiles are experiencing large declines worldwide and turtles are one of the most endangered groups according to the United States Fish and Wildlife Service, the Convention of International Trade in Endangered Species (CITES) and the International Union for Conservation of Nature (IUCN) (Gibbons et al. 2000). Although many factors have contributed to declines in reptile populations, emerging infectious diseases have been implicated in several cases (Jacobson et al. 1993; Herbst 1994; Jancovich et al. 1997). One such case is the gopher tortoise (*Gopherus polyphemus*), which has suffered range-wide reductions in population size due to habitat loss (USFWS 1987). Several studies now indicate that current declines may have also been associated with the emergence of an upper respiratory tract disease (URTD) (Brown et al. 1999; Seigel et al. 2003). This newly observed pathogen has caused concerns about the persistence of the remaining natural populations (Seigel et al. 2003), especially since populations in some parts of the range have been greatly reduced (Auffenburg and Franz 1982) and show lower levels of genetic variation (Clostio et al., *in prep*), making them potentially more vulnerable to disease (Spielman et al. 2004). URTD in gopher tortoises is associated with *Mycoplasma agassizii*, which produces a chronic infection similar to most pathogenic *Mycoplasma* species (Simecka et al. 1992; Brown et al. 1999; Frey 2002). Mycoplasmas are typically host specific and although mortality rates vary across species, lethality tends to be low (Frey 2002). One exception is the pathogen *M. alligatoris* which has been observed to cause 70% acute mortality in wild American alligator (*Alligator mississippiensis*) populations (Clippinger et al. 2000). Another mycoplasma species, *M. gallisepticum*, in house finches (*Carpodacus mexicanus*) causes high rates of morbidity but low rates of mortality (Kollias et al. 2004). Mycoplasmal infections can also be exacerbated by environmental stressors that lead to immune-suppression in the host (Simecka et al. 1992; Frey 2002). Although mycoplasmas are one of the most widely studied pathogens in domestic animals (Simecka et al. 1992; Frey 2002), their presence and potential impact in wildlife populations has only recently become more widely investigated (Brown et al. 1999; Clippinger et al. 2000; Hosseini et al. 2006).

Based on general knowledge of mycoplasma pathobiology it is therefore possible that the incidence of URTD in gopher tortoises may be potentially exacerbated by environmental stressors. Previous studies have noted that URTD is highest when tortoises are stressed by factors such as drought (Peterson 1994) and it has also been suggested that habitat degradation or overcrowding could exacerbate disease prevalence (Jacobson et al. 1991; Lederle et al. 1997). Moreover, population declines attributed to URTD in the desert tortoise have occurred during periods of drought (Peterson et al. 1994), suggesting that low precipitation is a stressor. In fact, another study in the desert tortoise found that during periods of drought individuals lost as much as 40% of their body mass (Peterson 1996). Habitat degradation caused by pine plantations has also been shown to influence gopher tortoise fitness (Aresco and Guyer 1999). These studies suggest that changes in temperature and precipitation as well as other factors associated with habitat quality may affect gopher tortoise health. Furthermore, recent observations in gopher and desert tortoises (*Gopherus agassizii*) have also found that the serological status of individuals can change over time (Lederle et al. 1997; Berry et al. 1999; Kahn and Mendonca 2005). Although it was suggested that changes in serological status may have been linked to environmental stressors in these aforementioned cases, it was not been explicitly investigated.

Only one previous study on the gopher tortoise has attempted to examine the influence of environmental covariates on the probability of infection by *M. agassizii* (Ozgul et al 2009). Environmental factors examined by Ozgul et al. (1999) included variables that were related to habitat quality such as the availability of basking sites, the abundance of exotic plants and factors related to food availability. Although none of these factors significantly influenced the probability of infection, the effect of climatic factors on URTD incidence and their interaction with determinants of habitat quality is yet to be investigated. The first objective of this study was therefore to examine whether direct measures of habitat quality such as soil and land cover type, rather than related factors investigated by Ozgul et al. (1999), influenced the serological status of individuals. The second objective was to determine if periods of high temperature and low precipitation, which are expected to lead to stress, were associated with an increase in the incidence of gopher tortoises testing positive for exposure to *M. agassizii*. The results of this study are important to population management because by determining the environmental factors

related to the incidence of *M. agassizii* will provide wildlife managers with predictive power to anticipate potential disease outbreaks or mortality events.

