GENETIC STRUCTURE AND PHYLOGEOGRAPHY OF THE FOX SQUIRREL, *SCIURUS NIGER*, AS INFERRED FROM A MITOCHONDRIAL GENE

by

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DEDICATION

Squirrels for nuts contend, and, wrong or right for the world's empire kings ambitiously fight. What odds? To us 'tis all the self-same thing: A nut, a world, a squirrel, and a king. ~ Charles Churchill

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ABSTRACT

GENETIC STRUCTURE AND PHYLOGEOGRAPHY OF THE FOX SQUIRREL, *SCIURUS NIGER*, AS INFERRED FROM A MITOCHONDRIAL GENE

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Sciurus niger (Rodentia: Sciuridae) is a large tree squirrel which inhabits the southeastern portion of North America. Currently there are ten recognized subspecies which are distinguished based on differences in morphology and ecology. While molecular work has been undertaken for a few subspecies of *S. niger*, the patterns of genetic differentiation of the entire species have yet to be examined. This study attempts to characterize the genetic structure of *S. niger* in order to help determine the validity of current subspecies designations and offer insight into the post-glacial colonization patterns of the species. A 296 base pair fragment of the mitochondrial control region (d-loop) was sequenced from 55 specimens of *S. n. vulpinus,* 13 samples of *S. n. niger,* and 13 samples of *S. n. rufiventer*. Fifteen previously reported haplotypes (Lance et al. 2003) representing *S. n. cinereus*, *S. n. rufiventer*, and *S. n. vulpinus* were incorporated into the analysis. Additionally, a data set of 89 sequences generated at the Van Den Bussche Laboratory of Molecular Systematics and Conservation Genetics were added to this data

set. These sequences included representatives of the following 8 subspecies: *S. n. bachmani, S. n. cinereus, S. n. limitis, S. n. ludovicianus, S. n. niger, S. n. rufiventer, S. n. subauratus,* and *S. n. vulpinus.* The compiled data set of 258 individuals belonging to 8 subspecies yielded 125 unique haplotypes, indicating extremely high levels of diversity in the control region. Several tree-based methods recovered two distinct shallow clades which do not correspond to geographic regions or subspecies. A parsimony-based minimum spanning network revealed two haplotype clusters which correspond to the two clades found in the tree-based methods. The haplotypes are closely linked in a star-shaped phylogenetic network; several of the most frequent haplotypes were internal, while the majority were unique to single populations and presented distal positions in the network. Overall there was a lack of genetic structure amongst populations with most of the variance explained by within population genetic diversity. Despite poor branch support, the congruent recovery of the two *S. niger* clades via both clustering-based and optimality criterion-based methods supports the separation of haplotypes into two major haplogroups. These results indicate that the currently recognized subspecies based on alpha taxonomic characters are not concordant with the mitochondrial history of *S. niger*. Instead, my findings suggest that the control region haplotype distribution in fox squirrels may be the result of repeated and rapid habitat expansions/retractions during glacial events in the Pleistocene. The shallow divergence between haplotypes across wide geographic distances suggest that the patterns of morphological and ecological differentiation the we observe within *S. niger* may have occurred much more recently than previously thought.

1. Introduction

Sciurid rodents (Rodentia: Sciuridae) have inhabited North America for approximately 17 million years (Black 1972). The genus *Sciurus* first appears during the Claredonian age (9.8 million years before present (mybp)) from Lyon County, Nevada (Emry et al. 2005). *Sciurus niger*, first described by Linnaeus in 1758 in *Systema Naturae* (Hall 1981), is a large (507-1361 g; Flyger and Gates 1982) scatter-hoarding tree squirrel with a range extending across the eastern and central United States (Fig. 1) that has a complex natural history (see Appendix A).

Based on pelage, size, and geographic location, ten fox squirrel subspecies are recognized: *S. n. avicennia*, *S. n. bachmani*, *S. n. cinereus*, *S. n. limitis*, *S. n. ludovicianus*, *S. n. niger*, *S. n. rufiventer*, *S. n. shermani*, *S. n. subauratus*, and *S. n. vulpinus* (Hall 1981; Fig. 1; Appendix B). These ten subspecies designations have been the subject of investigation in several studies. Based on cranial features, Turner and Laerm (1993) challenged the validity of *S. n. bachmani* and suggested it represents a clinal variant of *S. n. niger*. These authors also found that coat color, while traditionally used to delineate

subspecies, is not a reliable diagnostic character (Turner and Laerm 1993). Data from a cranial morphometric study of specimens across the entire range of *S. niger* indicates that individuals fall into 6 statistically significant groups, only two of which correspond to current subspecies designations (Roe 1994). Of the molecular phylogenetic studies on *S. niger*, little to no genetic structuring of populations and subspecies has been observed (Lance et al. 2003; Moncrief 1987; Moncrief 1993; Moncrief 1998; Moncrief and Dueser 2001). Allozyme analysis of *S. n. bachmani*, *S. n. limitis*, *S. n. ludovicianus*, *S. n. rufiventer*, *S. n. limitis*, *S. n. cinereus*, and *S. n. vulpinus* specimens indicate that the Mississippi river has played a role in current genetic structure of these groups, however, except for *S. n. subauratus*, allozyme character differentiation is not congruent with subspecies designations (Moncrief 1993; Moncrief 1998; Moncrief 1987). Mitochondrial markers have been used to investigate genetic structure in other sciurids (Arbogast et al. 2001; Barratt et al. 1999; Hale et al. 2004; Oshida et al. 2006; Oshida and Masuda 2000). Of these studies, only Lance et al. (2003) has utilized these markers to examine variation in *S. niger*. Using the rapidly evolving mitochondrial control region, these authors found populations of *S. n. cinereus*, along with neighboring populations of *S. n. vulpinu*s and *S. n. niger*, appear to have little genetic structuring, likely a result of incomplete lineage sorting (Lance et al. 2003).

Many questions about the ecology and morphology of fox squirrels have been addressed, yet only a handful of studies have focused on the phylogenetics of *S. niger* (Lance et al. 2003; Moncrief 1993; Moncrief 1998; Moncrief and Dueser 2001; Roe

1994). Many authors have suggested that additional work be undertaken to clarify the interspecific relationships of fox squirrels (Lance et al. 2003; Roe 1994). To date, no molecular study has included all subspecies of *S. niger*.

Nucleic acid sequencing is one of the most commonly used modern techniques for inferring phylogenies (Hillis et al. 1996) and is an effective tool for assessing genetic divergence (Parker et al. 1998). Mitochondrial DNA (mtDNA), which is maternally inherited and nonrecombining, is often utilized to answer questions regarding population-level genetic processes (founder effects, hybridization, introgression, dispersal patterns, habitat fragmentation, and population bottlenecks) in vertebrates (Arbogast et al. 2001; Barratt et al. 1999; Hale et al. 2004; Hillis et al. 1996; Moritz et al. 1987; Oshida et al. 2006; Oshida and Masuda 2000; Peterson and Stewart 2005; Shipp-Pennock et al. 2005). To date, only one study has utilized mtDNA markers, specifically the control region, to examine variation in *S. niger* (Lance et al. 2003).

The control region, also known as the displacement loop or d-loop, is a small, noncoding region of mtDNA which serves as the origin of heavy strand replication and mutates on average 4 times faster than other mitochondrial regions (Larizza et al. 2002). The extremely high rate of mutation in this region, especially among rodents (Larizza et al. 2002), makes this marker valuable for evaluating intraspecific variation (Barratt et al. 1999; Ducroz et al. 2005; Lance et al. 2003).

