GENETIC AND PHENOTYPIC VARIATION AMONG FOX SQUIRRELS IN EASTERN NORTH CAROLINA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

H_O: Observed Heterozygosity
H_E: Expected Heterozygosity
F_{ST}: Measure of population differentiation due to genetic structure
F_{IS}: Inbreeding Coefficient
PCR: Polymerase Chain Reaction
A: Number of total alleles
Rare: Number of alleles occurring at a frequency less than or equal to 0.05

ABSTRACT

GENETIC AND PHENOTYPIC VARIATION AMONG FOX SQUIRRELS IN EASTERN NORTH CAROLINA

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The longleaf pine (*Pinus palustris* Mill) ecosystem serves as habitat for the eastern fox squirrel (*Sciurus niger* L) in the southeastern United States and has been reduced in size and fragmented. Fragmentation often leads to loss of genetic diversity and an increase in population structure of species. To determine if this is happening in the fox squirrels of North Carolina, five microsatellite loci and phenotypic variation were used to compare geographic variation among fox squirrel populations. Fox squirrels showed a low level of population subdivision indicated by F_{ST} values of 0.010 to 0.017. In contrast, F_{IS} values were higher (0.222 to 0.230) indicating that inbreeding could be causing a loss of genetic diversity. Linear regression showed a positive correlation between individual weight and longitude and ANOVA analysis revealed squirrels were significantly heavier and shorter west of 78°W longitude, which runs just east of Wilmington N.C. North Carolina fox squirrels were found to be less heterozygous than those of the Midwestern United States, and one locus (FO-41) showed a major decrease in heterozygosity since 1983. Future management of fox squirrels should focus on maintaining habitat and population numbers sufficient to avoid inbreeding.

Introducing individuals from other areas may help to increase overall genetic diversity which should also conserve the overall fitness of North Carolina's fox squirrels as it has with other species.

CHAPTER 1. INTRODUCTION

Loss and fragmentation of a species' habitat, often reduce the size of animal populations (Fischer and Lindenmayer 2007), increase extinction risk (Lampila et al. 2009), and thus are a threat to biodiversity. The eastern fox squirrel may have been negatively impacted by the reduction and fragmentation of longleaf pine (*Pinus palustris* Mill) forests due to human activities (Frost 1993). Fox squirrels typically prefer forests, such as longleaf pine, with open understories and are displaced by gray squirrels (*Sciurus carolinensis* L) when species composition shifts to create forests that are more dense (Weigl et al. 1989). Fox squirrels are ecologically important because they distribute seeds and mycorrhizae fungi, and facilitate succession of grasslands into forests (Weigl et al.1989, Koprowski 1994).

Declines in population size can be accompanied by loss of genetic diversity. For example, when the grassland habitat of the European ground squirrel (*Spermophilus citellus* L.) became fragmented by agriculture and development, variation in the major histocompatablity complex genes (Říčanová et al. 2011) and among microsatellite loci (Slimen et al. 2012) declined. Also population declines and loss of genetic diversity have been documented in the Eurasian red squirrel (*Sciurus vulgaris*, L.) where similar loss of habitat and competition from the introduced eastern gray squirrel has occurred (Ogden et al. 2005, Barratt et al. 1999). Further, individual genetic heterozygosity has been related to fitness in the Siberian flying squirrel (*Pteromys volans* L) where more heterozygous individuals were able to disperse greater distances (Selonen and Hanski 2010), and the European alpine marmot (*Marmota marmota* L) where more

heterozygous individuals had higher survival rates (Da Silva et al. 2005). If genetic diversity is similarly related to fitness in fox squirrels, a loss of genetic variation could lead to reduced fitness.

Increased inbreeding occurring as population sizes decline can lead to lower heterozygosity within individuals and populations. Increased inbreeding can also lead to inbreeding depression which is caused by increased genetic load and expression of deleterious recessive alleles in populations (Reed et al. 2003). In species that are already declining in number due to habitat loss or degradation, loss of fitness from inbreeding depression can increase the risk of extinction (Charlesworth and Charlesworth 1987). This phenomenon has been extensively documented in large mammals such as red wolves (*Canis rufus* Audubon and Bachman) (Brzeski et al. 2014) and the Florida panther (*Puma concolor coryi* Bangs) (Hostetler et al. 2013). While not as widely reported as in the more charismatic animals, negative inbreeding effects have been documented in small mammals such as the black footed ferret (*Mustela nigripes* Audubon and Bachman) (Wisely et al. 2008) and deer mice (Schwartz and Mills 2004).

Because of the impact genetic and phenotypic changes can have on population health, information about these patterns within a species can inform management decisions and benefit conservation plans. For example, to improve transplantation success to save the federally endangered *Delmarva* fox squirrel subspecies, researchers and managers used knowledge of genetic data of donor and recipient populations to increase genetic diversity in introduced populations (Lance et al. 2003). When genetic factors are not considered, management efforts can be adversely

affected. For example, Wisely et al. (2008) found reintroduced populations of black-footed ferrets with lower genetic diversity populations had greatly reduced growth rates and had to be supplemented annually to persist.

The purpose of my research was to describe genetic and phenotypic variation of fox squirrels in eastern North Carolina.

Specifically:

1. I examined phenotypic variation between fox squirrel populations across geographic regions to determine if genetic or environmental differences were re4lated to variation between the fox squirrels inhabiting different areas of the eastern part of the state.
2. I used Hardy-Weinberg equilibrium to determine if allele frequencies were changing in fox squirrel populations of eastern North Carolina.
3. I compared genetic diversity between the geographic regions to determine if fox squirrels were experiencing population subdivision or inbreeding.
4. I compared gene diversity over time to determine if alleles have been lost over time.
5. I compared heterozygosity levels between fox squirrels of eastern North Carolina and an apparently healthy fox squirrel population from the Midwestern United States to determine if heterozygosity may have been lost in the fox squirrels of North Carolina.

CHAPTER 2: LITERATURE REVIEW

Genetic Variation in Natural Mammal Populations

Genetic diversity in wild populations is subject to change due to mutation, natural selection, genetic drift, non-random mating, and migration. Mutation and migration increase genetic diversity while genetic drift decreases it. Natural selection can decrease genetic diversity by removing alleles which are less fit, or it can increase or maintain genetic variation in cases where heterozygotes have an advantage. Higher individual fitness of heterozygotes compared to homozygous genotypes has been documented in some genes such as the major histocompatablity complex (Oliver and Piertny 2012). Non-random mating can decrease genetic diversity in the case of inbreeding, or increase genetic diversity when less related mates are selected more often (Conner and Hartl 2004). In species of conservation concern, loss of alleles due to inbreeding and genetic drift from to declining population sizes are usually the greatest genetic problems. These factors are especially problematic where fragmentation has led to decreased migration as has been documented in some small mammal species (Ćosić et al. 2013, Marchi et al. 2013).

Genetic drift effects are greater in smaller populations due to decreased gene pools and fewer numbers of offspring being produced each generation. In small populations, genetic drift can randomly result in alleles being passed on to the next generation at rates different than the parental generation, leading to alteration in gene frequencies over time (Conner and Godbois 2002). In the absence of mutation or migration, random changes in allele frequencies move alleles toward fixation even if

natural selection is selecting against the allele in question. This is illustrated in the Chatham Island black robin (*Petroica traversi,* Buller) where minisatellite DNA showed genes thought to be under balancing selection had become monomorphic at non-neutral genes due to drift (Miller and Lambert 2004). In populations lacking of gene flow from outside populations, genetic drift will eventually lead to loss of allelic diversity and differentiation between populations such as observed for the European common vole (*Microtus arvalis* L) (Fischer et al. 2014). If loss of diversity occurs at loci where it is important for fitness, population declines and increased extinction risk are more likely. For example, the major histocompatablity complex gene family is involved in vertebrate immune responses where more heterozygous individuals may have greater immune function against a greater range of pathogens and parasites than homozygotes (Lenz et al. 2009, Penn et al. 2002).