## METHODS

### *Study area and sample collection*

Samples were collected from gopher tortoises at 11 sites within the western portion of their range over a four year period from 2006-2009. One to 2 cc of blood was sampled from the brachial vein or the subcarapacial venous sinus of each tortoise and placed into a tube containing lithium heparin. Samples were then stored on ice in the field and refrigerated until they could be processed in the lab. Latitude and longitude coordinates were taken at the capture site of each individual and all individuals were marked to ensure that they were not sampled twice. In the laboratory, blood samples were centrifuged at 13,000 g for 30 seconds in order to separate the plasma from blood cells. Each plasma sample was then transferred to a 2 ml vial and stored at -20°C for a maximum of two weeks or at -80° for up to four months before being analyzed for antibodies to *M. agassizii*. Samples were analyzed at the University of Florida, Mycoplasma Research Lab using an enzyme-linked immunosorbent assay (ELISA) specifically developed to measure *M. agassizii* antibodies in the plasma (Schumacher et al. 1993). Based on these results, individuals were then classified as seronegative, suspect or seropositive.

### *Environmental variables*

Data pertaining to habitat quality was extracted for each individual sample point from a 30 m resolution land cover layer obtained from the Southeast Region Gap Analysis Program ([http://www.basinc.ncsu.edu/segap/datazip/region/lc\\_segap.zip](http://www.basinc.ncsu.edu/segap/datazip/region/lc_segap.zip)). Soil layers were obtained from the U.S. Department of Agriculture, Natural Resources Conservation Service (<http://SoilDataMart.nrcs.usda.gov>) for each county or parish from which the sample was collected. Only a single county (Greene Co., MS) lacked available data for detailed soils, which in this case data was obtained from a general soils layer that placed similar soil types into broader categories. Land cover and soil types were ranked then according to suitability. The habitat types examined in this study and their ranking from most suitable to least suitable was as follows: longleaf pine (1), utility swath and developed open space (2), clear cut (3), successional

scrub (4), and pine plantations (5). Soil types were classified and ranked as priority (1), suitable (2) or marginal (3).

Temperature and precipitation data for sites included in this study were obtained from the U.S. National Oceanic and Atmospheric Administration, National Environmental Satellite Data and Information Service (NESDIS). Data was acquired from weather stations located closest to the individual sample point (< 25 km). If multiple stations were present, then an overall average was taken. Although laboratory studies have shown that tortoises mount a measurable immune response post infection within 4-8 weeks (Brown et al. 1999), no previous data exists on either the time required for tortoises to mount a response to *M. agassizii* in the wild or the time that it takes for individuals to re-produce these specific antibodies during subsequent infections. Given this uncertainty, average temperature (°F) and precipitation (inches) was calculated for three time periods: two weeks, 1 month and 2 months prior to the sampling date.

### *Statistical Analysis*

Two types of statistical analyses were carried out to better understand the relationship between serological status and various environmental variables. An ordinal regression analysis was first carried out in R package (R Core Development Team 2009) to examine the effect of environmental variables on the three possible levels of serological status (negative, suspect and positive). The independent variables included in this model were site, year, season, sex, habitat type, soil and the three different temporal measures of temperature and precipitation. A possible interaction between temperature and precipitation were also evaluated because these two factors may be related. In the ordinal regression analysis, it is important to identify cases where responses were missing for a given categorical predictor. Categorical predictor variables that displayed zeros for one or more categories of the response variable were either removed from the analysis or the categories were defined more broadly to remove zero values. Model fit was evaluated with the Chi-squared goodness-of-fit test and by their corresponding pseudo-R<sup>2</sup> values. Data was also examined to ensure that the proportional odds assumption was met. A backwards regression approach was used to determine the best combination of variables in the final model. To circumvent collinearity among measures of temperature and precipitation taken at the three temporal scales, one of each of these three temporal measures was added separately to the model



in order to explore all possible combinations of both variables. Only the most significant measure of temperature and precipitation was retained in the final model.