In order to evaluate whether the ten recognized subspecies of *S. niger* are reflective of this species' evolutionary history, I examined the patterns of mtDNA differentiation from tissue samples collected across the entire species range. Genetic structure was assessed by the number of divergent subunits (haplotypes), which permitted the quantification of intraspecific divergence and assignment of these individuals to putative populations (haplogroups). In addition, these data were combined with geographic information in order to characterize the phylogeography of this species (Avise et al. 1987), including gene flow patterns (Slatkin and Maddison 1990), post-glacial colonization routes (Rowe et al. 2004), and biogeographic barriers (Soltis et al. 2006). Specifically, the following hypotheses were tested: (1) Geographic and genetic distance are correlated; (2) Eastern and western fox squirrel populations represent descendants of the proposed Florida and Texas glacial refugia, respectively; and (3) Current *S. niger* subspecies designations based on morphological characters are concordant with mtDNA haplogroup structure.

2. Materials and Methods

2.1 Sampling and DNA Extraction

Small pieces of liver tissue stored in lysis buffer were obtained from collections made by Dr. Nancy Moncrief (Virginia Museum of Natural History). A total of 81 samples were sequenced, including 55 *S. n. vulpinus*, 13 *S. n. niger*, and 13 *S. n. rufiventer*. 89 sequences that were previously generated at the Van Den Bussche Laboratory of Molecular Systematics and Conservation Genetics were incorporated into the data set. 15 of these sequences were duplications of individuals that were sequenced in our lab. These data represented 9 *S. n. bachmani* specimens, 8 *S. n. cinereus*, 10 *S. n. limitis*, 9 *S. n. ludovicianus*, 9 *S. n. niger*, 17 *S. n. rufiventer*, 11 *S. n. subauratus*, and 16 *S. n. vulpinus* specimens (Table 1). One tissue sample from an *S. carolinensis* specimen collected in Fairfax County, Virginia for use as an outgroup based on its close phylogenetic relationship with *S. niger* (Steppan et al. 2004). DNA was extracted using Qiagen DNeasy© Tissue Kit (Qiagen, Valencia, CA, USA) following manufacturer protocols.

2.2 Amplification and Sequencing

(5'TGATGATTTCACGGAGGTAGG-3'; Lance et al. 2003). Polymerase chain reaction (PCR) was carried out in a 20 µl reaction mixture containing 10 mM dNTP's, 1 µM each of forward and reverse primer, 2 mM of $MgCl_2$ buffer, 1 unit (.01µL) of *AmpliTaq*© DNA polymerase, and 2 µl of extracted DNA. A negative control was included in each PCR run. A thermal profile of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 30 sec at 50°C, and 2 min + 5 sec extension at 72°C was utilized. All amplifications were conducted using a PTC-100 Programmable Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplified PCR products were quantified on a 1% TAE agarose gel with a 500 bp ladder. PCR products were then cleaned using AMPure® magnetic beads (Agencourt, Beverly, MA, USA) according to manufacturer protocols. Sequencing reactions were performed using BigDye™ Terminator Cycle Sequencing Ready Reaction mix (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were cleaned using Sephadex® G-50 (Sigma-Aldrich Inc., St. Louis, MO, USA) and sequenced using a SpectruMedix® multi-capillary genetic analyzer (SpectruMedix LLC, State College, PA, USA). Both heavy (H) and light (L) strands were sequenced and then deconvoluted and examined with GenoSpectrum™ software (SpectruMedix LLC, State College, PA, USA).

2.3 Data Analysis

An additional data set of 89 sequences generated at the Van Den Bussche's Laboratory of Molecular systematics and 15 previously reported haplotype sequences

from 103 individuals (Lance et al. 2003) were incorporated into the data analysis. These published sequences were used to verify the sequences obtained for this study. Sequences generated for this study were proofed, edited to 296 base pairs, and aligned using Sequencher 4.0b (GeneCodes Corp., Ann Arbor, MI, USA) and alignments were checked by eye using Mesquite 1.12 (Maddison and Maddison, 2006). Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) trees were generated using PAUP v. 4.0b (Swofford 2000). For likelihood based analyses, the model of evolution was chosen using AIC criteria in ModelTest 3.06 (Posada and Crandall 1998). Trees were generated both with and without transition:transversion (ts:tv) character weighting and bootstrapped for 1,000 replicates. In addition, a Bayesian MCMC analysis was employed using MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). A coalescent-based Bayesian MCMC (Drummond et al. 2002) analysis was run in BEAST 1.4.2 (Drummond and Rambaut 2003) to deduce the most likely genealogy for the present-day haplotypes (Rosenberg and Nordborg 2002). The Bayesian skyline model (Drummond et al. 2005) was employed with a relaxed molecular clock model (Drummond et al. 2006) and a normal prior for 50,000,000 generations. Trees were sampled every 1000 generations, and then re-sampled using LogCombiner (Drummond and Rambaut 2003), with the first 5000 trees discarded as burn-in. The mean nucleotide substitution rate was input as 3.9 per million years based on average mammalian nucleotide substitution rates for the d-loop (Pesole et al. 1999). Effective sample size was monitored using TRACER (Rambaut and Drummond 2004) and

exceeded 260 for all parameters. A minimum spanning tree (Rohlf 1973) was constructed using the TCS program (Clement et al. 2000) at a 96% confidence level.

3. Results

3.1 Sequence Analyses

Of the 296 characters, 56 were found to be parsimony informative. Chi-square test found all base frequencies across taxa was equal (p=1.000). We recovered 125 haplotypes from the 258 individuals in the total data set (Table 1). The mean number of individual samples per haplotype was 2.06. *S. n. bachmani* samples were the most diverse, with a mean of 1 sample per haplotype; the next most highly diverse subspecies groups were *S. n. niger* (mean=1.03), *S. n. subauratus* (mean=1.22), and *S. n. vulpinus* (mean=1.29). *S. n. cinereus* was the least diverse by far with a mean of 9 samples per haplotype, and *S. n. limitis* samples was the next least diverse with 3.33 samples per haplotype. Six haplotypes were found in two or more subspecies. The ts:tv ratio was 2.70. The Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with gamma distribution and invariant sites (HKY+I+G) was selected for likelihood-based analyses by Modeltest.

3.2 Phylogenetic reconstructions

An MP analysis, along with ML and Bayesian MCMC analysis run with the HKY+I+G model all failed to resolve intraspecific relationships of *S. niger*. A NJ tree

was constructed with a character-weighted step-matrix. In this analysis, transitions were down-weighted with respect to transversions at a ratio of 1:10 to correct for a high ts:tv ratio, which may obscure phylogenetic relationships as a result of homoplasy (Avise et al. 1987). The NJ tree was bootstrapped for 1,000 generations and recovered two mixed clades within a shallow phylogeny (Fig. 3). Bootstrap support for these clades was poor with a value of 53 for clade I and 20 for clade II. Clade I, the larger clade, is composed of 92 haplotypes belonging to all 8 subspecies designations. *S. n. ludovicianus* and *S. n. limitis* are unique to this clade. In addition, all *S. n. rufiventer* haplotypes except for one (NRV2) are restricted to clade I, as well as all *S. n. subauratus* haplotypes except for one (S3). Clade II contains 31 haplotypes belonging to six subspecies.