In addition to drift, inbreeding can also result in decreased genetic diversity in small populations. Inbreeding occurs when the alleles present in a mating pair of a sexually reproducing species tend to be more identical by descent than what is expected in randomly mating populations. Inbreeding can lead to loss of heterozygosity, even if the overall allele frequency in a population does not change (Kimura and Crow 1963). In the case of heterozygote advantage, loss of heterozygosity can lead to a loss of fitness at both an individual and population level. Along with decrease in heterozygosity, inbreeding can also lead to increased expression of deleterious alleles throughout the population (Jaquiéry et al. 2009). Deleterious alleles change phenotypes and lead to decreased fitness by lowering survival and/or reducing fecundity (Reed et al. 2003). Inbreeding depression in small populations has been documented in several

species including the Florida panther (*Puma concolor coryi* Bangs) (Roelke et al. 1993), red wolves (*Canis rufus* Audubon and Bachman) (Brzeski et al. 2014), and California sea lions (*Zalophus californianus* Lesson) (Acevedo-Whitehouse et al. 2003). In small mammals, inbred black-footed ferrets (*Mustela nigripes* Audubon and Bachman) have smaller limbs and body sizes (Wisley et al. 2008) and inbred deer mice (*Peromyscus maniculatus* Wagner) have lower survival (Schwartz and Mills 2004).

There are numerous examples in small mammals where genotypic diversity is related to fitness. Examples in the Sciuridae family include the Siberian flying squirrel (*Pteromys volans*, L) and the European alpine marmot (*Marmota marmota*, L). Selonen and Hanski (2010) reported a correlation between individual genetic heterozygosity and dispersal in the Siberian flying squirrel. Da Silva et al. (2005) found a positive correlation between juvenile survival and individual heterozygosity in the European alpine marmot. Radwan et al. (2010) found some species vertebrates with low genetic diversity in MHC genes also had decreased immunity to pathogens while populations with a higher diversity of MHC genes had higher survival rates when exposed to pathogens (Penn et al. 2002). This alteration in resistance to pathogens could be important in rodent populations where it has been shown that an increased load of bacterial and viral parasites can lead to a decrease in litter size (Bordes et al. 2011).One species where low diversity in the MHC genes is likely having a deleterious effect on survival of the entire species is the Tasmanian devil (*Sarcophilus harrisii*, Boitard) where low genetic diversity may be contributing to the inability of the species to cope with the devil facial tumor disease which has caused significant mortality (Cheng et al. 2012).

Genetic diversity can play a major role in determining both the likelihood of persistence and the evolutionary trajectory of a species or a population. The path evolution follows depends on the specific assortment of alleles present in a population at a given time, and any alteration of gene frequencies in a population could affect the chances of a population or even a species as a whole surviving in the long-term (Lacy 2014). Species which are of conservation concern tend to have smaller population sizes, making them more vulnerable to changes in allele frequencies due to genetic drift and inbreeding (Buskirk and Willi 2006). These changes in allele frequencies can alter a population's ability to respond to natural selection in an adaptive way, especially if alleles become genetically fixed and thus populations are less able to evolve with changing environmental conditions (Wright 1932). Lack of adaptability due to loss of genetic diversity is thought to increase the likelihood of extinction in populations already experiencing declines in population size (Frankham 1995). A well established case of this type of species loss is the thylacine (*Thylacinus cynocephalus*, Harris) where low levels of genetic diversity were documented prior to the extinction of the species (Menzies et al. 2012). In populations which are already declining in numbers due to habitat loss or other factors, decreased fitness due to loss of genetic variation can impair the ability of the population to return to its original size even if environmental conditions become more favorable. This can lead to an extinction vortex where decreasing population sizes leads to a loss of genetic diversity and increased inbreeding, which leads to further declining population sizes which decreases genetic diversity and fitness even more (Blomqvist et al. 2010).

The increased susceptibility of smaller populations to loss of alleles and decreased heterozygosity can be offset by gene flow from neighboring populations. Unfortunately, migration is often prevented by fragmentation which poses barriers to movement between inhabited areas. For example, fragmentation has isolated populations of Eurasian red squirrels (Barratt et al. 1999). Even when recolonization occurs, if only a small number of individuals immigrate there can have low genetic diversity due to a “Founder's Effect”. If gene flow from the parent population(s) is not maintained, the newly established populations may not remain viable (Nei et al. 1975). Additionally, allele frequencies after random migration events without continued gene flow can differ based on chance from the population form which the founders came. For example, in the California Channel Islands founder effects and lack of continued gene flow resulted in low diversity within islands and high genetic differentiation between islands and the mainland in island spotted skunks (*Spilogale gracilis amphiala*, Merriam) populations (Floyd et al. 2011). Immigrating populations with low diversity can lack ability to adapt to new environmental pressures and are vulnerable to declines and/or extinction. While the skunks in the Floyd et al. (2011) study became separated from each other on naturally occurring islands, anthropogenic factors such as roads and urbanization can also lead to creation of artificial "islands" of habitat which are separated from each other by "seas" of uninhabitable areas which are unsuitable for many species to move across.

Non-Genetic Phenotypic Variation

In addition to genetic factors, environmental factors can also influence an individual's phenotype and can create geographic or temporal variation in populations.

Environmental conditions that can influence phenotypes include food availability and quantity, temperature, and predation pressure (Fietz and Weis-Dootz 2012). For example, roe deer (*Capreolus capreolus* L) occupying rich oak (*Quercus spp.*) forests had a higher body mass than those occupying more resource poor beech (*Fagus spp.*) forests (Pettorelli et al. 2002). Alteration of body size could impact fitness through its effect on ability to compete for mates, thermoregulate, escape from predators, or obtain food. Higher quality habitat has been related to increased fecundity of individuals and greater survival of offspring produced such as seen in Spanish imperial eagles (*Aquila adalberti,* Brehm) (Ferrer and Bisson 2003). In Gunnison's prairie dogs (*Cynomys gunnisoni* Baird) lower quality habitat was related reduced body mass, increased age of first reproduction, and increased age of dispersal (Rayor 1985). It follows that any degradation of habitat quality has the potential to alter the phenotype of individuals within that habitat.

Fox Squirrel Ecology and Life History

The eastern fox squirrel (*Sciurus niger*, L) is a medium to large tree squirrel occurring throughout the United States east of the Rocky Mountains as well as in northern Mexico and southern Canada. The coat color is variable with black forms being the most common in the southeastern United States (Steele and Koprowski 2001). It is the largest and most brilliantly colored tree squirrel in North America and thus is a favorite of nature watchers and hunters (Weigl et al. 1989). Thorington et al. (2012) list 10 subspecies of fox squirrels with *S. n. niger* being the subspecies in eastern North Carolina. While fox squirrels have been documented to survive up to 13 years in captivity, a lifespan of over 7.5 years is rare in the wild (Koprowski et al. 1988). Fox

squirrels feed primarily on conifer and hardwood tree seeds but their diet also includes fruits, buds, flowers, fungi, herbs, insects, and bird eggs and nestlings (Thorington et al. 2012). Fox squirrels are scatter hoarders; caching seeds in multiple locations to provide food for themselves during the winter (Koprowski 1994). Their ability to forage on the ground in open environments allows fox squirrels to use farm clearings and forest edges for both food and dispersal corridors better than gray squirrels (Goheen et al. 2003). In contrast to co-occurring eastern gray squirrels, fox squirrels are more energetically efficient at thermoregulation and can exploit a greater variety of food sources (Steele and Weigl 1993).

Fox squirrels typically occur in lower population densities than the eastern gray squirrel (Koprowski 1994). For the sandhills area of North Carolina, Weigl et al. (1989) estimated a mean population density of 5 squirrels per km^2 in the years 1985-1986. Some higher density populations exist such as on Spring Island, South Carolina where there are 78.8 fox squirrels per km^2 (Lee et al. 2005). This is the highest density of fox squirrels in the southeast and is most likely due to the variety of food sources on the island, maintenance of open habitat through prescribed fire and mowing, and the fact that hunting is not allowed (Lee et al. 2009). Typical fox squirrel home ranges are less than 0.08 km^2 but can reach upwards of 0.15 km^2 for squirrels in lower density populations of the southeastern United States (Koprowski 1994). Greater overlap among individuals occurs with these larger home ranges (Steele and Koprowski 2001).

Mating of fox squirrels peaks in November-February and April-July. Multiple males chase a single female and a female may copulate with multiple males. Gestation lasts 44- 45 days and a few females are able to produce two litters a year. Juvenile fox

squirrels disperse rapidly from their natal area after weaning (Thorington et al. 2012). Hansen et al. (1986) reported adult females seem to play the largest part in regulating population densities by preventing the recruitment of juveniles into the breeding population. Dispersal rates are higher in fox squirrels than in other squirrel species (Koprowski 1992 and 1994).