A second analysis based on a binary logistical regression was also carried out in SPSS to examine the effects of environmental differences on serological status. For this analysis, only seronegative and seropositive statuses were considered. Two separate analyses were conducted. The first analysis did not consider individuals that were suspect for exposure to URTD whereas in the second analysis suspect individuals were grouped together with individuals found to be seropositive. The same independent variables and interaction terms investigated in the previous analysis were used in the binary regression analysis. To determine the combination of variables that best explained the data, a backwards regression procedure was used in which variables were removed sequentially from the full model using the likelihood ratio test. The backward logistic regression approach was selected over the forward logistic regression because previous studies have suggested that the latter may fail to include important variables (Leung and Tran 2000). The Hosmer and Lemeshow test (1989) was used to examine overall model fit as variables were removed.

## **RESULTS**

A total of 125 males and 106 females were included in the study. Of these, 84% of individuals were found to be seronegative, 7% suspect and 9% seropositive. Temperature ranged from 77.57°F to 99.07°F for the time periods preceding sampling and precipitation ranged from 0.04 in to 0.33 in. One variable (site) had to be removed from the ordinal regression model because many sites lacked suspect or seropositive individuals and the variable habitat type had to be condensed. Ten individual samples for the year 2006 and one site sampled in 2008 were also removed due to missing data for one or more exposure classes for a total dataset of 165 individuals. For the final ordinal regression model, the following predictor variables were found to be significant: season, year, average temperature one week prior to sampling and average precipitation one month prior to sampling (Table 1). Increasing temperature and decreasing precipitation were both significantly associated with an increased probability of seropositive status. Season was also found to be significant in this study and the negative coefficient indicates that earlier in the season tortoises were more likely be seropositive. In this study, samples were

collected across three seasons; spring, summer, and fall. Examination of the data revealed that the disease is most prevalent during the summer season with 90% of seropositive individuals cases being sampled during this season.

Table 1. Ordinal regression model of seroprevalence for samples from 2007 to 2009. Significant factors are indicated in bold.

Predictor variable	Coefficients	SE	Wald Z	P
Sex	0.488	0.478	1.02	0.308
<b>Season</b>	<b>-4.179</b>	<b>1.320</b>	<b>-3.17</b>	<b>0.002</b>
<b>Year</b>	<b>-2.355</b>	<b>0.410</b>	<b>-5.75</b>	<b>0.000</b>
Soil	-0.088	0.499	-0.18	0.860
Habitat type	0.174	0.287	0.61	0.543
<b>Temp1*</b>	<b>0.232</b>	<b>0.082</b>	<b>2.81</b>	<b>0.005</b>
<b>Prep2*</b>	<b>-12.534</b>	<b>5.987</b>	<b>-2.09</b>	<b>0.036</b>

\* 'Temp1' and 'Prep2' are the averages for one week and one month prior to sampling

In fact, 76% and 81% of seropositive individuals occurred when average temperatures were above 92°F and average precipitation was below 0.15 in, respectively. Although soil type was not found to be a significant variable, 95% of individuals that tested positive for exposure to URTD were sampled on non-priority soil types. Similarly, seropositive individuals were also spread across both high and low quality habitat types. Because year was found to be a significant variable data within years were also examined in more detail. However, it was only possible to carry out an individual analysis for year 2007 since this was the only one with no missing cells for any of the ordinal response categories.

A total of 104 samples were included in the 2007 dataset. For the final model only average temperature and average precipitation one month prior to sample date were significant (Table 2). Based on the magnitude of the regression coefficients, the highest effect was found for precipitation while temperature showed only a small effect. The direction of the relationship for serological status with temperature and precipitation was the same pattern as in the model including all data. Further examination of the data showed that 83% of seropositives occurred at average temperatures >92°F (Figure 4). Precipitation showed the opposite trend with 89% seropositives occurring when average precipitation was <0.15 in (Figure 5). There was no significant interaction found for temperature and precipitation in any of the ordinal regression analyses.

Table 2. Ordinal regression model of seroprevalence for samples from 2007. Significant factors are indicated in bold.

