EVALUATING TAXONOMIC CLASSIFICATION OF TWO FOX SQUIRREL SUBSPECIES (*SCIURUS NIGER NIGER* & *S. N. SHERMANI*) USING MOLECULAR GENETICS

by

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(Under the Direction of Campbell J. Nairn & Steven Castleberry)

ABSTRACT

Sciurus niger niger and *Sciurus niger shermani* share similar habitat and have similar morphology, raising questions about the validity of the subspecies designations. *Sciurus niger shermani* is of conservation concern in Georgia and Florida, primarily due to habitat loss and uncertainty about population status. Clarification of the taxonomic relationship between these taxa is critical in developing appropriate conservation strategies. I developed novel microsatellite markers and sequenced the mitochondrial d-loop region to investigate genetic diversity and differentiation among populations of the subspecies in Georgia and Florida and examined genetic support for the taxonomic distinction. Consistent with previous studies, I found a lack of phylogeographic structure, but genetic variation among populations showed evidence of population structure. The observed population structure could be due to isolation by distance or it could be an artifact of small sample sizes from several populations. My results are not consistent with a separate taxonomic distinction at the subspecies level, but further studies are needed to examine the genetic variability documented in these populations in the context of other southeastern U.S. subspecies and populations.

INDEX WORDS: Fox squirrel, *Sciurus niger niger*, *Sciurus niger shermani*, microsatellite, mitochondrial sequencing, phylogeography

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B.S.F.R. Forest Resources, University of Georgia, 2011

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

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Electronic Version Approved:

Dean Of the Graduate School
The University of Georgia
December 2013

ACKNOWLEDGEMENTS

This project would not have been possible without the funding provided by the Georgia Department of Natural Resources. I would like to thank the people who helped me collect samples: David Mallard at Fort Benning and the students at the Jones Ecological Research Center. Thank you to Daniel Greene and his advisor Dr. Robert McCleery and all of their volunteers who assisted with sample collection in Florida.

Thank you to my advisors Dr. Steven Castleberry and Dr. Campbell J. Nairn for securing funding of this project and their guidance throughout this experience. I would like to thank my committee members Dr. Mike Conner for his trapping advice and Dr. Bob Warren for valuable insights in preparing this document. Thank you to Brian Shamblin for his guidance in analysis.

A special thank you to family and friends who were there for moral support and guidance.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Although there are many species concepts in the literature, the biological species concept is the most widely used definition to classify vertebrate organisms at the species level. It describes a species as one that is reproductively isolated from other closely related groups and able to produce fertile offspring within the group. However, the same criteria cannot be used to differentiate groups at the subspecies level.

Similar to species, there are multiple definitions of subspecies and no one definition of subspecies has been applied consistently across taxa (Haig et al. 2006). Traditionally, subspecies were classified based on variation in morphological characteristics (Haig et al. 2006). There are many issues with using minor morphological differences as they can lead to uncertain taxonomic classifications and misclassifications (Pampouile & Danielsdottir 2008). Another definition describes subspecies as populations that are on a trajectory towards speciation (Frankham et al. 2004). That is, the populations are on their way to being reproductively isolated, but have not yet reached that point. Consequences of inaccurate taxonomy include extinction of unrecognized endangered species or subspecies and denial of legal protection for endangered species or subspecies (Williams & Clemer 1989, Frankham *et al.* 2004), or alternatively, protecting a species or subspecies that does not warrant protection resulting in inefficient use of available resources (Laerm *et al.* 1981, Frankham et al. 2004).

Perhaps the most appropriate concept for classification of both species and subspecies is the genetic species concept. It is a modern approach that relies on genetic isolation rather than reproductive isolation by considering speciation, genetically defined phylogroups, hybridization, and genetic differentiation between phylogroups that are morphologically indistinct (Baker & Bradley 2006). This concept uses molecular genetic techniques to examine the genetic composition of individuals, and therefore, provides a way to calculate genetic variation and examine relationships at the subspecies and population levels. Genetic distance and genetic differentiation are used to measure the differences between and among populations. However, a limitation of this approach is that no threshold distance or differentiation values exists across taxa; therefore, values appropriate for a given taxon are determined from published literature.

My study focuses on the genetic diversity of two described subspecies of fox squirrel (*Sciurus niger*), the eastern fox squirrel (*S. n. niger*) and Sherman's fox squirrel (*S. n. shermani*) (Moore 1957). The two subspecies are morphologically very similar and share overlapping ranges in southern Georgia and northern Florida. My objective was to examine diversity and differentiation among populations of both subspecies in Georgia and Florida and examine genetic support for the taxonomic distinction. Information regarding genetic variability within and among populations of the two subspecies can be used to develop appropriate management strategies.

LITERATURE REVIEW

Sciurus niger Taxonomic Status

Currently, there are ten recognized subspecies of *S. niger* in the United States ranging from Delaware to Florida, west to Texas, and north to South Dakota. There are also introduced populations in California. Eastern subspecies average about 300 g larger than the western subspecies on average and are often gray, agouti, or sometimes all black in coloration as opposed to reddish brown in coloration (Weigl *et al.* 1989). In the Southeast, *S. niger* prefers mature pine forests and mixed pine-hardwood forests (Perkins *et al*. 2004) to provide food and nesting habitat (Weigl *et al.* 1989, Kantola & Humphrey 1990).

Southeastern populations of *S. niger* are generally considered to be declining due to a loss of habitat (Loeb & Moncrief 1993). *Sciurus n. niger* and *S. n. shermani* utilize mixed pine and hardwood forests including the longleaf pine ecosystem (Perkins 2004, Brockway *et al.* 2005) and it is estimated that longleaf pine exists in less than 3% of its historic range (Brockway et al. 2005). *Sciurus n. shermani* has been declared a “species of concern” by the Florida Fish and Wildlife Conservation Commission (Florida Fish and Wildlife Conservation Commission, 2011) and is listed as a High Priority Species in Georgia’s State Wildlife Action Plan (Georgia Department of Natural Resources, 2005). However, the closely related subspecies, *S. n. niger*, located in the northern portion of Florida and throughout Georgia, South Carolina and North Carolina, is not listed by any state. The two subspecies have adjacent ranges and similar pelage patterns creating confusion in subspecies differentiation (Moore 1956).

As a species, the pelage patterns of *S. niger* represent a wide spectrum ranging from solid black, tan agouti, gray, or any combination in eastern populations (Calahane 1961; Moore 1956) to a light reddish brown in western populations (Lowrey 1974). However, distinguishing eastern subspecies populations can be difficult using pelage pattern alone (Moore 1956).

Sciurus niger shermani was first described as a separate subspecies by Moore (1957). He described the range as most of peninsular Florida and probably into southeastern Georgia. Morphologically he described *S. n. shermani* as having a tan common phase color as opposed to gray-white common phase color typically found in *S. n. niger* populations. He also described the ear tips as white or tan and the feet as tan or occasionally buff. He compared skull characteristics of 11 *S. n. niger* and 87 *S. n. shermani* an concluded that *S. n. shermani* had slightly larger skull measurements. Although he stated that the skull morphological differences between *S. n. niger* and *S. n. shermani* were insufficient to differentiate the two subspecies, he included the measurements to show the extent of variation that exists between them.

Clarifying Misclassification Using Molecular Genetics

Without accurate classification, effective conservation and management of species or subspecies is difficult (Cotterill 1995). To avoid negative consequences of incorrect taxonomic assignment, molecular genetics can be used to accurately determine taxonomic relationships of organisms under study when morphological differences are insufficient to determine proper classification. Molecular genetic techniques are commonly used to examine or re-examine taxonomic classifications (Williams and Clemmer 1989, Larem *et al.*1981).

The genetic species concept is a common approach in examining taxonomic classifications. In the case of the now endangered Alabama Sturgeon (*Scaphirhynchus suttkusi*), the species had similar morphology to another species in the genus *Scaphirhynchus* living in the same habitat (Williams & Clemer 1989). The Alabama Sturgeon was misclassified as Shovelnose Sturgeon (*Scaphirhynchus platorhyncus*) until

1989 when molecular genetic techniques were used to declare *S. suttkusi* as a separate species (Williams & Clemer 1989). The species was then listed as Endangered in 2009 (U.S. Fish and Wildlife, 2009). In this example, molecular genetics was useful in protecting a species that had previously been grouped with a morphologically similar species.

A similar case of taxonomic misclassification occurred with two pocket gopher species, *Geomys pinetis* and *Geomys colonus*, living in adjacent ranges. The species were distinguished by pelage pattern and minor cranial differences (Bangs 1898). The population size of *G. colonus* was determined to be approximately 100 individuals occupying a small area (Ford 1980), and was subsequently listed as endangered (Odom *et al.* 1977). Using molecular genetic techniques, Laerm *et al.* (1981) determined that genetic differentiation values to be too low to discriminate at the subspecies level. Although genetic structure existed among populations, it was not consistent with the taxonomic distinction of *G. colonus* at the species level. The information permitted the Georgia Department of Natural Resources (DNR) to utilize resources previously dedicated for protection of the putative endangered *G. colonus* subspecies and allocate them towards truly endangered species (Laerm *et al.* 1981).

Previous *Sciurus niger* Genetic Studies

Previous studies have used molecular genetics to examine differentiation in *S. niger* at the species and subspecies level. At the species level, Moncrief *et al.* (2006) identified a diagnostic marker to differentiate *S. niger* from *S. caronlinensis*. They compared ten microsatellite loci across individuals in both *S. carolinensis* and *S. niger* populations. One locus consistently amplified as polymorphic alleles in *S. carolinensis* and as a monomorphic allele in *S. niger*. Although the marker differentiated the two species, it was monomorphic for all subspecies of *S. niger*. Thus, the marker was not informative on the subspecies level.