Fox Squirrels and the Longleaf Pine Forest

Fox squirrels prefer forests with a more open understory structure compared to the more common gray squirrel. In eastern North Carolina, fox squirrels are associated with longleaf pine ecosystems where fire historically maintained an open understory (Weigl et al. 1989),dominated by grass and with little woody debris (Loeb 1999). Prior to the arrival of Europeans longleaf pine forests occurred more or less continuously and covered 36% of the Coastal Plain where hardwood species existed only in relatively small patches of specialized habitats. Frequent, low intensity fires burned the understory of longleaf forests without harming the adult trees, and aided in the regeneration of longleaf pine and other fire-adapted tree species (Glitzenstein et al. 1995). Where changes in elevation were present in the landscape more pure stands of longleaf pine would occur on the south facing slopes, and the north facing slopes and higher elevations had more mixed forests (Frost 1993). The presence of some hardwood trees may be important to fox squirrel habitat. Perkins et al. (2008) found that Sherman's fox squirrels (*S. n. shermani* Moore) were most likely to use habitat that had 11.8% hardwood cover. On Spring Island, S.C. fox squirrels were also found to prefer habitat with some hardwood overstory versus pure pine habitat (Lee et al. 2009). Fox squirrels used hardwood trees for daytime refugia in a longleaf pine forest at the Joseph

W. Jones Ecological Research Center in Georgia (Conner and Godbois 2002). These studies suggest that maintaining some hardwood trees within the longleaf pine matrix benefits fox squirrel populations.

Along with maintaining the open understory habitat preferred by fox squirrels, fire may have also influenced the coat color of the fox squirrels because individuals occupying longleaf pine ecosystems have a higher frequency of melanism (darker colored hairs) compared to fox squirrels from other regions of the country. The dark fur may better camouflage squirrels from predators as they forage on the ground frequently blackened by fires (Kiltie 1989). In addition to a greater frequency of melanism, fox squirrels of the southeastern United States are larger which allows them to more effectively compete with gray squirrels for cavity nest sites than in the Midwest where fox squirrels are smaller. The larger body size may also help fox squirrels exploit the large, hard longleaf pine cones as a food source (Weigl et al. 1989).

Benefits of Fox Squirrels

Fox squirrels serve ecological functions by facilitating succession from grasslands to forests when they move through open areas burying tree seeds and distributing mycorrhizae fungi (Koprowski 1994, Moore and Swihart 2007). Black walnut (*Juglans nigra* L) is a preferred food of the fox squirrels in the Midwestern United States. Black walnut seeds germinated farther from the edge of the forest into grassland than other tree species which were eaten less often by fox squirrels, suggesting that walnuts buried by the fox squirrels colonized the edges of the prairie (Stapanian and Smith 1986). The size of tree squirrel populations can be indicator for the health of the forests they inhabit (Koprowski 2005a). Since the longleaf pine ecosystem inhabited by the fox

squirrel in North Carolina is one of the most endangered forest types in the country (Frost 1993), abundance of fox squirrels may indicate how successful forest restoration efforts are (Weigl et al. 1989, Edwards et al. 1998).

Fox Squirrel Declines

In eastern North America many subspecies of fox squirrels have undergone population declines (Weigl et al. 1989). This decline is thought to be the result of fire suppression and other management practices that reduced the amount of open forest habitat, allowing the eastern gray squirrel to encroach into areas that would have once been dominated by the fox squirrel (Edwards et al. 1998, Lee et al. 2009). In forests with a denser understory the eastern gray squirrel outcompetes fox squirrels for food resources. In areas where the two species are sympatric, niche differentiation is observed with fox squirrels occupying more open forests and edge habitats and gray squirrels occupying areas of more dense forest (Derge and Yahner 2000). Compared to where fox squirrels occur alone, fox squirrels occurring sympatrically with gray squirrels occupy a narrower ecological niche which could negatively affect populations by limiting resource availability in these areas (Edwards et al. 1998). An analogous case is seen in the Eurasian red squirrel (*Sciurus vulgaris*, L.) which has declined over much of its range due to the loss of its preferred forest habitat and competition from the introduced eastern gray squirrel (Ogden et al. 2005, Barratt et al. 1999).

One subspecies of fox squirrels which has been greatly affected by the loss of habitat to urbanization is the Delmarva fox squirrel (*S. n. cinereus*), which has declined to the point where it has been listed as federally endangered. The range of the Delmarva fox squirrel has been fragmented and reduced by 90% due to human

changes in the Delmarva Peninsula in Delaware, Maryland, and Virginia. Human-mediated translocations of individuals to areas of unoccupied but suitable habitat have been done to prevent extinction of the subspecies (Lance et al. 2003). Loss of habitat has also been suggested to have caused declines in populations of other fox squirrel subspecies in the southeastern United States, including in eastern North Carolina (Weigl et al. 1989).

Squirrel Population Genetics

A high rate of individuals dispersing from their natal area such as reported for fox squirrels (Koprowski 2005b), should maintain a low level of genetic divergence between different populations and minimize inbreeding assuming that the population size is large enough (Gaines and McClenaghan 1980). A low amount of allozymic variation among fox squirrel populations has been reported from widely separated areas of the southeastern United States (Moncreif 1998). Thus little genetic differentiation between populations is expected in the Sandhills and Coastal Plain of North Carolina unless some factor has prevented the movement of squirrels. Conservation genetic research has documented problems resulting from loss of gene flow populations in several species of squirrels. The Eurasian red squirrel (*Sciurus vulgaris,* L) has undergone a large population decline in Europe due to habitat loss, fragmentation, and invasion by the eastern gray squirrel, and the resulting smaller, isolated populations have low genetic variation (Ogden et al. 2005). Barratt et al. (1999) found genetic divergence between the red squirrel populations of the United Kingdom and continental Europe and concluded these differences were due to genetic drift within the small UK populations rather than evolutionary divergence. Due to habitat fragmentation the European ground

squirrel (*Spermophilus citellus* L) has also experienced a loss of genetic diversity and higher levels of population differentiation compared to ground squirrel species with similar ecological requirements (Slimen et al. 2011). Isolated populations of the northern flying squirrel (*Glaucomys sabrinus* Shaw*)* in the Appalachian Mountains have lower genetic diversity than flying squirrels from more continuous populations (Arbogast et al. 2005, Garroway et al. 2010). The Delmarva fox squirrel also has decreased genetic variation in isolated populations (Moncrief and Dueser 2001, Lance et al. 2003).

CHAPTER 3: MANUSCRIPT

GENETIC AND PHENOTYPIC VARIATION AMONG FOX SQUIRRELS OF EASTERN NORTH CAROLINA

Introduction

The longleaf pine (*Pinus palustris* Mill) ecosystem in North Carolina has been significantly reduced in size by climate change and human activities (Frost 1993). Declines in fox squirrel (*Sciurus niger* L.) populations in the southeast may be due to these changes since they prefer the more open canopy forests of the longleaf pine ecosystem and are displaced by eastern gray squirrels (*Sciurus carolinensis* L.) in more dense forests (Edwards et al. 1998, Lee et al. 2009, Weigl et al. 1989). Loss of its preferred forest habitat concomitant with competition from the introduced eastern gray squirrel resulted in declines in Eurasian red squirrel (*Sciurus vulgaris* L.) populations (Ogden et al. 2005, Barratt et al. 1999). Genetic and phenotypic changes related to population declines from habitat loss can increase extinction risk of species which are already endangered (Lampila et al. 2009). Genetic diversity changes from increased inbreeding and drift, and lack of gene flow often accompany and exacerbate declines in population sizes. For example, variation in MHC genes (Říčanová et al. 2011) and microsatellite loci (Slimen et al. 2012) declined in European ground squirrels (*Spermophilus citellus* L.) due to habitat loss which were related to decreased fitness and increasing extinction risk (Frankham 1995). Fitness declines and extinction risk are related to loss in heterozygosity and inbreeding depression (Reed et al. 2003, Charlesworth and Charlesworth 1987). Evidence of inbreeding depression has been

documented in red wolves (*Canis rufus* Audubon and Bachman) and the Florida panther (*Puma concolor coryi* Bangs) (Brzeski et al. 2014, Hostetler et al. 2013). While not as well studied as some more charismatic animals, inbreeding depression has been documented in small mammals such as the black footed ferret (Wisely et al. 2008), and deer mice (Schwartz and Mills 2004). Wisely et al. (2008) found populations of black-footed ferrets (*Mustela nigripes*, Audubon and Bachman) with lower genetic diversity also had greatly reduced growth rates, and these populations had to be supplemented annually to persist.