Predictor variable	Coefficients	SE	Wald Z	<i>P</i>
Sex	0.70	0.72	0.97	0.334
HabitatType	0.14	0.41	0.34	0.737
Soil	0.33	0.63	0.53	0.597
<b>Temp2*</b>	<b>0.45</b>	<b>0.16</b>	<b>2.8</b>	<b>0.005</b>
<b>Prep2*</b>	<b>-56.35</b>	<b>11.70</b>	<b>-4.81</b>	<b>0.000</b>

\* ‘Temp 2 ‘and ‘Prep2’ are the averages one month prior to sampling

For the logistic regression analysis, the final reduced model included the variables site, season, year, soil, average precipitation measured one month and two months prior to individual sample date (Table 3). All variables included in the final reduced model were significant except soil type. Precipitation measured two months prior to mycoplasma tests had the highest effect, followed by season and year. Therefore, as precipitation decreased, the odds of an individual testing seropositive increased. Because the variable year was again significant, the 2007 dataset was also examined independently. Using this dataset, site and average precipitation one month prior to sampling were the only significant variables in the final model. As was the case for the complete dataset, there was no significant interaction between temperature and precipitation..

Table 3. Factors associated with exposure status in the final binary logistical regression model for the total dataset using only seronegative and seropositive data.

Predictor variable	Coefficients	SE	Wald Z	<i>P</i>
<b>Site</b>	<b>1.169</b>	<b>0.504</b>	<b>2.318</b>	<b>0.020</b>
<b>Season</b>	<b>-7.474</b>	<b>2.263</b>	<b>-3.302</b>	<b>0.000</b>
<b>Year</b>	<b>-3.996</b>	<b>0.898</b>	<b>-4.449</b>	<b>0.000</b>
Soil	1.828	1.106	1.652	0.090
<b>Prep2</b>	<b>-46.935</b>	<b>12.310</b>	<b>-3.813</b>	<b>0.000</b>
<b>Constant</b>	<b>8016.999</b>	<b>1775.593</b>	<b>20.386</b>	<b>0.000</b>

For the second logistic regression analysis, suspect samples were pooled with seropositive samples (Table 4). The final model included three significant variables season, year, and average precipitation one month prior to sampling. A parallel analysis was carried out including only samples from 2007. The only significant variable in the final model was average precipitation measured one month prior to sampling ( $p = 0.004$ ). Again, there was no significant

interaction between temperature and precipitation for either the complete dataset or the 2007 dataset.

Table 4. Factors associated with exposure status in the final binary logistical regression model for the total dataset using grouping suspect with seropositive data.

Predictor variable	Coefficients	SE	Wald Z	P
<b>Year</b>	<b>-2.125</b>	<b>0.383</b>	<b>30.795</b>	<b>0.000</b>
<b>Season</b>	<b>-3.366</b>	<b>0.938</b>	<b>12.879</b>	<b>0.000</b>
<b>Prep2</b>	<b>-26.682</b>	<b>5.122</b>	<b>27.139</b>	<b>0.000</b>
<b>Constant</b>	<b>4276.442</b>	<b>770.713</b>	<b>30.788</b>	<b>0.000</b>

## DISCUSSION

The gopher tortoise is a federally threatened species (USFWS 1987) and populations have been declining over at least the last 100 years due to habitat loss (Auffenburg and Franz 1982; Lohoefer and Lohmeier 1984). More recently, local mortality events have been attributed to *M. agassizii* and URTD (Gates et al. 2002; Rabatsky and Blihovde 2002; Seigel et al. 2003). However, in most of these cases, tortoises were often not tested for exposure to *M. agassizii* prior to or during population declines and observed symptoms were used as the only sign of disease. Moreover, the symptoms of URTD can also be confused with overheating, further confounding the diagnosis of the disease (pers. comm. L. Wendland). Other conditions such as habitat quality or climatic factors were not addressed in these studies yet could play an important role in the increased incidence of this disease. Due to the current uncertainties concerning the origin and transmission pattern of *M. agassizii* wildlife managers in some states are required to destroy individuals that test positive for exposure to the pathogen even when the tortoise showed no symptoms of infection. Therefore, a better understanding of environmental factors related to the occurrence of this pathogen is needed to improve management practices.