Fike and Rhodes (2009) characterized 26 microsatellite markers for use in examining genetic diversity in *S. niger* and examined cross-utilization in gray (*S. carolinensis*) and the red squirrels (*Tamiasciurus hudsonicus*). They found that of the 26 loci that were polymorphic in *S. niger*, 22 were polymorphic in *S. carolinensis* and seven were polymorphic in *T. hudsonicus*. Fike *et al.* (2013) developed 11 microsatellite markers for *S. carolinensis* and tested them for cross utilization in *S. niger* and *T. hudsonicus*. Of the 11 loci polymorphic in *S. carolinensis*, eight were polymorphic in *S. niger* and four were polymorphic in *T. hudsonicus*. However, to date these markers have not been utilized to examine genetic diversity and structure in *S. niger* at the population or subspecies level.

Moncrief *et al.* (1993) attributed a recent expansion during the Pleistocene as the reason for low genetic differentiation among populations of *S. niger* in the lower Mississippi River valley, including individuals from both the west and east sides of the Mississippi River. They assayed 35 loci, of which, three exhibited east to west patterns of genetic divergence. Differences in morphology also were observed between eastern and western individuals suggesting a geographic barrier to gene flow has increased genetic differentiation. Currently, there are no known microsatellite markers in the literature that have been used to differentiate *S. niger* at the subspecies level.

Moncrief *et al*. (2010) sequenced a 402 nucleotide segment of the mitochondrial cytochrome-*b* gene in 102 *S. niger* individuals from a variety of populations, including *S.*

n. niger and *S. n. shermani* and found high haplotype diversity across the entire sampling population. They found a total of 22 haplotypes, of which four were frequent and widely distributed. The remaining 17 haplotypes were considered to be unique, however, these tended to only differ from the more common haplotypes by one or two nucleotide substitutions. The lack of structured nucleotide diversity was again presumed to be due to the recent and rapid population expansion following the glacial retreat in the late Pleistocene. They concluded that there is insufficient evidence to support phylogeographic structure in *S. niger*, that is, the subspecies lack evidence of different historic geographic ranges.

Moncrief *et al.* (2012) conducted a similar study to examine sequences from both the cytochrome-*b* gene as well as a 486 base pair region of the mitochondrial D-loop for 81 *S. niger* individuals sampled from throughout the range, excluding subspecies from Florida. Similar to Moncrief *et al*. (2010), they found high haplotype diversity. By expanding the sequencing region to include a portion of the D-loop they were able to define 55 haplotypes. The haplotypes that occurred in more than two localities were classified into three groups consisting of 16, six, and nine haplotypes, respectively. These were found in as few as two and as many as 16 localities. The haplotypes that did not fit into the groups were found throughout *S. niger*'s range. They were able to again conclude that as a whole, *S. niger* lacks phylogeographic structure.

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CHAPTER 2

Nuclear Microsatellite Marker Development for *Sciurus niger*[1]

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[1] Todd, R. R., Nairn, C. J., Castleberry, S. B, Conner, M., Shamblin, B. M. To be submitted to Conservation Genetics Resources

ABSTRACT

The eastern fox squirrel (*Sciurus niger*) has 10 subspecies throughout its range, most of which are found in the eastern United States. However, recent studies have found little evidence of genetic structure among *S. niger* as a whole leading to difficulty in confirming subspecies classification. We identified 8 polymorphic loci that could be used to examine genetic diversity and structure in *S. niger* populations. Observed heterozygosity ranged from 0.20 to 0.68 with a mean of 0.53. The microsatellite suite has a combined non-exclusion probability index of 5.56×10^{-5}. These markers will be informative in differentiating individuals on a subspecies level and other population-focused analyses.

INTRODUCTION

The eastern fox squirrel (*Sciurus niger*) consists of 10 described subspecies located throughout the eastern half of the United States as well as introduced populations in the west (Koprowski 1994). Some subspecies of *S. niger* have been differentiated based on minor morphological differences (Moore 1957); however, within the eastern subspecies there is little evidence of significant morphological differences (Moore 1956). Both pelage patterns and skull measurements have been shown as a poor determining characteristics of subspecies identification (Moore 1957). Sympatric taxa differentiated by minute morphological differences can lead to difficulty in identification and classification and consequently inefficient use of available management resources (Ford 1980).

Sciurus niger populations have declined in parts of the distribution resulting in designation of some subspecies as endangered or of conservation concern (Loeb and

Moncrief 1993). As a first step in developing effective and efficient management strategies for these subspecies, a robust assessment of the validity of subspecies designations is needed. Additionally, characterizing the distribution of genetic diversity within and among populations will also contribute to the overall understanding of processes affecting gene flow and population structure. We developed 8 novel polymorphic microsatellite markers for *S. niger* to facilitate examination of genetic diversity of *S. niger* subspecies and populations.

METHODS

Ear or organ tissue was taken from 25 road killed- individuals at the Jones Ecological Research Center in Baker County, Georgia. Samples were stored at -20^o C until genomic DNA was isolated from the tissue. Total genomic DNA was isolated from a female fox squirrel from the sample population at the Jones Ecological Research Center near Albany, Georgia using the DNEasy Blood and Tissue Kit (Qiagen). Methods described by Glenn and Schable (2005) with minor modifications were used to enrich genomic DNA for microsatellite loci. Restriction endonucleases RsaI and HpyCH4V (New England Biolabs) were used to digest DNA at 37°C. Digested genomic DNA was ligated to double stranded SuperSNX linkers (Glenn and Schable 2005) overnight at 16°C. Linker-ligated genomic DNA was amplified by the polymerase chain reaction (PCR) on an Applied Biosystems 9700 thermal cycler. Amplification products were analyzed by agarose gel electrophoresis for verification. The PCR amplified DNA was hybridized to 2 different mixtures of biotinylated oligonucleotide probes (Integrated DNA Technologies, mix 3: (AAAC)6, (AAAG)6, (AATC)6, (AATG)6, (ACAG)6, (ACCT)6, (ACTC)6, (ACTG)6; mix 4: (AAAT)8, (AACT)8, (AAGT)8, (ACAT)8, (AGAT)8). Streptavidin-coated magnetic beads (DynaBeads, Invitrogen) were used to capture biotinylated probes and hybridized DNA. Captured DNA was washed and eluted by dissociation from the biotinylated probes at 95°C. The enrichment process was repeated to increase the efficiency of microsatellite array capture. After the second enrichment DNA was used as a template for amplification by two rounds of PCR amplification. These products were then ligated into the pCR 2.1-TOPO vector and transformed into *Escherichia coli* TOP 10 competent cells (Invitrogen). Transformation reactions were then plated on LB containing 100 µg/ mL ampicillin and 2 µg Xgal/ mL for selection.

Colonies containing insert DNA were sampled using sterile toothpicks and placed in 10 µl ddH_20. Colonies were amplified by PCR in 20 µl reactions containing 10 mM Tris, pH 8.4, 50 mM KCL, 0.5 µM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U AmpliTaq Gold polymerase (Applied Biosystems). Cycling parameters were set at 95°C for 2 minutes, then 45 cycles of 95°C for 20 s, 55°C for 30 s, 72°C for 1 minute 45 s, and a final extension time of 10 minutes at 72°C. Reactions were then treated with Exo-Sap-IT (New England Biolabds) and sequenced using BigDye v.3.1 chemistry (Applied Biosystems). Sequencing reaction clean up and removal of unincorporated dyes was carried out by passage of reaction over Sephadex G-50 fine (Sigma Alrich). Reactions were analyzed on an Applied Biosystems 3730*xl* DNA Analyzer. Geneious v.6.1.2 (Biomatters) was used to align and edit sequences. Contigs containing simple sequence repeats were identified and amplification primers for 44 novel loci were designed using PRIMER3 (Rosen and Skaletsky 2000).

Amplification of microsatellite loci was performed in 10 µl reactions consisting of 10 mM Tris pH 8.4, 50 mM KCL, 0.5 µl GTTT "pig-tailed" locus specific primer, 0.05 µM CAG (CAGTCGGGCGTCATCA) or M13 (GGAAACAGCTATGACCAT) tagged locus specific primer, 0.45 µM fluorescently labeled CAG or M13 tag (Boutin-Ganache *et al.* 2001), 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.5 U AmpliTaq Gold polymerase, and approximately 2-5 ng of diluted genomic DNA template. CAG and M13 labeled primers included VIC, PET, NED (Applied Biosystems), and FAM (Integrated DNA Technologies) fluorophores. Cycling parameters (Don et al. 2001) were 95°C for 5 min, 20 cycles of 95°C for 30s, 60°C minute 0.5°C per cycle for 30s and 72°C for 1 min and a final extension of 72°C for 10 min. Size standard LIZ500 (Applied Biosystems) was added to reactions and fluorescently labeled amplicons were analyzed on an 3730*xl* DNA Analyzer (Applied Biosystems). Allele sizes were scored using GeneMarker v.2.20 (SoftGenetics).

Data analyses and formatting were carried out using GeneMarker® (SoftGenetics) to score and size alleles. Genotype data were used to calculate allelic richness (k), observed heterozygosity (H_o), expected heterozygosity (H_E), and deviations from Hardy-Weinberg Equilibrium (HWE), using CERVUS 3.0 (Kalinowski et al. 2007). Linkage disequilibrium (LD) was calculated using GENEPOP (Raymond and Rousset 1995), as well as sequential Bonferroni correction (Rice 1989).