Coalescent analysis also recovered a shallow phylogeny with the same two clades as the neighbor-joining analysis, albeit with a slightly different topology (Fig. 4). The likelihood values for the phylogeny was -1961.59, but failed to converge on a stable distribution despite an effective sample size value of 2000. The root height of the tree was estimated at 510,000 years at 95% HPD based on the input of 3.9 substitutions per million years. The time to most recent common ancestor (tmrca) for *S. niger* samples was estimated as 11,000 years, which is on the more recent end of fossil estimates for the appearance of *S. niger* (Kurten and Anderson 1980). The mean covariance value of .0036 indicates a lack of autocorrelation of rates in the phylogeny. The mean ts:tv value averaged 10.4 and the proportion of invariant sites was 47%.

The distribution of haplotypes within the minimum spanning network (Fig. 5) is congruent with the clades recovered in the tree-based analyses. The base of the network is comprised of two star-shaped clusters with a mixed *rufiventer*/*subauratus*/*vulpinus* clade positioned as the founder haplotype. These clusters correspond with Clade II of the tree-based analyses. A single *S. n. niger* haplotype (N10) connects the founder haplotype for clade II to the rest of the haplotypes, which correspond to Clade I of the previous analyses.

4. Discussion

4.1 Genetic Structure

The failure of traditional parsimony and likelihood methods to resolve the phylogeny, and the failure of Bayesian MCMC analyses to converge on a stable tree likelihood value suggests that the control region in fox squirrels is highly variable and saturated with transitional nucleotide substitutions. The extremely large number of haplotypes recovered from the data set reflects great genetic diversity in this region which in turn may reflect historically large effective population sizes. Yet squirrels are poorly preserved in the fossil record, suggesting that squirrel populations have been historically low in North America (Emry et al. 2005). These haplotypes form topologies which do not only not reflect collecting locality or region, but also do not reflect current subspecies designations. Both haplogroups contain haplotypes from six out of the eight subspecies examined. The only apparent structure is the confinement of *S. n. limitis*, *S. n. ludovicianus* and *S. n. rufiventer* (with one exception) haplotypes to haplogroup (Clade) I.

4.2 Pleistocene Glaciations

The Pleistocene glaciation period occurred in North America between 1.8 mybp and 11,550 ybp (Kurten and Anderson 1980). The Laurentide ice sheet encroached upon the northern half of North America, extending south of the Catskills into Pennsylvania

between 40° and 41° N, and into North Carolina along the southern Appalachian ridge to 36° N (Berkland and Raymond 1973). Three glacial cycles occurred during this period, causing repeated retraction and expansion of temperate flora and fauna (Bennett 1998; Graham 1999; Williams et al. 1998). The most recent glacial maximum occurred at approximately 18,000 ybp, during which time palynological evidence suggests a pocket of hardwood forest was located in the lower Mississippi river delta and another in northern Florida, both of which acted as refugia for temperate taxa (Davis 1981, Delcourt and Delcourt 1984; Magni et al. 2005). The genetic footprint of the Pleistocene is extensive; 67% of fish, 57% of herpetofauna, and 50% of mammalian speciation events resulting in extant sister species originated during this epoch (Avise et al. 1998).

It has been suggested that the genetic structure of fox squirrels has been impacted by Pleistocene glaciations (Moncrief 1993; Roe 1994; Weigl et al. 1989). *S. niger* fossils dating from the Rancholabrean (500,000 to 10,000 ybp) have been found in Arkansas, Missouri, eastern Texas, central Florida, Tennessee, and Kentucky (Kurten and Anderson 1980). The estimated timing of mitochondrial diversification within *S. niger* (11,000 ybp), is consistent with previous estimates, although on the more recent end of fossil evidence for the appearance of *S. niger* (Kurten and Anderson, 1980). Isolation in refugia during glacial maxima can result in allopatric speciation (Near and Benard 2004; Ribera and Vogler 2004), and in fox squirrels, cranial morphometric data suggest that southeastern fox squirrels recolonized from a Florida refugium and Midwestern squirrels dispersed from a Texas refugium (Weigl et al. 1989). While the Midwestern fox squirrel

subspecies, *S. n. rufiventer*, is virtually unique to haplogroup I in this analysis, southeastern fox squirrels (*S. n. niger* and *S. n. vulpinus*) were not isolated in either haplogroup. This may support contrasting evidence that temperate flora occurred at low densities further north in the Eastern United States (*Fagus* and *Acer*; McLachlan et al. 2005), which may have resulted in temperate fauna being distributed in a similar fashion, instead of in distinct isolated refugia (Bennett 1985; Jackson et al. 2000) or the existence of refugia in the upper central U.S. The possible persistence of deciduous forest trees in this region during the last glacial maximum was supported by a study with other temperate forest Sciurids (*Tamias striatus*; Rowe et al. 2004). Rowe et al. (2004) have documented patterns of genetic variation that suggest that some populations of deciduous forest vertebrates may have tolerated the climatic changes of glacial advance in cryptic northern refugia rather than migrating south to track favorable climates.

Sciurus niger limitis and *S. n. ludovicianus* are both found west of the Mississippi River, and their confinement in haplogroup I support the possibility that this river may have acted as a barrier to dispersal during post-glacial colonization, which was previously evidenced by electrophoretic data (Moncrief 1993). In addition, all *S. n. rufiventer* sequences except for one from Rooks County, Kansas, belong to haplogroup I (Fig. 7), which may also lend support to the importance of the Mississippi river as a barrier. The individual from Kansas belonging to haplogroup II also shares this haplotype with a specimen from Alleghany County, Maryland. This may be the result of a historical translocation for hunting purposes (Loeb and Moncrief 1993; Turner and Laerm 1993);

however, several other haplotypes are shared across large distances which may discount this supposition. Cranial morphometrics have indicated that other important physiogeographic barriers to dispersal include the Appalachian mountain chain, the Savannah River, and especially the Missouri River (Roe 1994), however the data examined herein do not support this hypothesis. Likewise, there is also no mitochondrial evidence that *S. n. subauratus* populations have been isolated from other populations of fox squirrel via specific habitat types associated with this subspecies (Roe 1994).

A review of the literature demonstrates that in North America, for most flora and fauna, the predominant trend is one of south to north post-glacial recolonization (Soltis et al. 2006), which may be evidenced by reduced levels of genetic variation in northern populations supporting expansions from southern refugia (Sage and Wolff 1986; Merila et al. 1996). Recent demographic expansion following the retraction of the Wisconsin glaciation approximately 10,000 ybp may account for the poorly supported topologies and lack of spatial structure of these mitochondrial haplotypes. This pattern has been observed in other taxa as a result of recent population expansion following the Pleistocene deglaciation (Pearce et al. 2004, van Vuuren and Robinson, 1997) and is consistent with the estimated tmrca of 11,000 ybp.

While the results presented here support a possible post-glacial range expansion of fox squirrel populations, the results did not, however, support a south to north post-glacial recolonization. Instead, the phylogeographic analyses presented herein indicate

that fox squirrel populations have recently expanded from refugia perhaps near the upper central U.S. The presence of more northern refugia in glacial landscapes would have important implications for resolving postglacial colonizations that are not concordant with southern Pleistocene refugia expanding northward. In these analyses, I also recovered two clades/haplogroups that were partitioned with a deeper structure among mtDNA lineages of fox squirrels suggesting that perhaps multiple (at least two) isolated refugia provided the source of postglacial colonists to the central and southeastern U.S. Thus, these results demonstrate that the demographic histories of species in glaciated landscapes are often more complex and variable than one would expect from a simple northward expansion form a southern glacial refugium.