The present study set out to test the hypothesis that certain environmental factors may have an influence on *M. agassizii* serological status in gopher tortoises. I found that a significant relationship did exist between seropositivity and changes in temperature and precipitation throughout the active season from April to September. Specifically, the probability of an

individual testing positive for exposure to the pathogen increased with temperature and decreased with precipitation. Based on this study, as well as previous studies on the effects of drought in the desert tortoise, precipitation appears to be the most important climatic factor determining seroprevalence. Precipitation had the highest effect on serological status in all analyses within the present study. This is interesting finding because previous research by Peterson (1994; 1996) has shown that changes in rainfall greatly influence tortoise fitness. Season was also significantly related to serological status with 90% of the seropositive tortoises observed in this study being sampled in the season with the highest temperatures and lowest precipitation

A previous study that examined the influence of environmental covariates on survival and seroprevalence at sites found that one measure of habitat quality, availability of basking sites, was associated with increased survival (Ozgul et al. 2009). However, there appeared to be no association between exposure status and factors attributed to habitat quality. This could be a result of the categorical classification scheme used here. A more continuous measure of habitat quality might better explain the relationship between habitat quality and seroprevalence. However, during this study I observed high seroprevalence at sites on suitable soils with open canopy long-leaf pine habitat. So it may be that habitat quality truly has no influence on the prevalence of *M. agassizii*. Microhabitat variables, which I was unable to measure here using GIS data, are likely to influence tortoise stress and disease incidence. For example, tortoises acquire much of their water through the consumption of plants but during periods of drought this water source is reduced. Therefore, food quality will be related to climatic factors and therefore should influence tortoise stress. Additional studies need to more explicitly examine the relationship between habitat quality, climatic factors and incidence of URTD in the gopher tortoise.

Periods of drought have already been shown to reduce tortoise body weight and metabolic rate. Therefore, it may be that these environmental factors are making tortoises more susceptible to infection through a suppressed immune response. However, it is difficult to show a direct link between host immune-suppression and an increase in disease incidence because individual stress levels were not measured in this study. In the future it will be important to

conduct health assessments on tortoises by examining white blood cell counts, plasma biochemistry and parasite load. This data may allow us to link tortoise health with serological status and climatic factors.

One major concern with wildlife diseases is the possibility of transmission between individuals or populations especially due to human-mediated translocations (Daszak et al. 2000). For tortoises, the most probable route of transmission for *M. agassizii* is via direct contact between tortoises (Brown et al. 1999). Therefore, an increase in activity patterns during periods of high temperature and low precipitation might elevate transmission. Yet, tortoise activity tends to decrease during periods of high temperature. McRae et al. (1981) found that tortoise activity was highest at approximately 86°F and that temperatures above this were associated with a decrease in activity. Also, periods of aestivation due to high temperatures have been observed in closely related *Gopherus* species (Voigt and Johnson 1976; Nagy and Medina 1986). Furthermore, Peterson (1996) and Duda et al. (1999) found that energy expenditure decreased in desert tortoises during periods of low rainfall. In this study, 83% of seropositive individuals were sampled when maximum temperatures were above 92°F, suggesting that activity levels were decreased. Studies that found a reduction in tortoise activity during periods of climatic stress are encouraging because it suggests that when these environmental stressors occur, infected individuals are likely to also display reduced movement and this will decrease the likelihood of transmission. However, the translocation of tortoises during these periods should be avoided because this action is likely to further stress tortoises as well as increase the chances of transmission.

One novel aspect of the present study is its utilization of GIS technology and spatial data to gain a better understanding of environmental factors affecting serological status in the gopher tortoise. This study is a first step in providing wildlife managers with environmental variables that can be used to predict disease incidence in the gopher tortoise. Spatial data is becoming increasingly more important to the understanding of wildlife diseases (Ostfeld et al. 2005). To date, this type of approach has been used to examine the relationship between landscape structure and hantavirus in deer mice populations (Langlois et al. 2001), analyze the effects of environmental factors on Lyme disease vectors (Das et al. 2002) and map areas of high risk for

West Nile virus (Theophilides et al. 2003). As the field of spatial epidemiology continues to expand, these spatial tools will give us the power to comprehend disease outbreaks, predict future occurrences and better manage wildlife disease in threatened and endangered species.

## CHAPTER 6: DISSERTATION CONCLUSIONS

The main objective of this study was to integrate methods from multiple disciplines to investigate how environmental factors influence population structure, gene flow and disease incidence in the gopher tortoise. The results from this dissertation suggest that landscape features influence both regional population structure and fine-scale dispersal patterns. However, the spatial scale at which specific landscape features affect gene flow appears to differ. The results also indicate that environmental variables can be used as predictors of disease occurrence. In addition to these general conclusions five broad questions were posed at the beginning of this study that can now be answered based on the results of this dissertation research.