The purpose of my study was to quantify phenotypic and genetic diversity of fox squirrel populations in eastern North Carolina.

Specifically:

1. I examined phenotypic variation between fox squirrel populations across geographic regions to determine if genetic or environmental differences were re4lated to variation between the fox squirrels inhabiting different areas of the eastern part of the state.
2. I used Hardy-Weinberg equilibrium to determine if allele frequencies were changing in fox squirrel populations of eastern North Carolina.
3. I compared genetic diversity between the geographic regions to determine if fox squirrels were experiencing population subdivision or inbreeding.
4. I compared gene diversity over time to determine if alleles have been lost over time.
5. I compared heterozygosity levels between fox squirrels of eastern North Carolina and an apparently healthy fox squirrel population from the Midwestern United States to determine if heterozygosity may have been lost in the fox squirrels of North Carolina.

I hypothesized that due to dispersal abilities of fox squirrels and the similarity of the environments they inhabit in North Carolina, phenotypic traits would not vary significantly between regions, and that levels of population subdivision should be low. In addition inbreeding levels should be higher than expected due to the suspected population declines and loss of habitat (Weigl et al. 1989). Inbreeding along with drift in the smaller populations would lead to gene diversity declining over time. Therefore, fox squirrel populations of eastern North Carolina should have lower heterozygosity than their Midwestern conspecifics.

Methods

Population Divisions

To determine if dispersal is providing sufficient gene flow to homogenize genetic and phenotypic patterns of fox squirrel populations, I compared traits across geographic areas with possible natural or man-made barriers. I divided populations between the Sandhills and Coastal Plain areas, populations east vs. west of 78°W longitude, north vs. south of 35°N latitude, and east vs. west of the interstate highway I-95. The Sandhills and Coastal Plain are widely accepted geographic regions in North Carolina, and have slightly different environments. The Sandhills occupy the southern portion North Carolina between the Piedmont and Coastal Plain, including Cumberland, Chatham, Harnett, Hoke, Lee, Montgomery, Moore, Richmond, Robeson, and Scotland counties. The Sandhills are at a higher elevation than the Coastal Plain with coarser soils, and have a higher percentage of hardwoods than the Coastal Plain (Frost 1993). The 78°W longitude division corresponds closely with sections of two interstates (I-95 and I-40) which could pose a barrier to squirrel movement. The 35°N latitude line runs

through an area of largely agricultural lands and developed areas with forests above and below this division. GPS coordinates and collection information for each squirrel sample are shown in Table 1, and a map of all phenotypic and genetic samples can be seen in Figure 1.

Phenotypic Variation

Body size and dark color are thought to be important to survival and/or reproductive success in fox squirrels and other mammals (Weigl et al. 1989, Kiltie 1989, Caro 2005). I obtained body weight, total length, and tail length from records of specimens at the North Carolina Museum of Natural Sciences in Raleigh, NC. I used a grid overlaid on a photograph of the dorsal surface of each study skin to quantify percentage of melanistic dorsal fur. I estimated dorsal fur color variation by counting the total number of patches that differed in color from the surrounding fur on each individual's dorsal surface.

Genetic Variation

I amplified DNA samples from 44 total squirrels (Table 1). Of these samples 18 came from frozen liver samples and 24 were from skin samples acquired from the palm pad of study skins from the Museum of Natural Sciences. One DNA sample was obtained from muscle tissue from a roadkilled squirrel, and one was from a fecal sample acquired from North Carolina State University. All samples were kept frozen until DNA extraction.I used Quigen DNA mini kits specific to the tissue type to extract DNA, and used Microsatellites to quantify the genetic composition of alleles from individuals from each population. I selected Seven microsatellite primer sets (Table 2) already developed for the fox squirrel that had high numbers of alleles per locus and high

individual heterozygosity levels reported for fox squirrels from the Midwest (Fike and Rhodes 2009). Of these, data for primers FO-11 and FO-26 did not reliably amplify DNA from the study skins or fecal sample I used in this study, so subsequently I only used five primer sets (FO-28, FO-33, FO-41, FO-45, and FO-63). All primers used in the study were purchased from Integrated DNA Technologies, Inc. and diluted with Tris-EDTA (TE) buffer for use.

I used 25 µL PCR reactions consisting of 16.3µL of H_2O, 0.5 µL of dNTP, 0.5 µL of $MgCl_2$, 2.5 µL of 10X buffer, 2.5 µL of both forward and reverse primers, 0.2 µL Taq polymerase, and 1 µL of extracted DNA. Amplification conditions consisted of 2 minutes at 94 °C followed by 94 °C for 30 seconds, 15 seconds at 54°C, and 72 °C for 15 seconds for 30 cycles, then 72 °C for 10 minutes and a final extension at 60 °C for 10 minutes to amplify DNA. I used gel electrophoresis to visualize PCR results on a 2% agarose gel to ensure successful amplification before continuing with DNA analysis. I used a TD 3130 DNA sequencer (Applied Biosystems) to determine amplified fragment length, and used GeneMapper version 3.1 software (Applied Biosystems) to determine the number of base pairs in each amplified DNA segment and used this to distinguish alleles present in each fox squirrel sample.

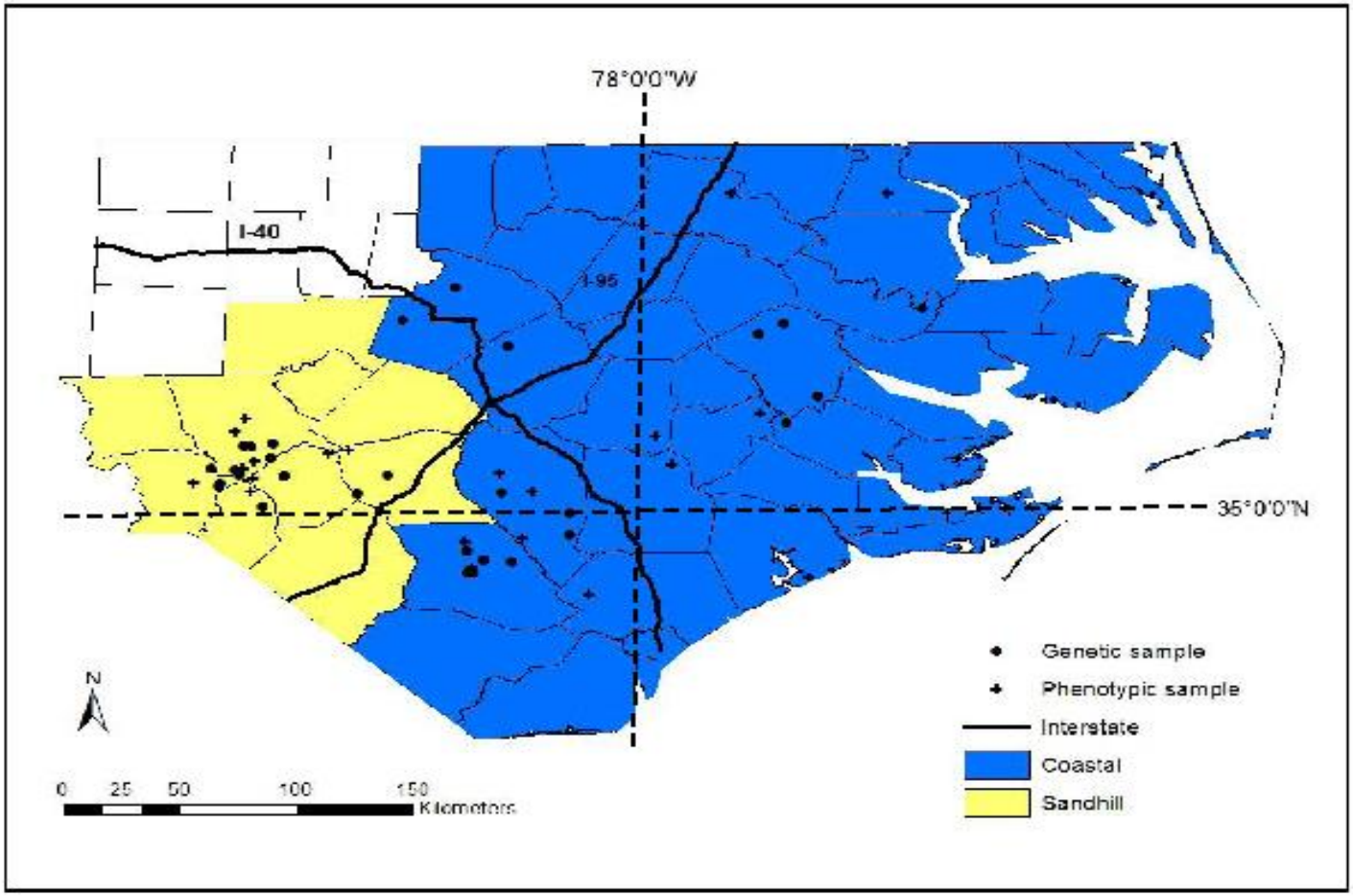

Figure 1. Map of geographic areas of locations of fox squirrel samples from North Carolina. Shaded counties represent areas of fox squirrel habitation