4.3 Future Directions

To further elucidate the role of Pleistocene glaciations and recolonizations on the present-day genetic structure of *S. niger*, two subspecies which were not available for this analysis should be included in this data set. *S. n. avicennia* and *S. n. shermani* are two subspecies which inhabit southern and northern Florida, respectively, and should provide additional insight into whether these areas were used as refugia as well as possible colonization routes. Further sampling of *S. n. rufiventer* east and west of the Mississippi River, and of *S. n. limitis* and *S. n. ludovicianus* would also help elucidate the validity of this river as a barrier to recolonization. Population size could be estimated using indices of genetic diversity in DnaSP (Rozas and Rozas 1999) or Arlequin (Excoffier and Schneider 2005). A mismatch distribution would establish the demographic history of

this species as well as estimate the timing of historical population expansions and contractions. A more thorough understanding of gene flow patterns and finer scale genetic structure could be provided by microsatellite analysis. Finally, sequencing radio-carbon dated fossils could be used to more effectively calibrate the molecular clock and better pinpoint divergence dates for *S. niger* (Saarma et al. 2007).

Table 1: Samples and their haplotype designations. Sample names refer to Virginia Museum of Natural History specimen numbers. Map numbers refer to Figure 5. White boxes represent haplotypes and sequences generated at George Mason University; light grey boxes represent haplotypes and sequences generated by Dr. Ronald Van den Bussche at Oklahoma State University; dark grey boxes represent previously published haplotypes (Lance et al. 2003); outlined boxes represent haplotypes and sequences generated by both George Mason University and Oklahoma State University.
*Smithsonian Museum of Natural History specimen

Haplotype	N	Sample name	Map	State	County/Parish	Subspp
B1	1	1426	12	AL	Covington	*bachmani*
B3	1	1318	10	LA	St. Tammany	*bachmani*
B4	1	1319	10	LA	St. Tammany	*bachmani*
B5	1	1320	10	LA	St. Tammany	*bachmani*
B6	1	1099	11	MS	Holmes	*bachmani*
B7	1	1100	11	MS	Holmes	*bachmani*
B8	1	1101	11	MS	Holmes	*bachmani*
B9	1	1104	11	MS	Holmes	*bachmani*
BR1	3	1091	11	MS	Holmes	*bachmani*
		1750	4	IN	Dubois	*rufiventer*
		2231	2	KS	Ellis	*rufiventer*
C1	22	Hap1	23	MD	Dorchester	*cinereus*
		1352	23	MD	Dorchester	*cinereus*
		1356	23	MD	Dorchester	*cinereus*
C2	22	Hap2	23	MD	Dorchester	*cinereus*
		1351	23	MD	Dorchester	*cinereus*
		1357	23	MD	Dorchester	*cinereus*
		1360	23	MD	Dorchester	*cinereus*
C3	22	Hap3	23	MD	Dorchester	*cinereus*
C4	23	Hap4	23	MD	Dorchester	*cinereus*
		1358	23	MD	Dorchester	*cinereus*
C5	3	Hap5	23	MD	Dorchester	*cinereus*
C7	1	Hap7	23	MD	Dorchester	*cinereus*
C8	1	Hap8	23	MD	Dorchester	*cinereus*
C9	1	1354	23	MD	Dorchester	*cinereus*
C10	1	1350	23	MD	Dorchester	*cinereus*
C11	2	Hap11	23	MD	Queene Anne's	*cinereus*

CV1*	2	1353	23	MD	Dorchester	*cinereus*
		1705	21	MD	Allegany	*vulpinus*
D2	2	1064	8	LA	Acadia	*ludovicianus*
		1071	8	LA	Acadia	*ludovicianus*
D3	3	1065	8	LA	Acadia	*ludovicianus*
		1066	8	LA	Acadia	*ludovicianus*
		1070	8	LA	Acadia	*ludovicianus*
D4	1	1068	8	LA	Acadia	*ludovicianus*
D5	1	1006	6	LA	Bossier	*ludovicianus*
D6	1	954	6	LA	Bossier	*ludovicianus*
D7	1	957	6	LA	Bossier	*ludovicianus*
L1	8	2234	3	TX	Tom Green	*limitis*
		2235	3	TX	Tom Green	*limitis*
		2237	3	TX	Tom Green	*limitis*
		2239	3	TX	Tom Green	*limitis*
		2240	3	TX	Tom Green	*limitis*
		2242	3	TX	Tom Green	*limitis*
		2243	3	TX	Tom Green	*limitis*
		2238	3	TX	Tom Green	*limitis*
L2	1	2236	3	TX	Tom Green	*limitis*
L3	1	2241	3	TX	Tom Green	*limitis*
N1	2	1557	13	GA	Jasper	*niger*
		1575	13	GA	Jasper	*niger*
N3	1	1539	13	GA	Jasper	*niger*
N4	1	1551	13	GA	Jasper	*niger*
N5	1	1571	13	GA	Jasper	*niger*
N6	1	1576	13	GA	Jasper	*niger*
N7	1	2904	14	SC	Aiken	*niger*
N8	1	2905	14	SC	Aiken	*niger*
N9	12	2906	14	SC	Aiken	*niger*
	1	2912	14	SC	Aiken	*niger*
N10	1	2908	14	SC	Aiken	*niger*
N11	1	2910	14	SC	Aiken	*niger*
N14	1	2914	14	SC	Aiken	*niger*
N16	1	2917	14	SC	Aiken	*niger*
N17	1	2918	14	SC	Aiken	*niger*
N18	1	2919	14	SC	Aiken	*niger*
N19	1	2920	14	SC	Aiken	*niger*
N21	4	Hap9	15	SC	Allendale	*niger*
			15	SC	Hampton	*niger*
N22	2	Hap12	15	SC	Allendale	*niger*

N25	1	Hap16	15	SC	Hampton	*niger*
N26	1	Hap17	16	SC	Beaufort	*niger*
N27	1	Hap19	16	SC	Beaufort	*niger*
N28	1	Hap20	16	SC	Beaufort	*niger*
		3660	20	VA	Sussex	*niger*
NV1	5	1711	21	MD	Allegany	*vulpinus*
		1715	21	MD	Allegany	*vulpinus*
		1544	13	GA	Jasper	*niger*
		1700	21	MD	Allegany	*vulpinus*
		1706	21	MD	Allegany	*vulpinus*
N30	4	Hap18	15	SC	Allendale	*niger*
		Hap10	16	SC	Beaufort	*niger*
		3615	1	SD	Clay	*rufiventer*
NRV1	5	1710	21	MD	Allegany	*vulpinus*
		1704	21	MD	Allegany	*vulpinus*
		1687	18	VA	Alleghany	*vulpinus*
		1748	4	IN	Dubois	*rufiventer*
		2903	14	SC	Aiken	*niger*
R1*	1	1742	4	IN	Dubois	*rufiventer*
R2	1	1743	4	IN	Dubois	*rufiventer*
R3	2	1746	4	IN	Dubois	*rufiventer*
		1747	4	IN	Dubois	*rufiventer*
R4	1	1744	4	IN	Dubois	*rufiventer*
R5	2	1751	4	IN	Dubois	*rufiventer*
		1753	4	IN	Dubois	*rufiventer*
R6	1	1752	4	IN	Dubois	*rufiventer*
R7	1	1756	4	IN	Dubois	*rufiventer*
R9	1	1758	4	IN	Dubois	*rufiventer*
R12	1	2218	2	KS	Rooks	*rufiventer*
R14	2	2216	2	KS	Rooks	*rufiventer*
		2217	2	KS	Rooks	*rufiventer*
R15	1	2221	2	KS	Rooks	*rufiventer*
R16	1	466	5	AR	Greene	*rufiventer*
R17*	1	467	5	AR	Greene	*rufiventer*
RV1	4	1749	4	IN	Dubois	*rufiventer*
		1759	4	IN	Dubois	*rufiventer*
		1754	4	IN	Dubois	*rufiventer*
		1705	21	MD	Allegany	*vulpinus*
RV2	2	1698	21	MD	Allegany	*vulpinus*
		2214	2	KS	Rooks	*rufiventer*