The first question asked was how population genetic structure was delineated across the range for the gopher tortoise and whether it appeared to coincide with geographic barriers. To answer this question, I utilized both mitochondrial and nuclear microsatellite markers to examine patterns of both historical and contemporary population genetic structure, respectively. For the mitochondrial sequence data, a deep phylogenetic divergence between eastern and western populations was observed that appeared to correspond to the Apalachicola River. The importance of the Apalachicola River as a historical barrier to gene flow for the gopher tortoise was further supported by evidence from nuclear microsatellites and reflects a deep phylogeographic break observed in other co-distributed taxa (Church et al. 2003; Pauly et al. 2007; Burbrink et al. 2008). This study also found a distinct haplotype present in individuals from western Georgia, which had not found in any previous study on gopher tortoises suggesting that populations in this region may be genetically distinct and of special conservation concern. Although genetic divergence between populations east and west of the Apalachicola River was substantial, the amount of mitochondrial genetic variation within each sub-region was low. This low haplotype diversity has also been found in other chelonian species (Edwards 2003; Velo-Antón et al. 2008) and may reflect the lower micro-evolutionary rate reported for the order Testudines (Avice et al., 1992). The slower rate of nucleotide substitution in tortoises may be related to their larger body size, lower metabolic rate and their long generation times (Martin and Palumbi 1993).



In contrast to the mitochondrial data, our microsatellite dataset revealed high genetic differentiation between numerous sample sites and evidence of regional population sub-structuring. As observed in the mitochondrial data, the greatest levels of nuclear genetic divergence were found between populations east and west of the Apalachicola River, further supporting this region as a strong historical barrier to gene flow. Based on Bayesian assignment methods, the true number of population clusters throughout the range appears to number either five or six. All populations sampled west of the Mobile River were grouped into one population unit separate from all other sample sites located further east. Within the federally listed portion of the range very little population structure existed. Populations in south-central Alabama, east of the Mobile River, appear to form an independent group although there was also some evidence of genetic exchange with populations from western Georgia that could be the result of male-mediated gene flow. Sample sites in western Georgia also grouped separately from other sites in the eastern portion of the range based on microsatellite data, further supporting the designation of populations in this region as a distinct management unit. Lastly, assignment methods placed all individuals sampled along the Atlantic coast in one group and all individuals sampled from Florida into another. Bayesian assignment tests indicated only a few long distance dispersal that events that might be related to human-mediated translocations. One individual sampled in South Carolina appeared to be a first generation migrant from south-central Alabama, while four individuals from eastern Georgia seemed to be second generation migrants from the federally listed portion of the range.

The second question posed here was whether the genetic data showed evidence of population declines. Populations in the western portion of the range displayed a lower number of alleles and decreased levels of heterozygosity relative to other populations in the eastern portion of the range of the gopher tortoise. However, BOTTLENECK found only one sample site out of 18 tested (DNF1,  $p = 0.027$ ) that displayed evidence of significant heterozygote excess using the Wilcoxon signed-rank test. In contrast, Garza and Williamson's  $M$  value, which may be able to detect much more historical bottlenecks, showed values below those normally observed in stable populations for all gopher tortoise populations. Furthermore, the Bayesian coalescence model implemented in MSVAR indicated a significant long-term decrease in population size for populations across the federally listed range around the time of the last glacial maximum. These

results suggest that while population declines began during the Pleistocene range contractions they may have continued to the present as a result of progressive habitat fragmentation across the range of the gopher tortoise.

The third question asked was how landscape features might influence regional population structure and gene flow within the western portion of the gopher tortoise range. A causal modeling approach was used to examine the importance of straight-line distance, a putative riverine barrier (the Chickasawhay River) and four major landscape features (elevation, land cover, soil, and major roads) on levels of population genetic differentiation across the federally listed range of the gopher tortoise. In order to quantify the relative importance of different landscape variables, I used two different methods to quantify landscape resistance: isolation by resistance (IBR) and least-cost path (LCP) analysis. My results showed that the Chickasawhay River did not appear to constitute a substantial barrier to gene flow. However, there was strong support for the effects of straight-line distance, elevation, and major roads on population structure. Resistance distances measured between sampling sites using IBR outperformed landscape resistance models based on LCP measures. When compared to a simple model of isolation by distance, the highest supported model of IBR improved model fit by 11% after controlling for the effects of straight-line distance. When compared to highest supported LCP model, IBR improved model fit by 20%. Furthermore, results from a multiple regression of distance matrices (MRDM) provided support for the same landscape variables as those revealed using a causal modeling approach and partial Mantel tests, showing that these were not model-dependent results.