Table 1. Location, collection year, and sample type of fox squirrel samples in eastern North Carolina

Sample Number	County	Latitude (°N)	Longitude (°W)	Year	Genetic Sample	Sample Type*
5128	Wayne	35° 14' 19.5"	77° 53' 3.04"		No	SS
8333	Bladen	34° 37' 45.55"	78° 36' 19.11"		Yes	SS
897	Moore	35° 7' 53.47"	79° 25' 46.2"	1928	Yes	SS
265	Sampson	34°59'30.19"	78 21' 33.76"	1950	No	SS
266	Johnson	35° 23' 23.42"	78 °31' 4.94"	1956	Yes	SS
737	Sampson	34° 47' 10.53"	78° 23' 39.11"	1962	No	SS
635	Moore	35° 15' 43.88"	79° 30' 7.66"	1966	No	SS
638	Moore	35° 11' 43.51"	79° 28' 10.19"	1966	No	SS
639	Moore	35° 11' 43.51"	79° 28' 10.19"	1966	No	SS
640	Moore	35° 11' 43.51"	79° 28' 10.19"	1966	No	SS
641	Moore	35° 11' 43.51"	79° 28' 10.19"	1966	No	SS
643	Craven	35° 17' 59.67"	77° 22' 34.1"	1966	Yes	SS
637	Pitt	35° 25' 6.16"	77° 15' 22.96"	1967	Yes	SS
645	Richmond	35° 1' 58"	79° 39' 51.29"	1967	No	SS
686	Bladen	34° 37' 44.61"	78° 35' 13.91"	1967	No	SS
687	Bladen	34° 37' 45.11"	78° 35' 13.45"	1967	Yes	SS
688	Bladen	34° 37' 45.11"	78° 35' 13.45"	1967	Yes	SS
690	Bladen	34° 37' 45.11"	78° 35' 13.45"	1968	Yes	SS
691	Sampson	34° 48' 4.53"	78° 12' 50.07"	1968	Yes	SS
692	Bladen	34° 41' 15.43"	78° 32' 37.1"	1968	Yes	SS
693	Bladen	34° 41' 15.43"	78° 32' 37.1"	1968	No	SS
694	Bladen	34° 41' 15.43"	78° 32' 37.1"	1968	Yes	SS
695	Bladen	34° 41' 15.43"	78° 32' 37.1"	1968	Yes	SS
697	Lenoir	35° 20' 28.96"	77° 28' 38.6"	1969	No	SS
696	New Hanover	34° 6' 35.53"	77° 57' 6.33"	1970	Yes	SS
1090	Moore	35° 5' 42.25"	79° 28' 20.24"	1970	No	SS
1662	Johnston	35° 38' 31.55"	78° 27' 5.32"	1971	Yes	SS
698	Halifax	36° 19' 42.49"	77° 35' 22.01"	1972	No	SS
2677	Wake	35° 45' 43.38"	78° 51' 32.93"	1975	Yes	SS
1901	Scotland	34° 59' 45.63"	79° 26' 47.86"	1976	No	SS
3051	Duplin	35° 6' 51.55"	77° 49' 8.03"	1977	No	SS
3575	Hoke	35° 3' 45"	79° 18' 44.02"	1981	Yes	SS
3576	Hoke	35° 3' 45"	79° 18' 44.02"	1981	No	SS
4071	Hoke	35° 3' 45"	79° 18' 44.02"	1981	No	SS
3746	Hoke	35° 3' 45"	79° 18' 44.02"	1982	No	SS
3788	Hoke	35° 3' 45"	79° 18' 44.02"	1982	No	SS

Table 1. Continued

3930	Bladen	34° 37' 45.55"	78° 36' 19.11"	1982	No	SS
3931	Hoke	35° 3' 45"	79° 18' 44.02"	1982	No	SS
4170	Moore	35° 4' 40.36"	79° 29' 13.7"	1982	No	SS
8055	Sampson	35° 4' 33.7"	78° 29' 7.72"	1982	Yes	SS
3984	Bladen	34° 46' 16.53"	78° 37' 17.47"	1983	No	SS
6433	Bladen	34° 39' 6.73"	78° 35' 25.04"	1983	No	SS
6426	Hoke	35° 3' 13.82"	79° 26' 0.88"	1986	No	SS
6247	Bladen	34° 37' 45.55"	78° 36' 19.11"	1987	No	SS
6257	Sampson	34° 53' 45.49"	78° 12' 46.22"	1989	Yes	SS
6906	Onslow	34° 36' 39.45"	77° 17' 24"	1991	No	SS
7262	Hertford	36° 19' 35.29"	76° 59' 21.22"	1992	No	SS
14995	Hoke-Cumberland	35° 9' 55.98"	79° 8' 29"	1994	No	SS
17304	Bladen	34° 43' 34.75"	78° 36' 31.93"	1998	Yes	LS
8566	Pitt	35° 41' 52.36"	77° 29' 3.87"	2000	Yes	SS
14996	Moore	35° 11' 43.51"	79° 28' 9.98"	2000	No	SS
8567	Moore	35° 19' 10.48"	79° 27' 58.17"	2001	No	SS
16876	Moore	35° 11' 39.04"	79° 26' 22.59"	2002	Yes	LS
16882	Bladen	34° 40' 35.11"	78° 26' 7.29"	2002	Yes	LS
14998	Scotland	34° 55' 24.16"	79° 23' 47.36"	2003	Yes	SS
16877	Moore	35° 12' 26.49"	79° 21' 20.19"	2004	Yes	LS
17301	Moore	35° 8' 42.21"	79° 22' 0.73"	2004	Yes	LS
13411	Moore	35° 8' 41.53"	79° 22' 0.98"	2005	Yes	SS
13412	Hoke	35° 10' 36.98"	79° 4' 6.02"	2006	No	SS
15489	Moore	35° 3' 53.56"	79° 29' 26.19"	2006	Yes	LS
15001	Richmond	35° 3' 52.02"	79° 37' 56.02"	2008	No	SS
14999	Richmond	35° 1' 4"	79° 33' 50"	2008	Yes	LS
15000	Moore	35° 5' 21.98"	79° 30' 2.98"	2008	Yes	LS
15490	Richmond	35° 1' 34.49"	79° 33' 36.36"	2008	Yes	LS
16878	Wake	35° 54' 22.64"	78° 39' 15.15"	2008	Yes	LS
16879	Martin	35° 48' 43.59"	76° 51' 3.88"	2008	No	LS
16880	Richmond	35° 5' 45.88"	79° 35' 37.78"	2008	Yes	LS
17307	Pender	34° 31' 53.4"	78° 8' 25.22"	2008	No	LS
17303	Moore	35° 8' 42.21"	79° 22' 0.73"	2009	Yes	LS
16881	Pitt	35° 44' 38"	77° 23' 15"	2010	Yes	LS
17308	Cumberland	34° 58' 55.95"	79° 1' 53.93"	2010	No	LS
17309	Cumberland	35° 3' 57.13"	78° 54' 54.53"	2010	No	LS
17305	Sampson	34° 59' 26.8"	78° 28' 29.2"	2011	Yes	LS
22314-02	Moore			2012	Yes	FS
22314-04	Cumberland -Sampson			2014	Yes	MT

*Sample types: SS= Study Skin, LS= Liver Sample, FS= Fecal Sample, MT= Muscle Tissue

Table 2. Information for forward (FW) and reverse (RV) primers and associated florescent labels used for genetic comparisons.