S1	1	1	9	LA	Iberville	*subauratus*
S2	1	2	9	LA	Iberville	*subauratus*
S3	1	3	9	LA	Iberville	*subauratus*
S4	1	4	9	LA	Iberville	*subauratus*
S5	1	867	9	LA	E. Baton Rouge	*subauratus*
S6	1	1049	9	LA	Iberville	*subauratus*
S7	1	1116	9	LA	Iberville	*subauratus*
S8	2	1131	7	LA	Madison	*subauratus*
		1133	7	LA	Madison	*subauratus*
S9	2	1135	7	LA	Madison	*subauratus*
		1136	7	LA	Madison	*subauratus*
V1	5	1708	21	MD	Allegany	*vulpinus*
		1709	21	MD	Allegany	*vulpinus*
		1665	19	VA	Rockingham	*vulpinus*
		1699	21	MD	Allegany	*vulpinus*
		1703	21	MD	Allegany	*vulpinus*
V2	2	1726	21	MD	Allegany	*vulpinus*
		1701	21	MD	Allegany	*vulpinus*
V3	1	1702	21	MD	Allegany	*vulpinus*
V4	1	1706	21	MD	Allegany	*vulpinus*
V5	1	1707	21	MD	Allegany	*vulpinus*
V6	1	1712	21	MD	Allegany	*vulpinus*
V7	1	1713	21	MD	Allegany	*vulpinus*
V8	1	1714	21	MD	Allegany	*vulpinus*
V9	1	569078*	22	MD	Montgomery	*vulpinus*
V10	1	1651	18	VA	Alleghany	*vulpinus*
V11	1	1660	18	VA	Alleghany	*vulpinus*
V12	1	1662	18	VA	Alleghany	*vulpinus*
V13	1	1690	18	VA	Alleghany	*vulpinus*
V14	1	1694	18	VA	Alleghany	*vulpinus*
V15	1	2205	18	VA	Alleghany	*vulpinus*
V16	1	2206	18	VA	Alleghany	*vulpinus*
V17	1	2207	18	VA	Alleghany	*vulpinus*
V18	1	2209	18	VA	Alleghany	*vulpinus*
V19	1	Hap21	18	VA	Augusta	*vulpinus*
V21	2	2164	17	VA	Bland	*vulpinus*
		2184	17	VA	Russell	*vulpinus*
V22	1	2165	17	VA	Bland	*vulpinus*
V23	3	2169	17	VA	Bland	*vulpinus*
		2166	17	VA	Bland	*vulpinus*

		2180	17	VA	Russell	*vulpinus*
V24	1	2167	17	VA	Bland	*vulpinus*
V25	1	2168	17	VA	Bland	*vulpinus*
V26	1	2170	17	VA	Bland	*vulpinus*
V27	2	2171	17	VA	Bland	*vulpinus*
		2172	17	VA	Bland	*vulpinus*
V28	1	2173	17	VA	Bland	*vulpinus*
V29	1	2174	17	VA	Bland	*vulpinus*
V30	1	2213	19	VA	Culpeper	*vulpinus*
V31	1	1786	19	VA	Fauquier	*vulpinus*
V32	1	1788	19	VA	Page	*vulpinus*
V33	1	1789	19	VA	Rappahannock	*vulpinus*
V34	1	1648	19	VA	Rockingham	*vulpinus*
V35	1	1791	19	VA	Rockingham	*vulpinus*
V36	1	1792	19	VA	Rockingham	*vulpinus*
V37	1	2185	19	VA	Rockingham	*vulpinus*
V38	2	2186	19	VA	Rockingham	*vulpinus*
		2188	19	VA	Rockingham	*vulpinus*
V39	1	Hap22	19	VA	Rockingham	*vulpinus*
V41	1	2175	17	VA	Russell	*vulpinus*
V42	1	2176	17	VA	Russell	*vulpinus*
V43	1	2177	17	VA	Russell	*vulpinus*
V44	1	2178	17	VA	Russell	*vulpinus*
V45	1	2179	17	VA	Russell	*vulpinus*
V46	1	2181	17	VA	Russell	*vulpinus*
V47	1	1794	19	VA	Shenandoah	*vulpinus*