RESULTS

Approximately 400 individual colonies were screened for genomic DNA inserts and sequenced. Forty-four primer pairs were designed and screened for amplification consistency using 25 individual samples from the study population of *S. niger*. Eight of these loci amplified consistently and were polymorphic (Table 2.1). None of the eight loci deviated significantly from HWE. No loci exhibited linkage disequilibrium following sequential Bonferroni correction (Rice 1989). The number of alleles per locus ranged from 2-6 with a mean of 3 across all 8 loci. The microsatellite panel has a combined non-exclusion probability of identity equaling 5.56×10^{-5}. The error rate calculated using GeneMarker was 2.0%. These nuclear microsatellite markers will be useful for studies examining genetic diversity and its distribution in subspecies and populations of *S. niger*.

ACKNOWLEDGMENTS

We thank students and staff at the Jones Ecological Research Center for assistance with sample collection. We thank D. Greene and R. McCleery from the University of Florida. The Georgia Genomics Facility assisted in the final steps of marker development. Funding was provided by the Georgia Department of Natural Resources through the State Wildlife Grants Program.

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Table 2.1 Characterization of microsatellite loci amplified in 25 *Sciurus niger* samples from the Jones Ecological Research Center, Baker County, Georgia

Locus	Primers	Repeat	N	k	Size Range	H_o	H_e	P_{HW}	Null
~~Sn13 F~~	~~GTTTGCTGCAGTCATCAATCCCAG~~	~~(GTTT)^9~~	~~25~~	~~3~~	~~165-173~~	~~0.64~~	~~0.56~~	~~0.828~~	~~-0.076~~
~~Sn13 R~~	~~**CAG**TCTCCCAGAAACCTCCAAC~~								
Sn19 F	GTTTCCACTCTATGTTGGCTTTCAATAGG	(CATA)^11	25	6	285-309	0.68	0.77	0.127	0.048
Sn19 R	**CAG**CCACTGATTTGGGAGGCTG								
Sn110 F	**CAG**CCTGGGTTCAATCGTCACTAC	(CAAAA)^7	25	2	150-155	0.36	0.30	0.652	-0.969
Sn110 R	GTTTGCAGCCTGAAGAGGGAGTTA								
Sn115 F	**CAG**TCAGGCTGAGTTCAATCCTCGTAAC	(TAAA)^8	25	2	172-180	0.32	0.47	0.193	0.180
Sn115 R	GTTTCAAGAAACAGTCCCTGTGTATCA								
Sn116 F	**CAG**TCATGGGCAGGTACTATGTCTTCTTA	(GT)^13	25	3	201-207	0.56	0.58	0.064	-0.004
Sn116 R	GTTTCCTCAGGAAATTCACCCTATTA								
Sn118 F	**CAG**TAGGCATGTTAACCGAGAATCT	(GT)^13	25	2	233-235	0.20	0.30	0.240	0.192
Sn118 R	GTTTAAACCAATCACCACCTTGTTAC								
Sn132 F	**CAG**CAAGAATCTGGAGGATTGACTGTA	(GT)^14	25	2	241-243	0.44	0.51	0.702	0.061
Sn132 R	GTTTGGAAAGCAACAGAGGCTTCTAA								
Sn134 F	**CAG**CAGTTAGAATGACAGCCATCAA	(TAGA)^9	24	4	228-240	0.63	0.71	0.535	0.057
Sn134 R	GTTTCACATCCTCTCCAGCATTTATT								

N is the number of individuals successfully amplified of 25 attempted. k is the number of alleles. H_O is the observed heterozygosity. H_E is the expected heterozygosity. P_{HW} is the probability the locus is in Hardy-Weinberg equilibrium. Null is the null allele frequency estimate.
CAG tag: CAGTCGGGCGTCATCA
All sequences will be submitted to Genbank for reference numbers

CHAPTER 3

EVALUATING TAXONOMIC CLASSIFICATION OF TWO FOX SQUIRREL SUBSPECIES (*SCIURUS NIGER NIGER* & *S. N. SHERMANI*) USING MOLECULAR GENETICS[1]

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[1] Todd, R. R., Nairn, C. J., Castleberry, S. B., Greene, D., McCleery, R. To be submitted to Conservation Genetics.

ABSTRACT

Fox squirrel (*Sciurus niger*) subspecies in the southeastern United States are difficult to distinguish based on external morphology, raising questions about subspecific designations. *Sciurus niger niger* and *S. n. shermani* are sympatric in parts of their range and share similar pelage patterns and morphology making discrimination difficult using physical characteristics. Proper management depends on accurate subspecies identification. *Sciurus niger shermani* has been listed as a species of concern in Florida and Georgia due to habitat loss and uncertainty about population status. We used novel microsatellite markers and mitochondrial D-loop sequencing to examine the genetic differentiation between populations of *S. n. shermani* and *S. n. niger*. Microsatellite analyses were consistent with a lack of overall phylogeographic structure reported in previous studies of *S. niger*. Using mitochondrial sequencing, we found 82 unique haplotypes throughout the sampling range showing high haplotype diversity, typical of small mammals. The lack of structure between the two described subspecies is not consistent with existing taxonomic distinction of *S. n. niger* and *S. n. shermani*.

INTRODUCTION

Currently, there are ten recognized subspecies of the fox squirrel (*Sciurus niger*) in the United States ranging from Delaware to Florida, west to Texas, north to South Dakota, with introduced populations in California. Eastern subspecies average 300 g larger than western subspecies on average and are often gray, agouti, or sometimes all black in coloration compared to a reddish brown coloration (Weigl *et al.* 1989). Southeastern populations of *S. niger* generally are considered to be declining due to a loss of habitat (Loeb and Moncrief 1993). *Sciurus n. shermani* has been declared a "species of concern" by the Florida Fish and Wildlife Conservation Commission (Florida Fish and Wildlife Conservation Commission, 2011) and is listed as a High Priority Species in Georgia's State Wildlife Action Plan (Georgia Department of Natural Resources, 2005). However, the closely related subspecies, *S. n. niger*, located in the northern portion of Florida and throughout Georgia, South Carolina and North Carolina, is not listed by any state. These two subspecies have adjacent ranges and similar pelage patterns creating confusion in subspecies differentiation (Moore 1956).

Sciurus niger shermani was first described as a separate subspecies based on morphology. Moore (1957) described *S. n. shermani* as having a tan common phase color as opposed to gray-white common phase color typically found in *S. n. niger* populations. He described the ear tips as white or tan and the feet as tan or occasionally buff. He also compared skull characteristics of 11 *S. n. niger* and 87 *S. n. shermani* and concluded that *S. n. shermani* had slightly larger skull measurements. Although he stated that the skull morphological differences between *S. n. niger* and *S. n. shermani* were insufficient to differentiate the two subspecies, he included the measurements to show the extent of variation that exists between them. The lack of significant differences in skull measurements and the presence of continuous variation in pelage coloration across the putative subspecies suggest that these morphologic differences may not provide a robust basis on which to differentiate the two putative subspecies.

As a species, *S. niger* lacks phylogegraphic structure. Moncrief *et al.* (1993) found low levels genetic differentiation in a study that examined overall genetic variation at 35 microsatellite loci across numerous populations in the lower Mississippi River valley. Using mitochondrial sequencing, Moncrief *et al.* (2010) and (2012) found high haplotype diversity among populations of *S. niger*, to which they again concluded there was a lack of phylogeographic structure.

Given the uncertainty regarding the taxonomic status of the subspecies, we examined genetic diversity and structure between *S. n. niger* and *S. n. shermani* populations using novel microsatellite markers and mitochondrial sequencing. Our results will provide information regarding patterns of genetic diversity in the two subspecies that can be used to inform science-based management decisions.

MATERIALS AND METHODS

Study Area

I collected *S. niger* tissue for genetic analysis from 11 fox squirrel populations ranging from southwestern Georgia to central Florida (Figure 3.1). The three *S. n. niger* populations were sampled in Chattahoochee (CHT; n = 17) and Baker (BKR; n= 24) counties, Georgia and Jefferson (JEF; n=5) county Florida. Samples from *S. n. shermani* populations were collected from Suwannee and Lafayette combined (SULA; n = 5), Clay (CLY; n = 5), Levy (LEV; n = 12), Putnam (PTNM; n = 16), Marion (MRN; n = 7), Citrus (CIT; n = 8), Hernando (HERN; n = 5), and Sumter (SUM; n = 5) counties, Florida. Only populations represented by a minimum of five samples were included in the analysis of nuclear microsatellite genotypes. However, miscellaneous locations represented by fewer than five individuals were used for examination of haplotype diversity.

Sample Collection

Tissue samples from ears or liver were collected from harvested or road-killed squirrels. Ear biopsy punches were taken from live-captured individuals. Samples were stored in 95% ethanol at room temperature. DNA extraction was accomplished using a DNEasy® Blood and Tissue extraction kit (Qiagen) according to the manufacturer's protocol. DNA was quantified using a Qubit™ fluorometer (Invitrogen). All samples were diluted to a standard concentration of 10ng/µl.