Locus	Primer Sequence	Fluorescent Label	Labeled Primer
FO-11*	FW: CCATTTATGAGGGAGGTAGGG RV: TTGAATCTGTAGATTGGGTAGTATGG	56-FAM	RV
FO-26*	FW: TTTAGAGTCTCGGCTGCTATCC RV: GCTATGGAACCAACCTAAGTGC	5-HEX	FW
FO-28	FW: CCAGGTCAGAATTTACTGGA RV: AGTTCTGGAATTCTCTGTCTCTT	5-HEX	RV
FO-33	FW: ATTTCCCTGGGTTCAATTCC RV: GTGGTTGCTTCCATAATGAGG	56-FAM	FW
FO-41	FW: AGCGTTCTTTAGAGAAACAGAACC RV: AGCCTGGAACGATATCATGG	5-HEX	RV
FO-45	FW: AATTTGTGAAGATCTAACCGAAGC RV: CTGTCTGCCTCTCACACTGC	56-FAM	FW
FO-63	FW: CATAGTCACTTTCAAAGACTATTGATT RV: TTGATTATGGGATACTCTGTAATTC	56-FAM	FW

All fluorescently labeled primers were labeled on 5' end.

* Primer pairs for loci FO-11 and FO-26 were unable to amplify lower quality DNA samples from museum study skins and fecal samples and were excluded from this study

Data Analysis

I compared phenotypic data between areas using ANOVA. I used linear regression to test for linear relationships between phenotypic traits and geographic location. For all statistical tests I used an α-value of 0.05 to determine significance. To look for patterns of allele distribution I plotted the occurrence of each allele of each locus by hand and looked for clusters of alleles.

I used a chi-square goodness of fit test to analyze microsatellite data for Hardy-Weinberg equilibrium by geographic region to determine if allele frequencies were changing, and calculated Wright's F-Statistics to compare the allele frequencies across regions. I calculated observed and expected heterozygosity, and used Fstat computer software (Goudet 2002) to calculate Wright's F-statistics. I used F_{ST} and F_{IS} to determine if there was genetic structuring or inbreeding in fox squirrel populations. I used bootstrap analysis conducted in Fstat to calculate 95% confidence intervals and determine significance of the F statistics. I compared F statistics of populations between the Sandhills and Coastal Plain regions, populations on the eastern and western sides of interstate I-95 which runs north-south through the region, and populations occurring on either side of the 35°N latitude line. F statistics were not used to compare squirrels east and west of 78° W longitude because only six genetic samples were available from east of that longitude. I also used linear regression to determine if gene diversity had changed over time. In addition I compared gene diversity between the samples collected from 1928-1983 (n=18) and those collected from 1986-2014 (n=24), to determine if gene diversity differed between modern samples. These years were chosen because the mid to late 1980's saw the construction of I-40 along with increased

development in eastern North Carolina. I compared observed heterozygosity values to those reported by Fike and Rhodes (2009) from Midwestern fox squirrels for the same microsatellite loci to attempt to determine if heterozygosity levels of North Carolina fox squirrel populations suspected to be declining differed from that of an apparently healthy and expanding population (Koprowski 2005 b). I used paired t-tests with each loci making up a pair to determine statistical significance of differences in observed heterozygosity and gene diversity was determined.

Results

Phenotypic Variation

Sandhills vs. Coastal Plain

Mean values for all phenotypic comparisons are summarized in Table 3. For the Sandhills region the mean weight of fox squirrels was 956.8 g and individual weights ranged from 444.6 g to 1594.2 g compared to a mean of 862.7 g and a range of 483.6 g-1134.0 g for the Coastal Plain. Mean total length for the Sandhills was 570.7 mm and the range was from 412.2 mm to 628.7 mm. For the Coastal Plain the mean total length was 582.3 mm with a range of 469.0 mm to 693.0 mm Mean tail length was 272.6 mm and the range was from 206.2 mm to 383.0 mm. For the Coastal Plain the mean was 257.2 mm and the range 150.0 mm to 308.0 mm. Mean heterozygosity was 0.50, and the mean percentage of melanistic fur was 34.2% for the Sandhills and 0.47 and 29.7% for the Coastal Plain. Fox squirrels of the Sandhills had on average 3.1 patches of different colored fur per individual while those of the Coastal Plain had on average 3.2. per squirrel. ANOVA revealed no significant ($p > 0.05$) differences in any of the phenotypic traits between the Sandhills and Coastal Plain regions.

East vs. West of 78°W Longitude

Fox squirrels west of 78°W had mean weight of 932.7 g with a range of 444.6 g to 1594.2 g and those east of 78°W had a mean of 749.1 g with a range of 483.6 to 990.7. Mean total length west of 78°W was 571.8mm and individual values ranged from 412.2 mm to 693.0 mm. East of 78°W total squirrel length had a mean of 607.5 mm and ranged from 541.0 mm to 691.0 mm. Tail length west of 78°W had a mean of 261.3 mm and ranged from 150.0 mm to 383.0 mm. For populations east of 78°W tail length had a mean of 277.6 mm and ranged from 260.0 mm to 305.0 mm. West of 78°W mean heterozygosity was 0.47 compared to 0.55 east of 78°W. The mean percentage of melanistic fur was 32.2% west of 78°W and 28.1% to the east. West of 78°W fox squirrels had on average 3.1 patches of different colored fur per individual compared to 3.3 to the east. ANOVA analysis showed significant differences in the weight ($p=0.05$), and total body length ($p=0.02$) of squirrel populations separated by the 78°W longitude line, with squirrels to the west being heavier and those to the east being longer.

North vs. South of 35°N

North of 35°N fox squirrels had a mean weight of 940.1 g with a range of 444.6 g to 1594.2 g. South of 35°N the mean weight was 862.5 g and the range was between 483.6 g and 1134.0 g. The mean total length north of 35°N was 575.7 mm and the range was from 412.2 mm to 691.0 mm. South of 35°N the mean total length was 580.2

Table 3. ANOVA results of phenotypic traits and observed heterozygosity for North Carolina fox squirrels.

	Sandhills vs. Coastal Plain			78°W Longitude			35°N Latitude			I-95		
	Sandhills (n=23)	Coastal (n=21)	P-Value	West (n=55)	East (n=16)	P-Value	North (n=28)	South (n=16)	P-value	East	West	P-Value
Weight (g)	956.8	862.7	0.11	932.7	749.1	0.05	940.1	862.5	0.21	860.5	954.7	0.12
Total Length (mm)	570.7	582.3	0.32	571.8	607.5	0.02	575.7	580.2	0.71	582.2	572.6	0.41
Tail Length (mm)	272.6	257.2	0.20	261.3	277.6	0.33	267.3	257.5	0.43	260.1	267.4	0.54
Heterozygosity	0.44	0.48	0.70	0.47	0.55	0.43	0.53	0.39	0.06	0.47	0.50	0.70
% Melanistic	34.2	29.7	0.57	32.2	28.1	0.69	32.5	29.5	0.715	31.5	31.4	0.99
Dorsal Fur Variance (Patches/Squirrel)	3.1	3.2	0.84	3.1	3.3	0.75	3.1	3.2	0.78	3.2	3.1	0.75

* Significant Values are outlined.

mm and the range was between 509.0 mm and 693.0 mm. Mean tail length north of 35°N was 267.3 mm and the range was from 150.0 mm to 383.0 mm. South of 35°N the mean tail length was 257.5 and the range was from 190.0 mm to 308.0 mm. Mean heterozygosity north of 35° N was 0.53 compared to 0.39 south of 35°N. The fox squirrels north of 35°N were on average 32.5% melanistic compared to 29.5% in the fox squirrels to the south of 35°N. Dorsal fur variance was 3.1 color patches per squirrel north of 35°N and 3.2 patches per squirrel south of 35°N. ANOVA showed that none of the phenotypic traits were significantly different between the regions north and south of 35°N. Heterozygosity, however, did show a near significant difference (p=0.06).