*CV1 is labeled CS1 in Figs. 2 and 3

*R1 is labeled R11 in Figs. 2 and 3

*R17 is labeled B2 in Figs. 2 and 3

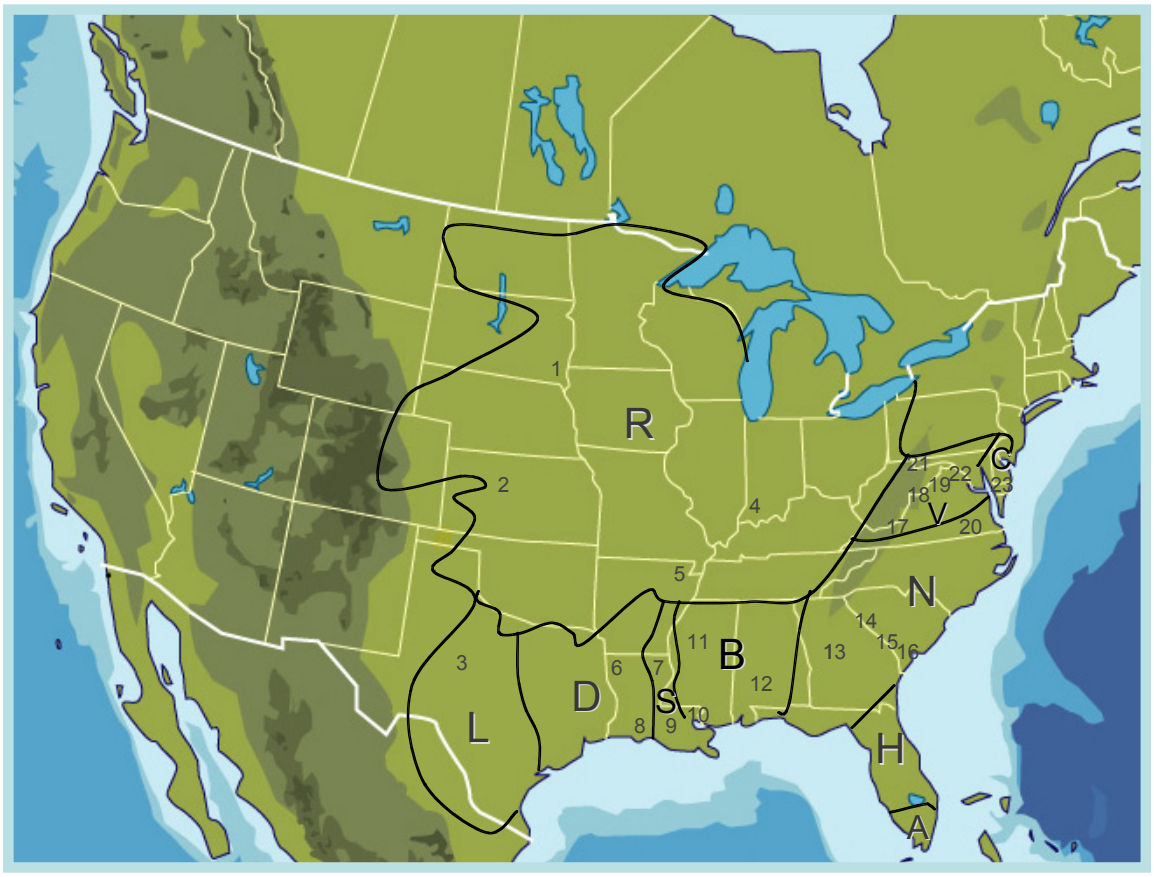

Figure 1: Distribution of *Sciurus niger*. Reproduced from Hall, 1981. Numbers represent collecting localities listed in Table 1. Letters represent subspecies as follows: A – *S. n. avicennia*, B – *S. n. bachmani*, C – *S. n. cinereus*, D – *S. n. ludovicianus*, H- *S. n. shermani*, L – *S. n. limitis*, N – *S. n. niger*, R – *S. n. rufiventer*, S – *S. n. subauratus*, V – *S. n. vulpinus*.

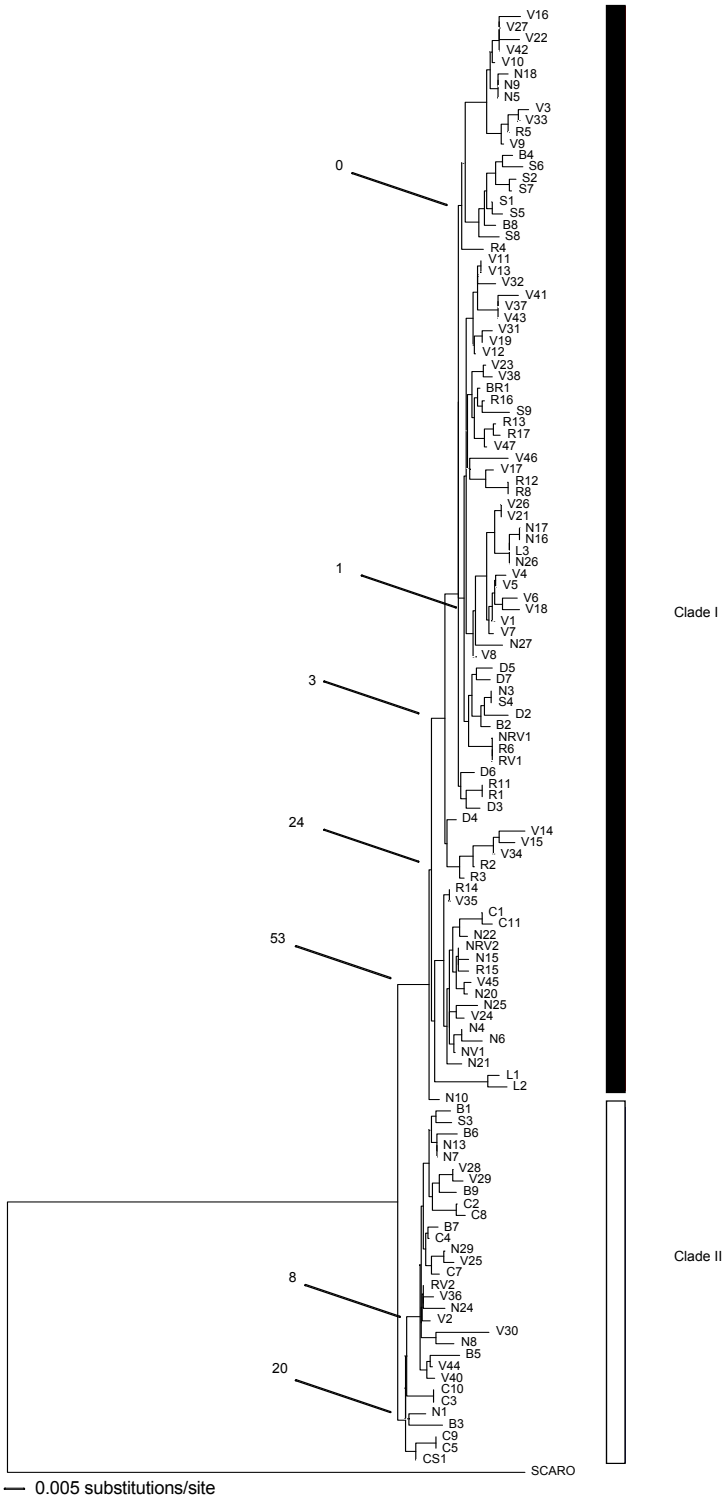
V16
V27
V22
V42
V10
N18
N9
N5
V3
V33
R5
V9
B4
S6
S2
S7
S1
S5
B8
S8
R4
V11
V13
V32
V41
V37
V43
V31
V19
V12
V23
V38
BR1
R16
S9
R13
R17
V47
V46
V17
R12
R8
V26
V21
N17
N16
L3
N26
V4
V5
V6
V18
V1
V7
N27
V8
D5
D7
N3
S4
D2
B2
NRV1
R6
RV1
D6
R11
R1
D3
D4
V14
V15
V34
R2
R3
R14
V35
C1
C11
N22
NRV2
N15
R15
V45
N20
N25
V24
N4
N6
NV1
N21
L1
L2
N10
B1
S3
B6
N13
N7
V28
V29
B9
C2
C8
B7
C4
N29
V25
C7
RV2
V36
N24
V2
V30
N8
B5
V44
V40
C10
C3
N1
B3
C9
C5
CS1
SCARO
0
1
3
24
53
8
20
Clade I
Clade II
0.005 substitutions/site

Figure 2: Neighbor-joining tree with 1:10 transition:transversion weighting, bootstrapped for 1000 replicates.

Figure 3: Bayesian Skyline analysis with relaxed molecular clock; 50 million replicates.