Mitochondrial Sequencing

We designed two primers (Sn-CYB-1F-TGAATTGGAGGACAACCAGTTGAA, Sn-12s-4r-GATGGAGATAGAGGGCATTCTCACTG) to amplify a 1496 bp nucleotide region representing the majority of the mitochondrial genome control region (D-loop). The mitochondrial control region was amplified from each sample of genomic DNA using PCR. Amplification was performed in 20 µl reactions consisting of 10 mM Tris, pH 8.4, 50 mM KCL, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 U of AmpliTaq Gold polymerase (Applied Biosystems), 5.0 µM forward amplification primer, 5.0 µM reverse amplification primer (Integrated DNA Technologies), and 10 ng of genomic DNA. Reactions were arrayed in 96-well plates and carried out using single or dual block 9700 thermal cyclers (Applied Biosystems). Cycling parameters were 5 minutes at 95°C then 30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute 45 seconds at 72°C and a final 10 minute extension at 72°C.

Mitochondrial DNA amplicons were evaluated by agarose gel electrophoresis and treated with ExoSAP-IT® according to the manufacturer's protocol (United States Biochemical Corporation). We designed six primers to sequence mitochondrial amplicons (SnMit-3f: AATTGGAGGACAACCAG, SnMit-472f: GTACATACATACTCATATAGTAC, SnMit-970f: TGTAGCTGGACTTTAGG, SnMit-628r: GATAGTCATTTGGACGA, SnMit-1035r: CTATGTCCATCAAGCATG, SnMit-1474r: AGATAGAGGGCATTCTC). Amplicons were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction mix (Applied Biosystems) and purified by passage over Sephadex G-50 Fine (Sigma-Aldrich). Sequencing reactions were analyzed using a 3730xl DNA Analyzer (Applied Biosystems) at the University of Georgia Genomics Facility.

Sequence Analysis

Sequence trace files were imported into Geneious© software, trimmed, and aligned to identify mitochondrial DNA sequence variants (haplotypes). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was also determined by Geneious© to determine haplotype relationships. Pairwise Φ_{ST} comparisons and genetic distance-based analysis of molecular variance (AMOVA) were conducted using the software Arlequin version 3.5 (Excoffier *et al.* 2005). Significance values for AMOVA were obtained from 10,000 permutations. Correlation between genetic and geographical distances was determined using a Mantel test in Arlequin 3.5 (Excoffier *et al.* 2005). Genetic distance was represented by $F_{ST}/(1\text{-}F_{ST})$.

Spatial analysis of Molecular Variance (SAMOVA) was conducted to define population groups by maximizing genetic partitioning among groups (Φ_{CT}) (Dupanloup *et al.* 2002). Population grouping was tested for a range of k values from 2-11, the latter representing the number of sampling locations. Optimal clustering was achieved when Φ_{SC} (the genetic differentiation partitioned among sample locations within groups) became negative.

Microsatellite Markers

Amplification of nuclear microsatellite loci was performed in 10 µl reactions consisting of 10 mM Tris pH 8.4, 50 mM KCL, 0.5 µM GTTT "pig-tailed" locus specific primer, 0.05 µM CAG (CAGTCGGGCGTCATCA) or M13 (GGAAACAGCTATGACCAT) tagged locus specific primer, 0.45 µM fluorescently labeled CAG or M13 tag (Boutin-Ganache *et al.* 2001), 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.5 U AmpliTaq Gold polymerase, and approximately 2-5 ng DNA template. Locus-specific primers for amplification were individually labeled with VIC, PET, NED (Applied Biosystems), or FAM (Integrated DNA Technologies) fluorophores. Touchdown cycling parameters (Don *et al.* 2001) were 95°C for 5 min, 20 cycles of 95°C for 30s, 60°C minus 0.5°C per cycle for 30s and 72°C for 1 min and a final extension of 72°C for 10 min. Size standard LIZ500 (Applied Biosystems) was added to the reactions and analysis conducted on an 3730*xl* DNA Analyzer (Applied Biosystems). Trace files were imported and allele sizes were scored using GeneMarker® v.2.20 (SoftGenetics).

Microsatellite Marker Analysis

GeneMarker® was used to score allele sizes and format data. Genetic structure was initially examined using a Bayesian clustering approach in program STRUCTURE (Pritchard *et al.* 2000). Several values of k were examined under a model assuming no admixture and correlated population allele frequencies. Sampling locations were included as a prior to inform the model of the true population groupings. All runs consisted of 10,000 iteration burn-in followed by 10,000 Markov Chain Monte Carlo (MCMC) repetitions. Analysis in STRUCTURE estimated the probability of populations for various numbers of assumed populations (k). The appropriate estimation of k has the lowest log likelihood (Ln P(D)).Pairwise F_{ST} comparisons and exact tests of population differentiation were calculated using Arlequin 3.5 software (Excoffier *et al.* 2005). Microsatellite F_{ST} values were significant at a p-value of 0.05. Exact tests of population differentiation were conducted with 100,000 permutations and 10,000 dememorization steps. Mantel testing was used to determine the correlation between genetic and geographical distances also using Arlequin 3.5. Genetic distance was represented by $F_{ST}/(1-F_{ST})$ (Slatkin 1995). Expected heterozygosity (H_E) was calculated using CERVUS 3.0 (Kalinowski et al. 2007).

RESULTS

We successfully genotyped 106 of the 109 samples at eight microsatellite loci. All 109 samples from microsatellite analyses as well as an additional 37 samples were successfully sequenced for the 1300 base pair D-loop.

Genetic Diversity

Of the 136 samples sequenced, 82 unique haplotypes with 108 variable positions were identified. Most haplotypes were observed in only one individual; however, some haplotypes were shared between two or more individuals. UPGMA analysis partitioned the 82 haplotypes into three major haplogroups (Figure 3.2). Of the 11 populations, Haplogroup A was only represented in CHT and BKR, Haplogroup B was represented in all populations except CIT and HERN, and Haplogroup C was represented in all populations (Figure 3.3). Haplotype diversity ranged from 0.893-1.000 and nucleotide diversity ranged from 0.005-0.012 (Table 3.1).

Allelic richness ranged from two to eight alleles per locus with a mean of 3.6 (Table 3.2). Average expected heterozygosities ranged from 0.4493 to 0.5472 (Table 3.2).

Population Structure

Of 163 pairwise Φ_{ST} comparisons, 25 were significantly different (Table 3.4). Mitochondrial Φ_{ST} showed that CHT differed from BKR, PTNM, CIT, and HERN. BKR differed significantly from all populations except JEF. Populations CIT and HERN both differed from CHT, BKR, SULA, LEV, PTNM, MRN, and SUM. Both PTNM and MRN had 5 significant differences (CHT, BKR, MRN, CIT, and HERN; BKR, CLY, PTNM, CIT, and HERN, respectively). Remaining populations had anywhere from one to four significant differences each. The mitochondrial distance data had 31 significant differences across the 163 pairwise comparisons that were generally consistent with differences seen in the pairwise Φ_{ST} calculations (Table 3.5).

Exact tests of differentiation showed 24 significant differences (Table 3.6). CHT was different from every other population. BKR differed from every population except for JEF consistent with Φ_{ST} comparisons. LEV was significantly different from all populations except MRN and SUM, and PTNM was different from CIT.

SAMOVA assembled the 11 populations into five groups using mitochondrial haplotypes (Figure 3.4) with $\Phi_{ST} = -0.02096$ ($p \leq 0.000$) and $\Phi_{CT} = 0.19661$ ($p \leq 0.000$). Group 1 consisted of CHT, Group 2 consisted of BKR, Group 3 consisted of JEF, CLY, and PTNM, Group 4 consisted of SULA, LEV, MRN, and SUM, and Group 5 consisted of CIT and HERN.

Of 163 pairwise microsatellite F_{ST} comparisons, 11 were significantly different. The CHT population significantly differed from BKR, SULA, LEV, CLY, PTNM, MRN, CIT, and SUM (Table 3.4). BKR was significantly different from CHT, LEV, and PTNM. The remaining populations had anywhere from zero to three significant differences. Four of the microsatellite pairwise distance values between CHT and CLY, PTNM, CIT, and SUM, were significantly different (Table 3.5). Exact tests of differentiation for microsatellite F_{ST} showed no significant differences (Table 3.6).

The lowest value of Ln P(D) determined by STRUCTURE was -1570.7 representing two populations (Table 3.8). All 17 Individuals from CHT were grouped as predominately Cluster 1 and the remaining 92 individuals from all other populations grouped as predominately Cluster 2 (Figure 3.5).

DISCUSSION

Accurate taxonomy is important from biological and management perspectives. *Sciurus n. niger* and *S. n. shermani* have been described as separate subspecies based on morphology and have not been reevaluated using comprehensive sampling across the subspecies ranges. Molecular approaches can provide resolution in cases of taxonomic ambiguity, particularly in instances where variation in morphological characters is limited. The effectiveness of genetic techniques has been shown in studies conducted on morphologically similar subspecies of *Mustela sibirica* and *Geomys pinetis* (Koh *et al.* 2012, Laerm *et al.* 1981).

Genetic Diversity

Genetic diversity observed in my results is consistent with previous studies examining *S. niger* populations (Moncrief *et al.* 2010, Moncrief *et al.* 2012). Using mitochondrial sequencing, we found 82 haplotypes across all 136 samples yielding high haplotype diversity ranging from 0.893 to 1.000. Moncrief *et al.* (2012) found total of 55 haplotypes with a lower range in haplotype diversity from 0.00 to 1.00. Barratt *et al.* (1999) and Trizio *et al.* (2005) found high haplotype diversities among populations of Eurasian red squirrels (*Sciurus vulgaris*) and state that it is common for rodents to have high haplotype diversity. Nucleotide diversity was also similar to previous studies. Nucleotide diversity ranged from 0.005 to 0.012 in my study, and Moncrief *et al.* (2010) and (2012) found nucleotide diversity ranging from 0.00-0.007 and 0.00 to 0.029, respectively.