East vs. West of I-95

East of I-95 fox squirrels had a mean weight of 860.5 g with a range between 483.6 g and 1134.0 g. West of I-95 the mean weight was 954.7 g and the range was between 444.6 g and 1594.2 g. Total length averaged 582.2 mm east of the interstate and ranged from 469.0 mm to 693.0 mm. West of I-95 the mean total length was 572.6 mm and the range between 412.2 mm and 641.4 mm. Tail length averaged 260.1 mm east of I-95 and ranged between 150.0 mm and 308.0 mm. West of I-95 tail length averaged 267.4 mm and ranged from 160.0 mm to 383.0 mm. Mean heterozygosity was 0.47 east of I-95 and 0.50 west of I-95. On average the dorsal surface of fox squirrels east of I-95 was 31.5% melanistic compared to 31.4% west of I-95. Fox squirrels east of I-95 had on average 3.2 distinct color patches per squirrel compared with 3.1 west of I-95. ANOVA showed no significant differences in phenotypic traits between these areas.

Table 4. Relationships between phenotypic traits and geographic location.

Comparison	R^2	P-value
Weight and Longitude	0.11	0.03
Total Length and Latitude	0	0.87
Total Length and Longitude	0.03	0.15
Heterozygosity and Latitude	0.09	0.06
Heterozygosity and Longitude	0	0.94
Dorsal Fur Variance and Longitude	0	0.54
Dorsal Fur Variance and Latitude	0.01	0.40
% Melanistic and Latitude	0.00	0.98
% Melanistic and Longitude	0	0.86
Tail Length and Longitude	0	0.82
Tail Length and Latitude	0	0.94

Relationships Between Phenotypic Traits and Geographic Location

Linear regression of weight vs. longitude showed a significant linear relationship (Table 4). As seen in Figure 2, there was a significant although slight positive relationship between body weight and west longitude (R^2=0.1067, p= 0.03). All other phenotypic traits did not show a significant relationship with either latitude or longitude. Heterozygosity did show a near significant linear (p=0.06) relationship with latitude with heterozygosity tending to be lower in fox squirrels further to the south.

Genetic Variation

Over the entire state plotting the occurrence of alleles showed alleles were evenly distributed, with only less common alleles being restricted by area.

Sandhills vs. Coastal Plain

The average observed heterozygosity for the Sandhills region was 0.44 and the average observed heterozygosity for the Coastal Plain squirrels was 0.48 (Table 5). The Sandhills had on average 5.6 total alleles and 1.8 rare alleles per locus and the Coastal Plain had an average of 7.0 total alleles and 2.4 rare alleles per locus. ANOVA analysis showed no statistically significant difference between the number of total alleles (p=0.17) or rare alleles (p=0.46) between the Sandhills and Coastal regions. A paired t-test indicated that the difference in H_O was not statistically significant (p=0.75). χ^2 goodness of fit tests (α=0.05) indicated that the Sandhills fox squirrel populations were in Hardy-Weinberg equilibrium only at locus FO-63. The Coastal Plain squirrels showed a departure from Hardy-Weinberg at the FO-33, FO-45, and FO-63 loci. Over all loci the fox squirrels of eastern North Carolina had an observed heterozygosity of 0.467.

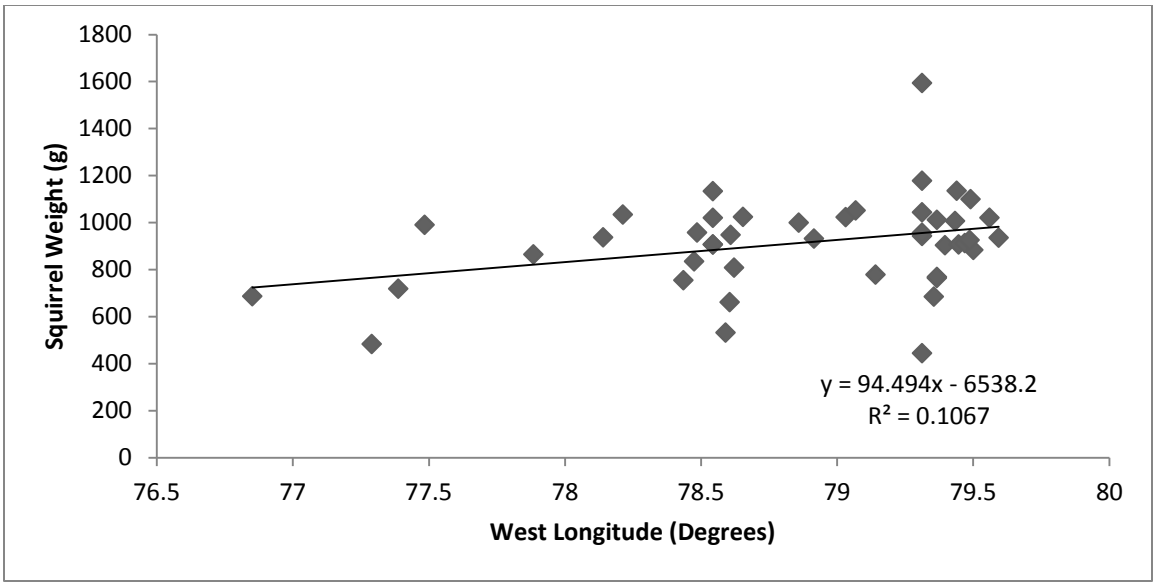

Figure 2. Relationship between squirrel body weight and west longitude

Table 5. Allele, heterozygosity and Hardy-Weinberg analyses for fox squirrel populations in different regions of Eastern North Carolina

Population/Locus	A	Rare	H_O	H_E	HW χ^2	HW P
Sandhills						
FO-28	4	0	0.59	0.68	42.25	0.03
FO-33	6	1	0.25	0.67	66.79	<0.01
FO-41	5	3	0.18	0.06	63.21	<0.01
FO-45	6	3	0.70	0.36	81.82	<0.01
FO-63	7	2	0.50	0.58	12.58	>0.99
Mean	5.6	1.8	0.444	0.470		
Coastal Plain						
FO-28	7	2	0.50	0.71	19.48	0.85
FO-33	6	1	0.47	0.87	36.09	0.02
FO-41	6	3	0.32	0.28	16.34	0.70
FO-45	6	2	0.43	0.35	52.20	<0.01
FO-63	10	4	0.69	0.83	119.00	<0.01
Mean	7.0	2.4	0.482	0.608		
North of 35°N						
FO-28	5	0	0.44	0.61	9.52	0.99
FO-33	6	2	0.39	0.76	16.86	0.66
FO-41	5	2	0.22	0.16	49.26	<0.01
FO-45	6	1	0.85	0.30	142.82	<0.01
FO-63	7	1	0.60	0.61	16.02	>0.99
Mean	5.8	1.2	0.500	0.488		
South of 35°N						
FO-28	7	1	0.56	0.76	15.36	0.96
FO-33	6	1	0.39	0.48	44.10	<0.01
FO-41	5	1	0.25	0.24	12.99	0.88
FO-45	5	0	0.37	0.35	41.86	<0.01
FO-63	8	2	0.37	0.71	36.4	0.97
Mean	6.2	1.0	0.388	0.508		
East of I-95						
FO-28	6	0	0.44	0.73	45.43	0.01
FO-33	6	1	0.50	0.48	43.09	<0.01
FO-41	5	1	0.38	0.32	58.58	<0.01
FO-45	5	3	0.37	0.35	168.80	<0.01
FO-63	9	4	0.37	0.78	13.61	>0.99
Mean	6.2	1.8	0.460	0.532		
West of I-95						
FO-28	6	2	0.56	0.63	16.35	0.95
FO-33	5	1	0.28	0.75	54.22	<0.01
FO-41	4	2	0.11	0.16	23.39	0.27
FO-45	6	2	0.75	0.30	38.40	<0.01
FO-63	6	1	0.60	0.51	44.32	0.85
Mean	5.4	1.4	0.410	0.470		

*A=Total number of alleles, Rare= Number of rare alleles (frequency less than or equal to 5%)

Table 6. Wrights F-Statistics for North Carolina fox squirrel populations

Comparison	F_{ST}	Lower 95% CI	Upper 95% CI	F_{IS}	Lower 95% CI	Upper 95% CI
Sandhills/Coastal	0.069	-0.007	0.042	0.304	0.014	0.421
Interstate 95	0.010	0.002	0.020	0.225	0.006	0.419
35°N	0.013	-0.004	0.029	0.222	-0.001	0.467

The Weir and Cockerham estimate of Wright's F_{ST} across all alleles sampled is 0.069, with 95 % confidence intervals of -0.012 and 0.029. The F_{IS} value is 0.231 with 95% confidence interval of -0.055 to 0.479 (Table 6). The Coastal Plain squirrel populations had a mean of 7.0 total alleles and 2.4 rare alleles per loci, while the squirrel populations of the Sandhills had a mean of 5.6 total alleles and 1.8 rare alleles per loci.