Surprisingly, this study was unable to determine any effect of land cover type on regional genetic structure. This might be due to a lag time in the ability of molecular markers to detect population structure due to recent changes in land use patterns and the long generation time of tortoises (40-60 years). Further work should seek to address whether historical land cover correlates better with current population structure. Although land cover features related to anthropogenic changes in land use were not found to influence gene flow, major roads were found to have a significant impact on population genetic structure. This result supports findings from other studies in reptiles that have shown the negative influence of human mediated habitat

alterations on natural population structure (Hauswald and Glenn 2005; Moore et al. 2008; Shepard et al. 2008).

The fourth question I posed was what habitat variables influence local population structure and whether the same landscape features that shape population structure at the regional level operate at the fine-scale level. To answer this question, I collected 169 individuals from a 14 x 35 km area within the Forrest and Perry Counties of the Desoto National Forest (DNF), located in southern Mississippi. Previous studies of movement indicated that the majority of gopher tortoise movements were restricted to foraging within 30 m of their burrow (Mc Rae et al. 1981) and that long distance movements were < 1km (Diemer 1982). Based on these observations, the spatial scale of the study should have been adequate to recover fine scale spatial genetic structure if it existed. A previous fine-scale study on another long-lived reptile found significant genetic structuring at distance < 500 m (Moore et al. 2008). However, no evidence of fine-scale spatial genetic structure or isolation by distance was found in the present study. Also, an assignment test found no indication of sex-biased dispersal. This was surprising because most movement studies have shown that males have larger home ranges and make long distance movements to find mating opportunities (Diemer 1992). However, many previous studies that utilized molecular markers to indirectly estimate gene flow have found that dispersal is often underestimated by direct studies (Koenig et al. 1996), suggesting that lifetime dispersal capabilities for the gopher tortoise is much greater than seasonal studies have indicated.

I also examined the effect of geographic distance, a riverine barrier and several landscape features (elevation, slope, roads, land cover, canopy density and soils) on genetic distances between individuals. Using IBR to determine landscape connectivity, I found that soil was the only landscape feature that significantly explained genetic distance between individuals of both sexes. In addition, canopy density also explained a small portion of the genetic structure in females whereas land cover types explained a portion of the genetic distances observed between males. Since the location of open canopy areas is important to females for nesting, it is likely that female movement is influenced by the availability of suitable soils and habitat with a low percent canopy cover. Males on the other hand, display larger home range sizes and greater dispersal

distances, which may require them to disperse through a variety of land cover types. Thus, land cover maybe a more important determinant of movement for males than females.

Land cover was not found to be significant at the regional scale and given the lag time of molecular markers it was surprising that current land cover patterns explained genetic distance for males at the local level. However, results from a fine-scale study conducted on another long-lived reptile found evidence that genetic structure was driven by recent habitat modifications (Moore et al. 2008). Regional studies also indicated that low elevation impeded gene flow. However, at the local level, there was no effect of topographic features such as elevation and slope, suggesting that these features may have been too subtle in the study area to have a substantial effect on local dispersal patterns. Finally, major roads were a significant barrier to gene flow in the regional study. However, at the fine-scale I found no evidence that unpaved roads or major roadways influenced the spatial genetic structure of this population. It may be that the roads within the local study were too recent for significant genetic differentiation to have developed or that traffic volume within the area was relatively low, as I observed a low incidence of road mortality over the course of the study. Therefore, roads in the study area may not have presented a substantial enough barrier to movement for tortoises to lead to significant local structure. Alternatively, it might be that human-mediated movement across major roadways might also explain the lack of genetic differentiation at the fine-scale level.