Microsatellite genotyping revealed a relatively low allelic richness of 3.6 alleles per locus, suggesting low nucleotide diversity among populations. This observation is also generally consistent with other *S. niger* microsatellite studies. Fike and Rhodes (2009) found a similar allelic richness at 4.7 alleles per locus for *S. niger*. At the subspecies level Moncrief and Dueser (2001) found allelic richness in *S. n. cinereus* to be between 1.0-1.2 alleles per locus. The low nucleotide diversity we observed could be due to random marker selection, meaning the colonies we picked to create our markers could have been those with fewer alleles. Low allelic richness can lead to increased levels of inbreeding and an overall loss of genetic diversity. Allelic richness should be continually monitored in *S. niger* populations to check for a loss in genetic diversity.

Phylogeography

Phylogeography is the distribution of individuals influenced by the historical processes that are responsible for the current geographic distribution. High haplotype diversity and low nucleotide diversity together suggest a lack of phylogeographic structure in the fox squirrel populations we sampled. Moncrief *et al.* (2010) and (2012) consistently saw high haplotype diversity and concluded that the populations they sampled had a lack of phylogeographic structure. High haplotype diversity is further supported in the distribution of mitochondrial haplotypes among populations. There are few shared haplotypes between populations (Table 3.1) and shared haplotypes within populations have few individuals. Generally, the populations consist of unique haplotypes, which is congruent with findings from Moncrief *et al.* (2010) and (2012).

We can infer the haplotype relationships using UPGMA. The absence of haplogroup A representation in other populations could potentially be due to low sample numbers in populations where only five individuals were sampled. Another possible explanation for haplogroup A only being represented in CHT and BKR is potential admixture from more western populations or subspecies. BKR had low representation of haplogroup B, but we speculate this may possibly be due to the habitat quality and landscape surrounding the population. The BKR population is surrounded by large expanses of center-pivot agricultural lands, which may act as a barrier to migration and dispersal. Populations CIT and HERN both have lower sample sizes, which could potentially explain the lack of haplogroup B representation. There are no obvious geographic barriers that would prevent haplogroup B haplotypes from being represented in these populations. Moncrief (1993) found evidence of east to west divergence of fox squirrel subspecies on either side of the Mississippi River suggesting that a geographic barrier has the ability to prevent gene flow between populations.

Population Structure

While there is a lack of phylogeographic structure, we detected evidence of population structure in my analysis. Population structure is the current distribution of populations and their genetic relationships. The eleven populations in my study exhibit genetic relationships. With SAMOVA, we determined five groups to be the optimum based on the most appropriate values of F_{SC} and F_{CT}, which is consistent with the patterns seen in the UPGMA analyses and haplogroup representation.

CHT was the most unique population and consistently differed from all other populations. The uniqueness of CHT was initially observed in the STRUCTURE analysis, which pulled out all individuals from CHT as a separate group from individuals of all other populations. CHT had eight significant microsatellite F_{ST} comparisons and was the only population that had significant differences in microsatellite distance analyses, further supporting the distinctiveness of CHT.

BKR shows low levels of microsatellite F_{ST} differentiation, but high levels of Φ_{ST} pairwise differentiation. For distance, BKR had ten significant differences in mitochondrial analyses, but no significant differences in nuclear microsatellite analyses. These differences in mitochondrial comparisons may be due to the high haplotype diversity seen among the eleven populations and possibly other factors such as the unique landscape surrounding the BKR population. BKR is surrounded by center-pivot agricultural lands and could act as a barrier to gene flow similar to what Moncrief (1993) found with populations spanning the Mississippi River.

There is only one remaining significant difference for microsatellite F_{ST}, which is between CLY and PTNM. There are no other significant microsatellite distance comparisons. We speculate that low sample numbers from some populations may have had an impact on the number of significant differences. Seven of the 11 populations had sample sizes of less than 10 individuals. The four populations with larger sample sizes were all significantly different from each other for F_{ST}, meaning that they were as different across subspecies as they were within subspecies. Populations with 10 or fewer individuals did not yield any additional significant differences in F_{ST}, which could be due to low sample numbers. However, for distance the only significant differences between large populations are CHT and PTNM suggesting that these two populations exhibit genetic differentiation due to isolation by distance.

For both mitochondrial Φ_{ST} and distance, there were numerous significant differences. This is consistent with the earlier findings of high haplotype diversity across the 11 populations. With such high haplotype diversity, we expected and saw greater levels of differentiation between populations in both Φ_{ST} and distance pairwise comparisons.

Summary

Overall, the eleven populations representing both *S. n. niger* and *S. n. shermani* do not exhibit phylogeographic structure, but do exhibit genetic structure among populations. Individuals in population CHT are distinct from the other individuals. More comprehensive sampling throughout the range of the species, including samples from Alabama and western Georgia would need to be conducted to see how CHT relates to geographically proximal populations. BKR was identified as a unique population, which may be due to the fragmented landscape and absence of suitable habitat surrounding the population. Because of high haplotype diversity, there was significant differentiation between many populations for both mitochondrial F_{ST} and mitochondrial distance analyses. These combined results of the analyses conducted with mitochondrial and nuclear microsatellite data are not consistent with separation of *S. n. niger* and *S. n. shermani* at the subspecies level. Future studies can compare the microsatellite and mitochondrial data from the populations in this study to additional southeastern populations to further examine potential population structure. Characterizing the genetic diversity and distribution across the full range of the species is needed and will facilitate a better understanding of genetic structure among *S. niger* populations and subspecies. This in turn will inform resource managers in their development of effective management strategies of *S. niger* at the subspecies and population levels.

ACKNOWLEDGMENTS

We would like to thank all of the students and staff at the Jones Ecological Research Center for assisting us in sample collection. Thank you to Daniel Greene, his volunteers, and his advisor Dr. Robert McCleery for their assistance in collecting samples from Florida. Mr. David Mallard and those at the Wildlife Resources Division at Fort Benning were very helpful in collecting samples. Thank you to the Georgia Genomics Facility for helping complete genotyping and sequencing. Thank you to Dr. Brian Shamblin for his guidance in analysis. Thank you to Georgia Department of Natural Resources for providing the funding for this project and the Warnell School of Forestry and Natural Resources for providing the resources to carry out this study.