North vs. South of 35°N Latitude

North of 35°N loci FO-28 and FO-33 were in Hardy-Weinberg equilibrium while the other three alleles all showed a departure from the expectations of the Hardy-Weinberg equation (Table 5). Average allelic richness was 5.8 alleles per loci north of 35°N and 6.2 alleles per loci south of 35°N. North of 35°N there were 1.2 rare alleles per loci and 1.0 rare allele per loci to the south of 35°N. ANOVA analysis showed no statistically significant difference between populations north and south of 35°N in total number of alleles (p=0.45) or in the number of rare alleles (p=0.69). The average observed heterozygosity in the northern populations was 0.50 and that of the southern squirrel populations was 0.39. A paired samples t-test revealed no significant difference in the average heterozygosity between the two areas (p=0.36). As seen in Table 6. Wright's F-statistics showed a F_{ST} value of 0.013 (Lower 95% CI= -0.004 and upper 95% CI= 0.029) and a F_{IS} value of 0.222 (95% confidence interval 0.00- 0.413).

East vs. West of Interstate 95

For populations of fox squirrels east of I-95 only FO-63 was in Hardy-Weinberg equilibrium. West of I-95 FO-28, FO-41, and FO-63 were in Hardy-Weinberg equilibrium, while FO-33 and FO-45 deviated from Hardy-Weinberg equilibrium (Table 5). Mean allelic richness was 6.2 and 5.4 for populations east and west of I-95 respectively. On average there were 6.2 rare alleles per loci to the east of the interstate

and 5.4 rare alleles per loci to the west. ANOVA analysis showed no statistically significant difference between populations separated by I-95 in either total number of alleles (p= 0.37) or number of rare alleles (p=0.80).

Average observed heterozygosity for squirrels east of I-95 was 0.46 and for squirrels west of the road average observed heterozygosity was 0.412. There was no significant difference between the average heterozygosity of the squirrels on either side of the interstate (p=0.70). Squirrel populations on either side of interstate I-95 had a F_{ST} value of 0.010 with a 95% confidence interval of 0.002 to 0.020, and a F_{IS} value of 0.225 with a 95% confidence interval between 0.006 and 0.419 (Table 6).

Gene Diversity 1926-1983 vs. 1986-2014

On average there were 5.8 alleles per loci prior to 1983 and 5.2 alleles per loci after 1983. Average gene diversity was 0.63 prior to 1983 and 0.57 after 1983. The comparison of gene diversity before and after 1983 showed no clear pattern of change in gene diversity (Table 7). A t-test comparing mean gene diversity also showed that there was no significant overall difference in mean allelic diversity between the two time periods (p= 0.56). However, FO-41 did show a nearly 5 fold loss of allelic diversity in modern samples, and two of the five total alleles found at the locus were only found prior to 1983.

Table 7. Gene Diversity 1926-1983 vs. 1986-2014

Locus	1926-1983		1986-2014	
	A	Gene Diversity	A	Gene Diversity
FO-28	6	0.775	6	0.678
FO-33	6	0.784	5	0.754
FO-41	5	0.517	3	0.104
FO-45	3	0.408	6	0.564
FO-63	9	0.673	6	0.748
Mean	5.8	0.631	5.2	0.498

Heterozygosity in North Carolina Fox Squirrels vs. Midwestern Fox Squirrels

Heterozygosity of 4 of 5 loci used in this study and the overall average were lower than that reported by Fike and Rhodes (2009) for the Midwestern fox squirrels in their study. Only FO-45 showed a higher heterozygosity in North Carolina than the Midwest (0.577 vs. 0.296) (Table 8).

Table 8. Observed heterozygosity in eastern North Carolina vs. that of Midwestern fox squirrels reported by Fike and Rhodes (2008), and mean observed heterozygosity from both regions.

Locus	H_O North Carolina	H_O Midwest (Fike and Rhodes, 2008)
FO-28	0.500	0.815
FO-33	0.350	0.741
FO-41	0.243	0.593
FO-45	0.577	0.296
FO-63	0.664	0.741
Mean H_O	0.467	0.637

Discussion

With the exception of weight and total length, phenotypic traits did not vary significantly between any of the regions compared. The lack of differences in tail length and fur coloration between regions is likely due to both the low level of genetic structuring found in this study (F_{ST}= 0.01-0.069) and the similarity of the environments inhabited by fox squirrels in eastern North Carolina. The increase in weight in the western portion of the study area may be due to increased habitat quality in the Sandhills compared to the Coastal Plain. Increased habitat quality has been shown to affect body size in other species (Pettorelli et al. 2002). The greater quality of the Sandhills habitats was also asserted by Weigl et al. (1989) based on greater density of fox squirrels in this area. This habitat quality difference would not explain why the more eastern squirrels are longer. The longer body length may represent adaptation to some environmental gradient such as temperature, which has been shown to affect body size in other mammalian species (Quin et al. 1996), or may be due to random differences. Although squirrels south of 35°N had a lower average heterozygosity than those to the north F_{ST} values between 0.010 and 0.069 indicate that population structuring is not occurring at a level to explain this difference. This difference may be the result of random variation between populations, that would be especially likely if the populations to the south of 35°N are small. Gene flow is apparently occurring across the region but inbreeding and/or drift could be resulting in increased homozygosity if populations are small.

Of the five microsatellite loci used, all were found to deviate from the expectations of the Hardy-Weinberg equation in at least one region across all the

comparisons. This departure from Hardy-Weinberg equilibrium means that allele frequencies are changing over time. In fox squirrel populations of eastern North Carolina, the most likely violations of the assumptions of the Hardy-Weinberg equation would come from the decline in fox squirrel populations which have been suggested by Loeb and Moncrief (1993) and Weigl et al. (1989) Decreasing population size would alter allele frequencies via the inbreeding found in this study and also genetic drift. The inbreeding levels found in this study (Overall F_{IS}=0.222-0.304) could also explain the decrease in average gene diversity in the squirrel samples from after 1983. If inbreeding due to declining populations is resulting in lower heterozygosity it may also help to explain why North Carolina populations had lower levels of observed heterozygosity than those reported by Fike and Rhodes (2008) for Midwestern fox squirrel populations which are expanding. While more research is needed to determine how inbreeding levels affect fox squirrels and how North Carolina's fox squirrels compare to other fox squirrels from the southeast, if inbreeding is causing a loss of heterozygosity it could make North Carolina fox squirrels more vulnerable to pathogens and parasites if relevant gene families are negatively impacted (Radwan et al. 2010). Red wolf populations with a mean inbreeding coefficient of only 0.154 were found to be experiencing inbreeding depression and lower fitness. Inbreeding depression has also been shown to decrease survival and fitness in the Florida panther (Hostetler et al. 2013), and small mammals such as deer mice (*Peromyscus maniculatus L*) (Schwartz and Mills 2004), and the common hamster (*Cricetus cricetus* L) (La Haye et al. 2011).

Overall, the level of genetic structure in North Carolina's fox squirrels populations seems to be low. The calculated F_{ST} value (0.069) indicate that genetic structuring

within the North Carolina fox squirrel populations was higher between the Sandhills and Coastal Plain compared to any of the other divisions tested. Along with environmental differences between the two areas, interstate highways, I-40 and I-95 run north to south near this boundary. This study could have underestimated population structuring since many of the samples used are older than the 1990 completion date of I-40 in eastern North Carolina. Even though fox squirrels disperse from their natal areas over great distances and varied terrain (Koprowski 1994, and 2005b), these interstates likely present more of a barrier to fox squirrels than any environmental differences. The F_{ST} value for squirrels divided by I-95 is less than that between the Sandhills and Coastal squirrels by more than a factor of six. This would seem to indicate that I-40 is likely posing a greater barrier to fox squirrel dispersal though the effects are likely cumulative. Despite these barriers it seems enough fox squirrels are able to move between populations to keep genetic structure low. This was also the case with the European ground squirrel where Ćosić et al. (2013) found that a small number of individuals moving between populations over as few as three generations. The lack of population structuring and allelic composition differences among regions suggests changes in genetic diversity are being driven by inbreeding and genetic drift. More research is needed to determine what, if any effects this is having on fox squirrel populations in eastern North Carolina.

CHAPTER 4: LITERATURE CITED

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