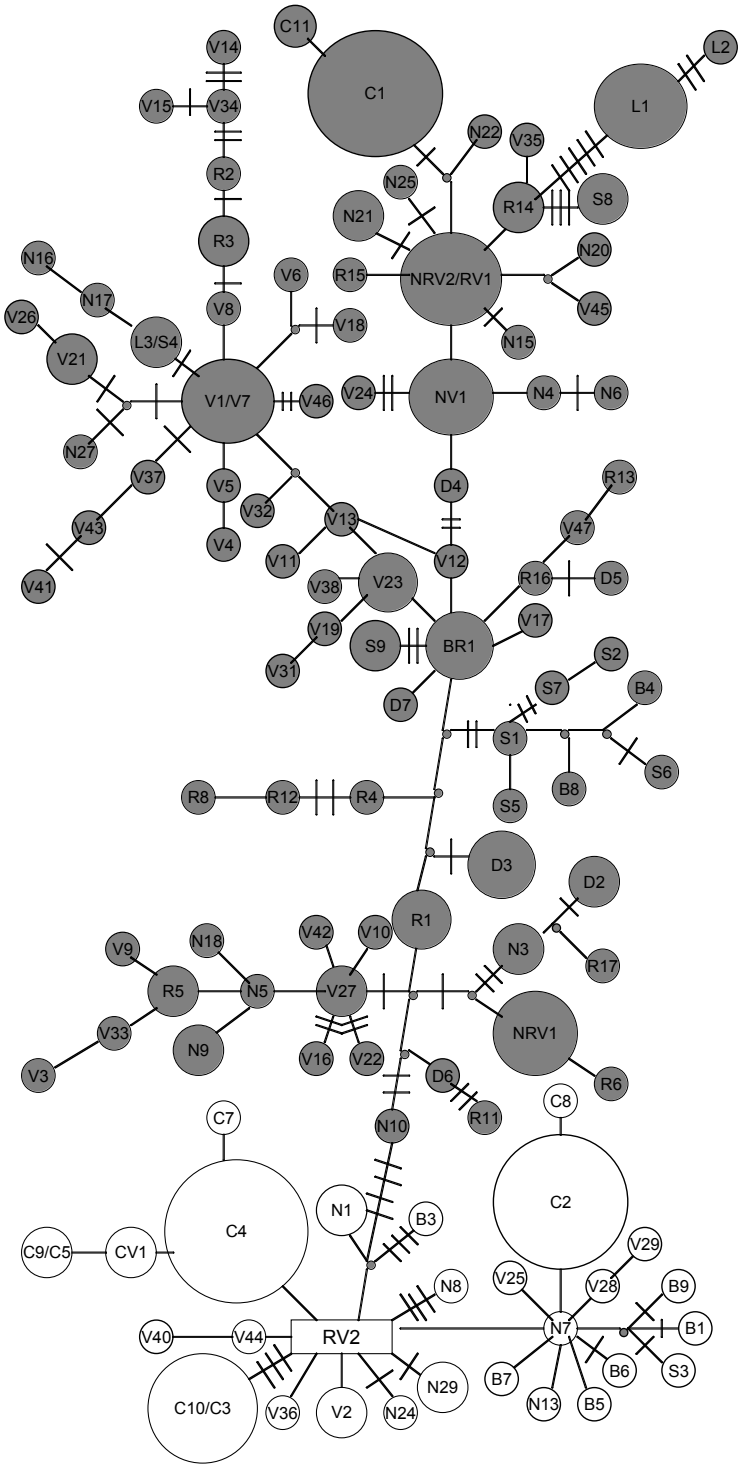

Figure 4: Minimum spanning network, 96% confidence. Hatch marks indicate base pair substitutions and size of haplotype nodes reflects number of individuals belonging to the haplotype. Black haplotypes correspond to Clade I and white haplotypes to Clade II.

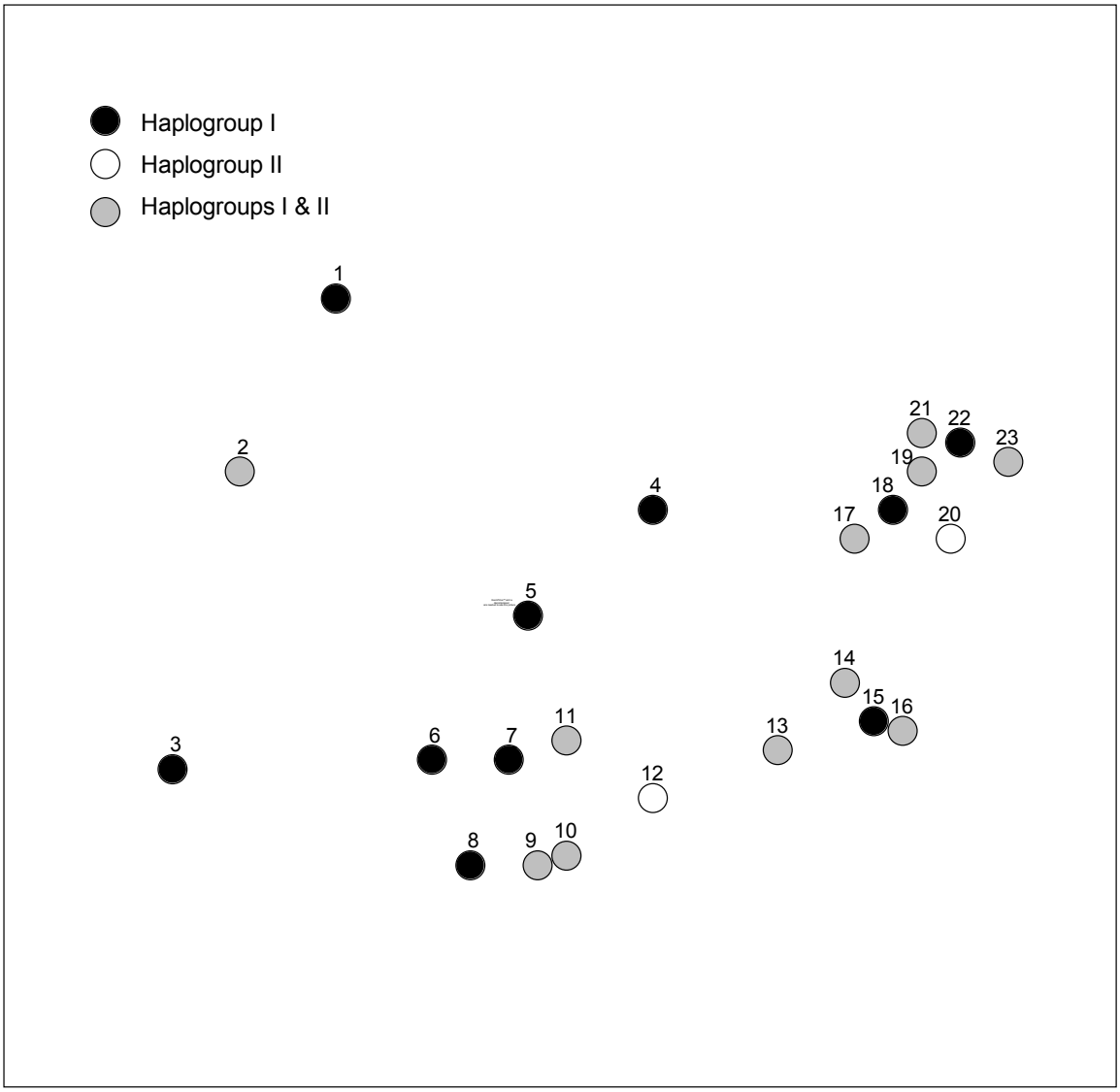

Figure 5: Haplogroup distribution map. Black circles represent localities in which haplogroup I haplotypes were found, white circles represent localities in which haplogroup II haplotypes were found, and striped localities represent localities in which both haplogroups were found.