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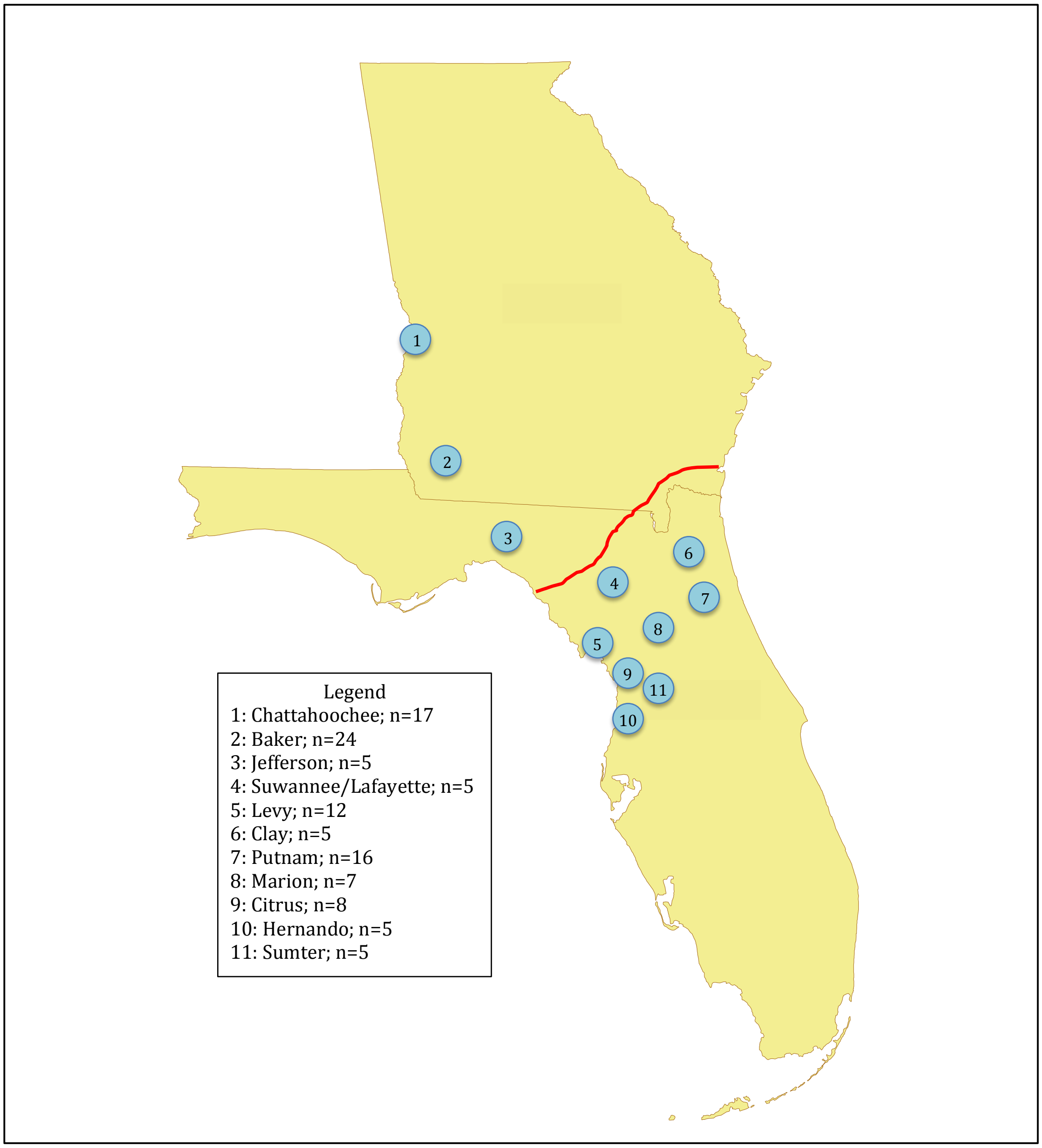
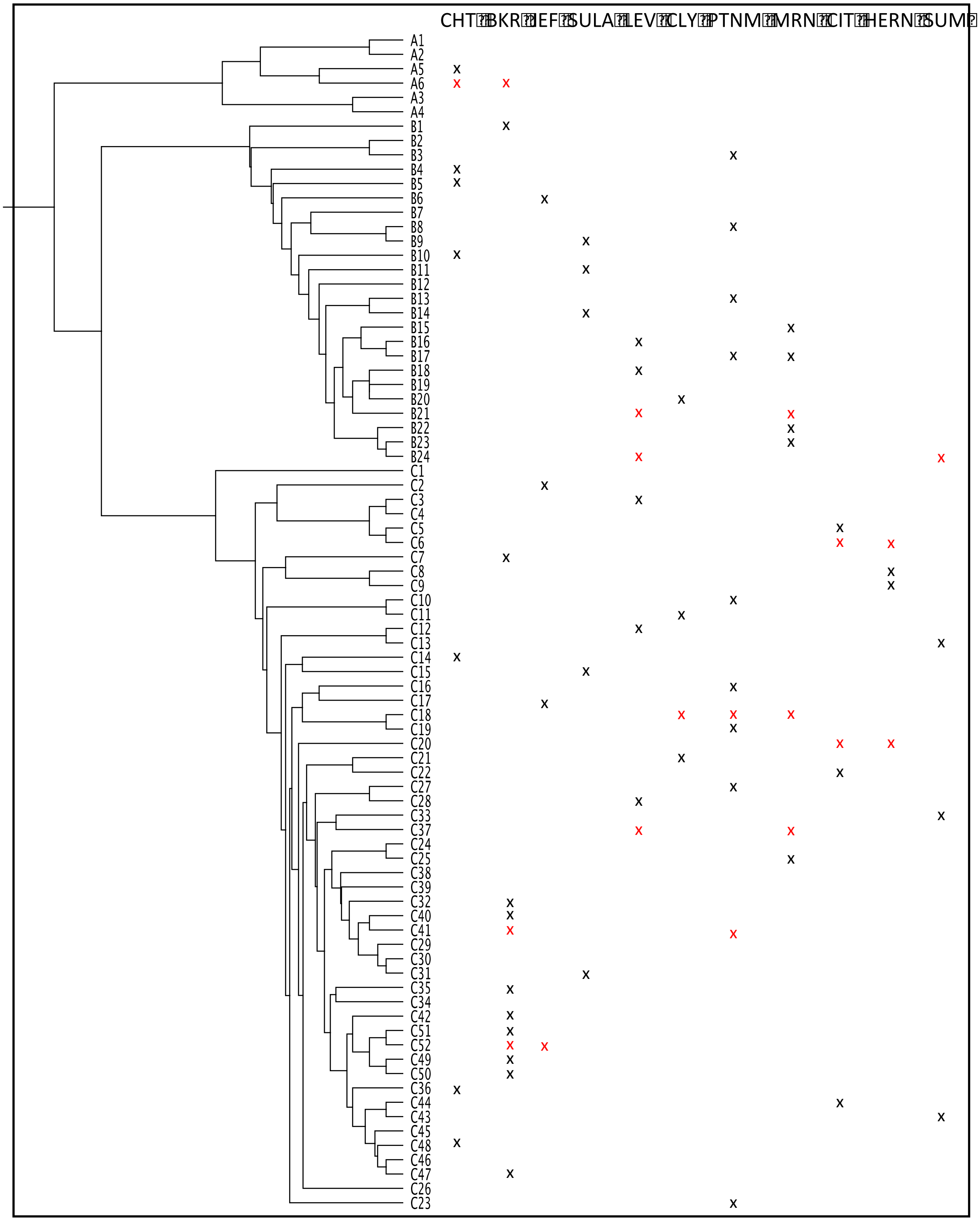


Figure 3.1 Locations of tissue collection sites in Georgia and Florida used in a comparison of genetic diversity and structure of *Sciurus niger niger* (sites 1-3) and *Sciurus niger shermani (*sites 4-11) populations, 2011-13. Range boundary, indicated by the red line, adapted from Hall 1981.



red. Sampling occurred between 2011-13.

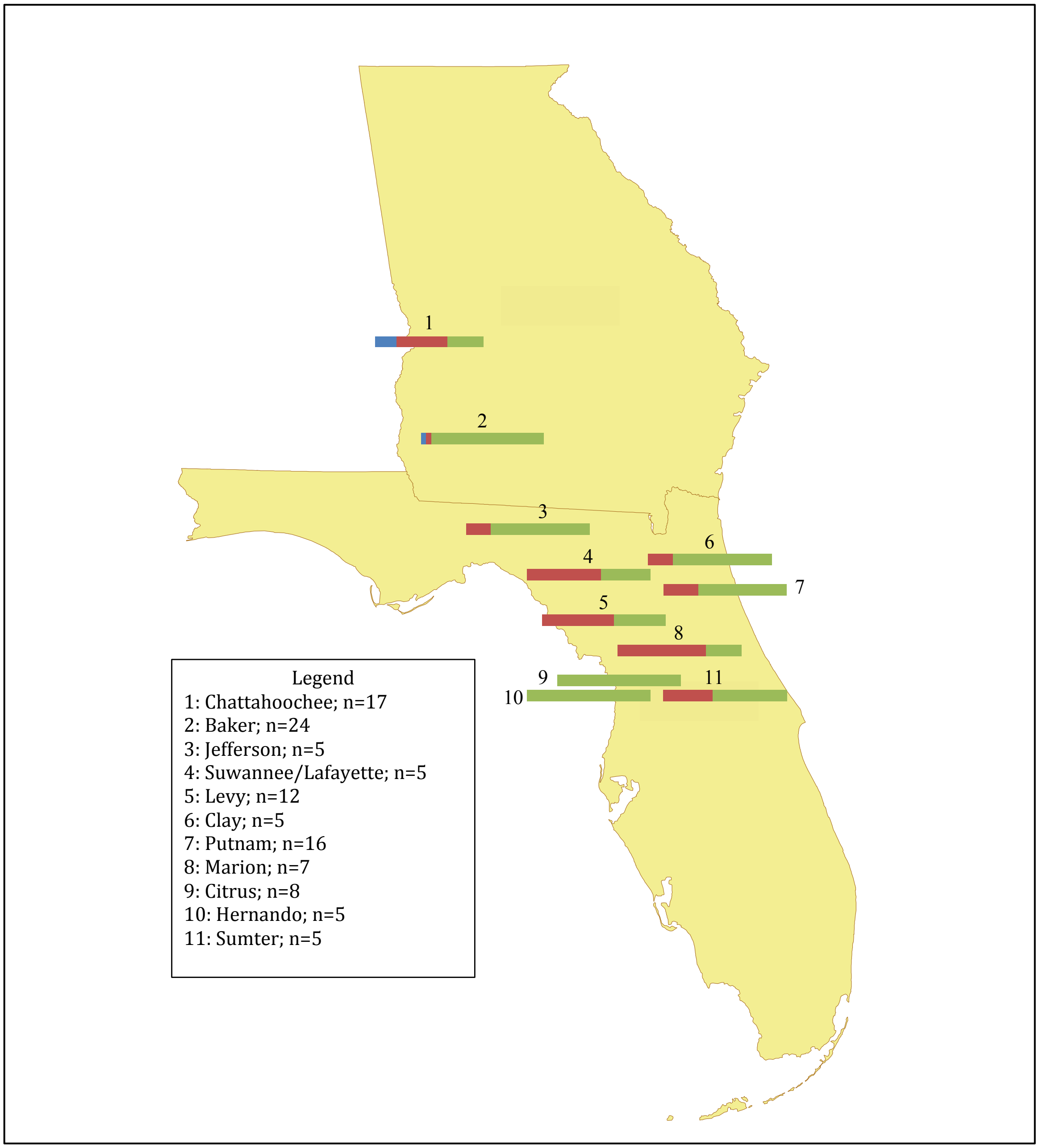


Figure 3.3 Haplogroup frequencies for the eleven population of *S. n. niger* and *S. n. shermani* sampled between 2011-13. Blue indicates Haplogroup A, red indicate Haplogroup B, and green indicates Haplogroup C.