The final question I posed was whether environmental variables influence the occurrence of the pathogen *M. agassizii* in gopher tortoises. The emergence of many wildlife diseases appears to be linked to changes in the environment that alter the relationship between the host and pathogen (Daszak et al. 2000; Rachowicz et al. 2005). One potential reason for this connection is that environmental changes can cause stress in the host and increased their disease susceptibility (Daszak et al. 2000; Bradley and Altizer 2006). I found that a significant relationship did exist between the incidence of seropositive tortoises and changes in temperature and precipitation throughout the active season. Specifically, the probability of an individual testing positive for exposure to the pathogen increased with higher temperature and lower precipitation values. It may be that during periods of stress, individuals are more susceptible to exposure and infection leading to an increase in the occurrence of seropositive individuals during

hot and dry seasons. Future work should focus on whether the two environmental factors (temperature, precipitation) consistently predict future disease occurrence across years and whether hot, dry periods lead to measurable increases in stress and attenuated immunocompetence.

The results for this dissertation study have the potential to improve management and recovery plans for the gopher tortoise. My evaluation of range-wide population genetic structure was able to identify geographic regions that constitute genetically distinct populations for conservation. Specifically, I recommend that populations East and West of the Apalachicola River should be protected as evolutionary significant units (ESUs) based on evidence of historical isolation. Additionally, populations in the federally listed portions of the range, south-central Alabama, western Georgia and those along the Atlantic coast should be recognized as distinct management units (MUs) based on microsatellite-based measures of genetic structure. Furthermore, by examining genetic diversity across the range of the gopher tortoise, I was able to show that the western portion of the range, which has experienced at least an 80% loss of habitat, displayed lower allelic richness and observed heterozygosity. This suggests that management in the western portion of the range should focus on increasing habitat area and restoring connectivity between populations to prevent further reductions in genetic diversity.

Human induced habitat loss and degradation have been linked to population declines in many reptiles (Kuo and Janzen 2004; Velo-Antón et al. 2008; Richmond et al. 2009), including the gopher tortoise (USFWS 1987). In this study I examined the effect of both anthropogenic and natural landscape features on genetic structure at regional and local scales. At the regional level, I found evidence that major roads influence population structure whereas at the fine scale, canopy cover and land use affect genetic distances in females and males, respectively. These results indicate that management strategies should seek to reduce the regional impact of roads by constructing wildlife corridors that traverse these features or through human-assisted translocation. At the fine scale, wildlife managers should focus on increasing open canopy habitat through the use of prescribed fire as well as maintain tracts of suitable habitat between populations as this will promote gene flow for both males and females.

This study was the first to find a link between climatic conditions and disease occurrence in the gopher tortoise. This research also suggests that wildlife managers should avoid translocating tortoises during periods of high temperature and low precipitation since these conditions are likely to lead to stress, suppressed immunity and an elevated risk of disease transmission. Additional studies should aim to explore this relationship further and determine the predictive power of these two variables across multiple years. It may also be possible to combine landscape genetic data with disease occurrence data to examine the effects of key landscape features on the transmission dynamics of *M. agassizzi*. Finally, this study showed that novel approaches in the field of landscape genetics and spatial epidemiology could be utilized to improve management for a threatened species. Based on this study it is likely that these approaches could be used to examine population genetic structure and disease occurrence in other threatened and endangered species.

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APPENDIX  
Animal subjects approval form

# Institutional Animal Care and Use Committee

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UNIVERSITY OF NEW ORLEANS

**DATE:** March 26, 2009

**TO:** Dr. Nicola Anthony

**FROM:** Steven G. Johnson, Ph.D.  
Chairman

**RE:** *IACUC Protocol # UNO-09-002*  
*Entitled: Influence of environmental variables on gene flow, population structure and disease transmission in the gopher tortoise *Gopherus polyphemus**

Your application for the use of animals in research (referenced above) has been approved beginning March 26, 2009 and expiring March 26, 2010.

## VITA

Rachel Wallace Clostio was born in Conroe, TX and received her B.S. in Biology from Baylor University in Waco, TX. She will continue on to a postdoctoral research position at Louisiana State University studying examining genetic relationships between pathogenic *Vibrio spp.*, evaluating how these bacteria respond to environmental factors using remote sensing data and investigating the role of Type III Secretion Systems in *Vibrio spp.* on host immune response.