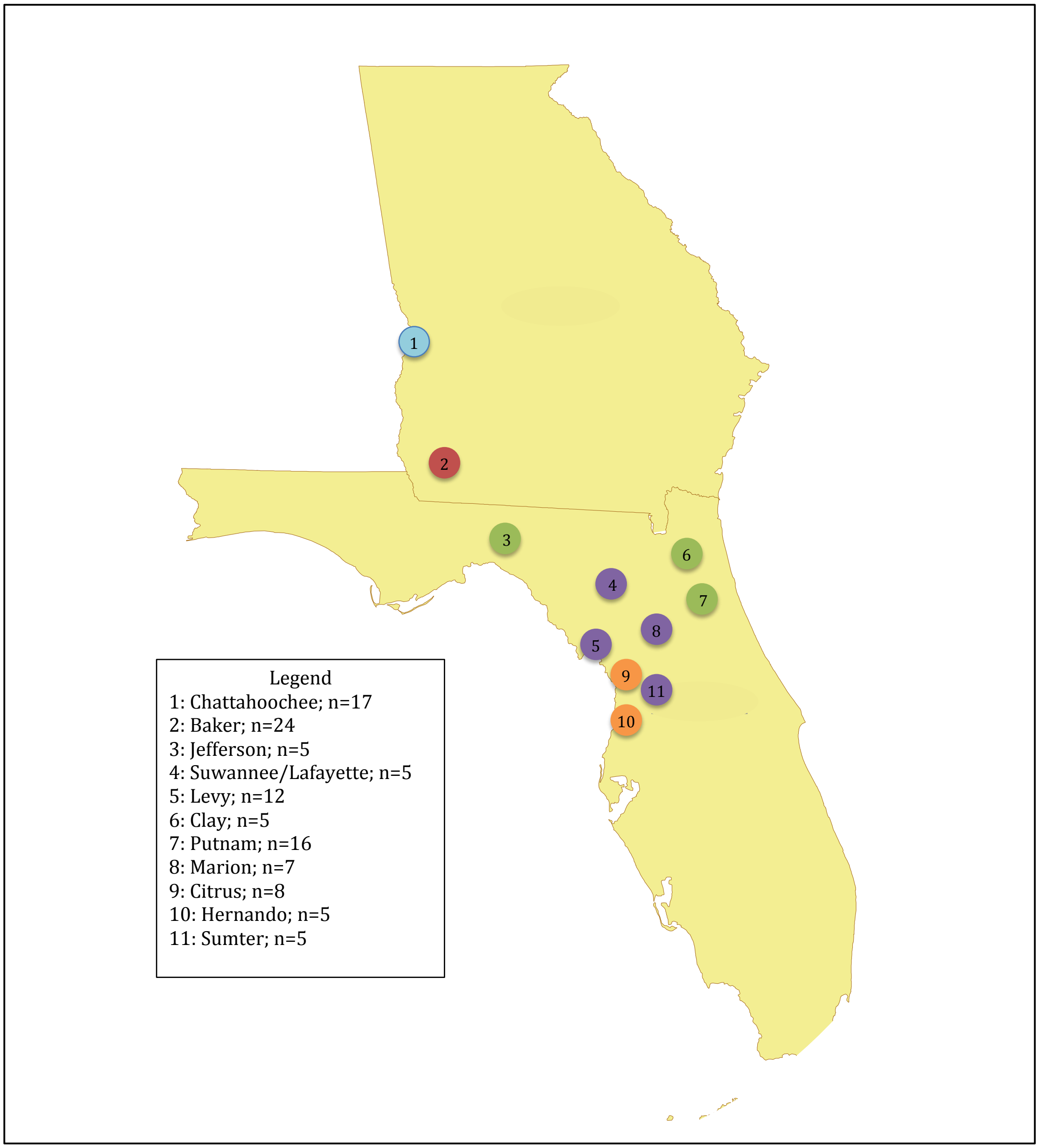


Figure 3.4 SAMOVA haplotype grouping for eleven populations of *S. n. niger* and *S. n. shermani*. Group 1 (blue) = Chattahoochee, Group 2 (red) = baker, Group 3 (green) = Jefferson, Clay, and Putnam, Group 4 (purple) = Suwannee/Lafayette, Levy, Marion, and Sumter, Group 5 (orange) = Citrus and Hernando. Sampling occurred between 2011-13.

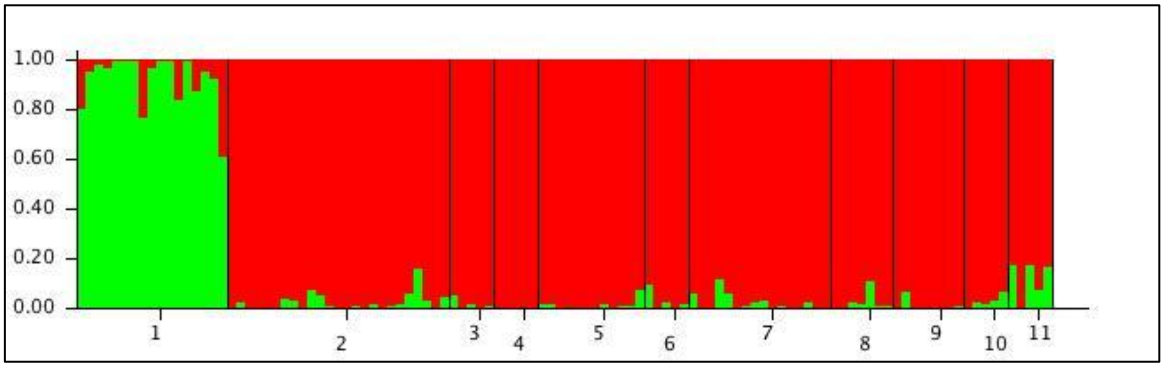


Figure 3.5 STRUCTURE analysis groupings for 109 genotyped samples of *S. n. niger* and *S. n. shermani* where Cluster 1 is shown in green and Cluster 2 is shown in red. Sampling occurred between 2011-13.

Table 3.1 Control region (D-loop) haplotype distribution between eleven populations of *S. niger* subspecies sampled from 2011-13. Sample number (n), number of haplotypes (H), haplotype diversity (h, standard deviation in parentheses) and nucleotide diversity (π, standard deviation in parentheses) is presented for each population included in the study.

			Shared										
Population	n	H	A6	B21	B24	C6	C18	C20	C37	C41	C52	h	π
CHT	17	8	2									0.905 (0.050)	0.012 (0.006)
BKR	24	13	1							2	7	0.906 (0.046)	0.005 (0.003)
JEF	5	4									1	0.900 (0.161)	0.009 (0.006)
SULA	5	5										1.000 (0.127)	0.010 (0.007)
LEV	12	8		2	3				1			0.924 (0.058)	0.009 (0.005)
CLY	5	4					1					0.900 (0.161)	0.010 (0.006)
PTNM	16	12					2			1		0.942 (0.048)	0.009 (0.005)
MRN	8	7		1			1		1			1.000 (0.076)	0.008 (0.005)
CIT	8	5				2		2				0.893 (0.086)	0.005 (0.003)
HERN	5	4				1		1				0.900 (0.161)	0.006 (0.004)
SUM	5	4			2							0.900 (0.161)	0.009 (0.006)

Table 3.2 Allelic richness and expected heterozygosity (H_E) of eleven sampled populations of *S. niger* subspecies across eight loci sampled from 2011-13. Total number of alleles per locus was obtained from nuclear microsatellite genotyping analysis.

Locus	BKR	CHT	JEF	SULA	CLY	LEV	PTNM	MRN	CIT	HERN	SUM	**Total Alleles/locus**
13	4	3	2	2	2	2	3	2	2	2	2	4
19	6	6	4	6	7	3	7	5	5	6	4	8
110	2	2	1	2	2	2	3	1	1	1	2	3
115	2	2	2	2	2	2	2	2	2	2	2	2
116	3	3	2	2	3	3	3	3	3	3	3	3
118	2	2	2	2	2	2	2	2	2	1	2	2
132	2	2	2	2	2	2	2	2	2	2	2	2
134	4	4	3	4	2	4	5	3	3	3	2	5
H_e	0.4757	0.5251	0.4722	0.5056	0.4493	0.5472	0.5045	0.4890	0.4979	0.4722	0.4750	

Table 3.3 F_{ST} values for the eleven populations of *S. n. niger* and *S. n. shermani* sampled between 2011-13. Values above the diagonal are F_{ST} values for microsatellite analyses and values below the diagonal are Φ_{ST} values for mitochondrial analyses. Significant pairwise values are in bold print. Statistical significance was accepted at p=0.05.

	CHT	BKR	JEF	SULA	LEV	CLY	PTNM	MRN	CIT	HERN	SUM
CHT		**0.076**	0.042	**0.083**	**0.084**	**0.998**	**0.107**	**0.050**	**0.132**	0.054	**0.095**
BKR	**0.265**		0.010	-0.026	**0.036**	0.017	**0.026**	0.007	0.037	0.008	0.031
JEF	0.079	0.053		-0.016	0.012	-0.014	0.029	-0.028	0.032	0.011	0.057
SULA	-0.017	**0.332**	0.048		0.002	-0.020	-0.011	-0.003	-0.005	-0.013	-0.001
LEV	0.021	**0.353**	**0.125**	-0.053		0.069	0.004	0.008	0.021	0.019	-0.004
CLY	0.887	**0.113**	-0.041	0.077	0.153		**0.045**	0.023	0.053	0.049	0.062
PTNM	**0.110**	**0.139**	-0.013	0.043	0.122	-0.017		0.019	0.010	0.030	-0.002
MRN	0.012	**0.451**	0.193	-0.076	-0.068	**0.227**	**0.161**		0.006	-0.012	0.016
CIT	**0.265**	**0.176**	0.096	**0.342**	**0.325**	0.105	**0.204**	**0.458**		-0.007	0.009
HERN	**0.238**	**0.184**	0.078	**0.266**	**0.318**	0.116	**0.129**	**0.425**	0.132		0.021
SUM	-0.031	**0.247**	-0.035	-0.094	-0.084	-0.003	-0.002	-0.050	**0.253**	**0.233**	

Table 3.4 Genetic distance-based pairwise values for the eleven populations of *S. n. niger* and *S. n. shermani* sampled between 2011-13. R_{ST} values from microsatellite analyses are above the diagonal. Φ_{ST} values from mitochondrial analyses are below the diagonal. Significant pairwise comparisons are in bold print. Statisical significance was accepted at p=0.05.

	CHT	BKR	JEF	SULA	LEV	CLY	PTNM	MRN	CIT	HERN	SUM
CHT		0.083	0.044	0.090	0.091	**0.111**	**0.119**	0.053	**0.152**	0.057	**0.104**
BKR	0.360		0.010	0.000	0.037	0.017	0.026	0.007	0.038	0.008	0.032
JEF	0.086	0.056		0.000	0.012	0.000	0.030	0.000	0.033	0.011	0.061
SULA	0.000	**0.496**	0.051		0.003	0.000	0.000	0.000	0.000	0.000	0.000
LEV	0.021	**0.545**	**0.142**	0.000		0.074	0.004	0.008	0.022	0.020	0.000
CLY	0.097	**0.127**	0.000	0.083	**0.180**		0.048	0.024	0.057	0.052	0.066
PTNM	**0.116**	**0.161**	0.000	0.045	**0.139**	0.000		0.020	0.010	0.031	0.000
MRN	0.012	**0.821**	**0.239**	0.000	0.000	**0.294**	**0.192**		0.006	0.000	0.016
CIT	**0.361**	**0.214**	**0.106**	**0.521**	**0.482**	**0.117**	**0.257**	**0.845**		0.000	0.009
HERN	**0.313**	**0.225**	0.085	**0.361**	**0.465**	**0.131**	**0.148**	**0.740**	**0.152**		0.021
SUM	0.000	**0.327**	0.000	0.000	0.000	0.000	0.000	0.000	**0.339**	**0.303**	

Table 3.5 Exact Test of differentiation values for the eleven populations of *S. n. niger* and *S. n. shermani* sampled between 2011-13. Significant pairwise values are in bold font. Differentiation values from microsatellite analyses are above the diagonal. Differentiation values from mitochondrial analyses are below the diagonal. Statistical significance was accepted at p=0.05.

	CHT	BKR	JEF	SULA	LEV	CLY	PTNM	MRN	CIT	HERN	SUM
CHT		0.129	0.234	0.230	0.116	0.230	0.112	0.160	0.162	0.218	0.213
BKR	**0.000**		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
JEF	**0.009**	0.172		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
SULA	**0.019**	**0.013**	0.446		1.000	1.000	1.000	1.000	1.000	1.000	1.000
LEV	**0.002**	**0.000**	**0.045**	0.086		1.000	1.000	1.000	1.000	1.000	1.000
CLY	**0.009**	**0.010**	0.203	0.442	**0.042**		1.000	1.000	1.000	1.000	1.000
PTNM	**0.000**	**0.000**	0.111	0.188	**0.004**	0.219		1.000	1.000	1.000	1.000
MRN	**0.013**	**0.007**	0.475	1.000	0.353	0.470	0.158		1.000	1.000	1.000
CIT	**0.001**	**0.001**	0.053	0.116	**0.006**	0.056	**0.011**	0.110		1.000	1.000
HERN	**0.011**	**0.009**	0.212	0.435	**0.041**	0.217	0.092	0.464	0.435		1.000
SUM	**0.011**	**0.011**	0.207	0.447	0.602	0.201	0.101	0.479	0.052	0.206	

Table 3.6 SAMOVA results from spatial haplotype analysis of eleven populations of *S. niger* subspecies sampled between 2011-13. SAMOVA was run with various assumptions of number of groups (k) to yield values of F_{SC} and F_{CT}. The appropriate value of k is determined when F_{SC} becomes negative.

k	F_{SC}	F_{SC} p-value	F_{CT}	F_{CT} p-value
2	0.05230	0.00000	0.19020	0.00196
3	0.04021	0.00000	0.18540	0.00196
4	0.00727	0.00000	0.18712	0.00000
5	-0.02096	0.00000	0.19661	0.00000
6	-0.03582	0.00000	0.20774	0.00000
7	-0.04956	0.00000	0.21309	0.00000
8	-0.05901	0.00000	0.21957	0.00000

Table 3.7 Log likelihood (Ln P(D))of number of populations (k) for eleven sample populations of *S. niger* based on nuclear microsatellite data obtained between 2011-13. The lowest value of Ln P(D) yields the highest probability of the correct number of populations.

k	Ln P(D)
1	-1594.9
2	-1570.7
3	-1577.7
4	-1646.0
5	-1647.9
6	-1691.3
7	-1767.2

CHAPTER 4

CONCLUSIONS AND MANAGEMEMNT IMPLICATIONS

Previous studies have consistently found a lack of phylogeographic structure among populations of *S. niger* (Moncrief *et al.* 2010, Moncrief *et al.* 2012). I examined the level of genetic differentiation between two described subspecies, *S. n. niger* and *S. n. shermani*, using modern molecular genetic techniques and also concluded that there is a lack of phylogeographic structure. Molecular genetic techniques allowed me to assess the level of genetic differentiation among populations and compare that with distribution of the respective ranges of the two subspecies as described in the literature. Accurate taxonomy is essential for proper management (Cotterill 1995), and because *S. n. shermani* is listed as a species of concern in Florida and Georgia, determining the level of differentiation is essential for creating appropriate management decisions.

I developed 8 unique polymorphic microsatellite markers. The markers yielded low allelic richness, which was found by other *S. niger* populations studies as well (Fike and Rhodes 2009, Moncrief *et al.* 2010) on. Low allelic richness observed could be due to the randomness of colony picking when developing the markers. Allelic richness levels should be continually monitored to look for losses in genetic diversity in *S. niger* populations. The markers will be useful in future studies examining other southeastern *S. niger* populations and subspecies in the southeastern U.S.

Application of these polymorphic markers in population analysis indicated a lack of phylogeographic structure between populations currently designated as *S. n. niger* and *S. n. shermani*. The most consistent difference found was between the Chattahoochee population, within the range of *S. n. niger*, and all other populations, which included both *S. n. niger* and *S. n. shermani*. These results might mean that Chattahoochee is more closely related to western subspecies or other proximal populations. There was genetic differentiation seen between Chattahoochee and Baker that may be due to isolation by distance, but we were not able to obtain samples from areas between these two locations to test our hypothesis. I was also not able to obtain samples from between the Baker county, Georgia population and Jefferson county, Florida population, which is the closest Florida population. The slight genetic differentiation seen between Baker and two Florida populations is again most likely due to isolation by distance. We can also speculate that low sample sizes in the Florida populations affect our ability to detect differentiation. While Chattahoochee was consistently grouped as a separate population in my analyses, more studies are needed to determine accurate subspecies representation. It is unclear if the Chattahoochee population is more closely related to another southeastern subspecies or if it is just a unique population in its current subspecies designation. Results of my analysis of genetic diversity and structure using microsatellite markers are not consistent with the current subspecies taxonomic distinction between *S. n. niger* and *S. n. shermani*. The four locations with the largest sample sizes (two in *S. n. niger* range and two in *S. n. shermani* range) are as different across subspecies as they are within subspecies suggesting that the current subspecies designations are irrelevant.

Moncrief *et al.* (2012) sequenced 486 bases pairs and found high haplotype diversity and 51 unique haplotypes. I extended the sequencing region to 1300 base pairs and found 82 unique haplotypes. My results are consistent with previous studies that found high haplotype diversity among *S. niger* populations. Chattahoochee is a unique population, which is consistent with microsatellite results. Baker is also a unique population, which may be due to the unique landscape that surrounds the population. The surrounding landscape consists of center-pivot agricultural lands that may act as a barrier to gene flow. The remaining nine populations were placed into three groups, but this may be due to low sample sizes obtained from many of the populations. Had I been able to collect some additional samples, the populations may have been clustered together in less groups as they might have been more similar to one another. It is possible that with more extensive sampling, haplogroup representations might be more similar between geographically proximal populations. The expected high haplotype diversity contributes to the genetic differentiation seen between populations, because high haplotype diversity yields greater genetic differentiation.

Similar to the microsatellite results, mitochondrial DNA results were consistent with the hypothesis that the level of genetic differentiation is among populations of *S. n. niger* and *S. n. shermani* is not consistent with subspecies classification. However, further studies that include a more comprehensive sampling strategy for populations throughout the range of the species are warranted.

MANAGEMENT IMPLICATIONS

This study alone cannot definitively suggest whether or not the two subspecies in question should be managed as separate subspecies. However, when paired with results from previous studies (Fike and Rhodes 2009, Moncrief *et al.* 2010, Moncrief *et. al.* 2012) the common findings of a lack of genetic differentiation suggest that the two subspecies in question are not genetically distinct. There is evidence of structure among populations of this study in that there are a few distinct populations, but populations across subspecies are as different from each other as they are within subspecies ranges. More studies need to be conducted sampling individuals from western Georgia and throughout Alabama as well as all other regions of Florida. More extensive sampling will allow for further examination of genetic differentiation and a more complete understanding of genetic relationships between populations. Ideally, future studies will sample across the range of both subspecies facilitating a more comprehensive population analysis. Information gained from a comprehensive analysis across the range of *S. niger* will facilitate development of effective management strategies for the species that considers genetic structure at the species, subspecies, and population levels.

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