Appendix A: *S. niger* Natural History

Adult fox squirrels are prey items for felids, canids, and predatory birds (Weigl et al. 1989), while smaller mammals and snakes predate nestlings and subadults (Loeb and Moncrief 1993). *S. niger* is distinguished from its sympatric congeners *S. aberti*, *S. carolinensis*, *S. griseus*, and *S. aureogaster*, by the absence of the third upper premolar (Hall 1981). The fox squirrel is also notable for its large size and highly variable coat color, possibly the most variable among North American mammals (Hall 1981, Weigl et al. 1989). Partial and complete melanism, erythrism, and albinism occur (Hall 1981; pers. obs.) Individuals weigh between 507-1361 g (Flyger and Gates 1982) and body length ranges from 454-698 mm (Hall 1981) with no sexual dimorphism in size or color (Koprowski 1994a). Fox squirrels have large home ranges compared to other sciurids, reported at 20-32 ha for males and 9-19 ha for females (Flyger and Smith 1980; Weigl et al. 1989) and may disperse as far 64.4 km during the fall season (Allen 1943). Fox squirrels play an important ecological role as dispersers of mast (Steele and Koprowski 2001) which aids in seedling recruitment (Barnett 1977; Haas and Heske 2005) and many characteristics of *Quercus* suggest a co-evolution of squirrels and trees (Smith and Follmer 1972; Steele and Koprowski 2001).

The distribution and abundance of *S. niger* is impacted by many factors, including habitat type, interspecific competition, and human influence (Derge and Steele 1999, Taylor 1974). Fox squirrels are found in forest patches of 40 hectares or more (Nixon

and Hansen 1987) and prefer low levels of understory (Steele and Koprowski 2001), although this varies among subspecies (Weigl et al. 1998). Fox squirrel densities in the eastern portion of their range are highest in mixed hardwood stands containing oak (*Quercus*), pine (*Pinus*), hickory (*Carya*) and walnut (*Juglans*), which provide winter-storable foods (Koprowski 1994a). In the western portion, fox squirrels are associated with riverine corridors of cottonwood (*Populus deltoides*; Knapp and Swendon 1986) and osage orange (*Maclura pomifera*) fencerows (Packard 1956). Most eastern and southern range extensions are associated with mixed pine-oak forests (Kantola and Humphrey 1990; Lowery and Davis 1942; Taylor 1974; Weigl et al. 1989), although two subspecies (*S. n. subauratus* and *S. n. subauratus*) specialize in bald cypress (*Taxodium distichum*) forests (Roe 1994; Williams and Humphrey 1979).

Anthropogenic influence has impacted fox squirrel distribution and abundance considerably. Native Americans and Europeans arriving in the 1600's hunted small game such as fox squirrels (Ewan and Ewan 1970; Loeb and Moncrief 1993). Currently, fox squirrels are hunted throughout most of their range, although the impact on population size appears to be negligible (Fies 1993). Colonization and expansion of settlements by Europeans increased deforestation and fragmentation of suitable habitat starting as early as the 1700's (Loeb and Moncrief 1993). Logging and managed forest practices may also negatively impact this species, as fox squirrel presence has been positively correlated with tree maturity (Conner and Godbois 2003; Taylor 1974). Moreover, mature forest may be the limiting factor in distribution of this species (USFWS 1993).

Interspecific competition with gray squirrels (*S. carolinensis*) is another important factor in fox squirrel distribution and abundance. Although feeding ecology between the two species is highly similar (Smith and Follmer 1972), fox squirrels prefer forests with mature trees and vines (Salsbury 2004), farmland-forest edge habitat (Derge and Yahner 2000), frequently burned forested areas and savannah (Flyger and Lustig 1976), as well as riverine corridors (Koprowski 1994a), while *S. carolinensis* is more likely to be found in interior forest areas with thick understory (Koprowski 1994b). Fox squirrel preference for edge habitat and greater use of terrestrial locomotion when compared to *S. carolinensis* (Adams 1976) has resulted in many modern *S. niger* populations thriving in suburban communities, parks, college campuses, golf courses and similarly modified habitats (Flyger and Lustig 1976). Delmarva fox squirrels (*S. n. cinereus*), however, have been negatively impacted by habitat fragmentation (Taylor 1974) and *S. n. vulpinus* populations have been extirpated over much of their former range in central Maryland and Virginia as a result of changing land use over the past century (Flyger and Lustig 1976; Moncrief 1998), while *S. carolinensis* populations in the same areas remain abundant. Despite available literature, it is still unclear whether gray squirrels typically outcompete fox squirrels in areas of sympatry (Loeb and Moncrief 1993).

Appendix B: *S. niger* Subspecies

Sciurus niger avicennia, first described in 1919, specializes in bald cypress, slash pine (*Pinus elliotii*), and mangrove (*Avicennia germinans*) swamp habitat in southern Florida (Fig.1; Williams and Humphrey 1979). The big cypress fox squirrel is listed as threatened by the Florida Game and Fresh Water Fish Commission as a result of habitat loss (Wood 1994).

Sciurus niger bachmani was described in 1942 as ferruginous with some black on the dorsum and white ears, toes, and rostrum (Lowery and Davis 1942); melanism is common. These squirrels are found in pine (*Pinus* spp.) forests in northern Georgia, Alabama, eastern Tennessee, eastern Mississippi and eastern Louisiana (Fig. 1; Roe 1994). The validity of this subspecies designation is in question (Roe 1994; Turner and Laerm 1993).

Sciurus niger cinereus Linnaeus, the Delmarva fox squirrel, was first described in 1758 as *Sciurus cinereus* (Koprowski 1994a) and is characterized by a steel gray color phase coupled with a smaller size. Found in mature hardwood and loblolly pine savanna in the Delmarva peninsula (Koprowski 1994), Delmarva fox squirrel population size has been declining since 1862 (Poole 1944) and is currently listed by the US Fish and Wildlife Service as endangered. *S. n. cinereus* was successfully reintroduced to its former range in Pennsylvania and parts of the Delmarva Peninsula including areas in

Maryland, Delaware, and Chincoteague Wildlife Refuge in Virginia following historical extirpations from these regions (Lance et al. 2003). These translocated populations have similar genetic and allozymic diversity when compared to source populations, suggesting the long-term viability of these populations is favorable (Lance et al. 2003; Moncrief and Dueser 2001).

Sciurus niger limitis Baird, described in 1855 as *Sciurus limitis*, is a smaller subspecies with a rufous venter which inhabits upland pine-oak-hickory woodlands (Goodrum 1937; Lowery and Davis 1942) from Colorado and Comanche Counties, Texas west to the Pecos River (Fig. 1; Lowery and Davis 1942).

Originally described in 1806, *Sciurus niger ludovicianus* Custis is found in pine-oak-hickory woodlands in Texas and Louisiana (Goodrum 1937; Lowery and Davis 1942).

Sciurus niger niger Linnaeus, the southeastern fox squirrel, is a large subspecies with a black head, with white ears, toes, and rostrum and high levels of melanism. The southeastern fox squirrel is associated with longleaf pine (*P. palustris*) and turkey oak (*Q. laevis*) forests (Weigl et al. 1989) in southeastern Virginia, North and South Carolina, eastern Tennessee, Georgia, and northern Florida (Fig. 1). *S. n. niger* has been recommended for listing as an endangered species in Virginia (Terwilliger 1991).

Geoffroy St.-Hilaire first described western fox squirrels as *Sciurus rufiventer* in 1803 (Koprowski 1994a). *S. n. rufiventer* occurs from northern New York to Tennessee, west to Oklahoma and North Dakota. These large squirrels are characterized by a tawny brown dorsum with a pale cinnamon venter (Lowery and Davis 1942). *S. n. rufiventer* has been expanding into North Dakota since 1935 (Hibbard 1956) and more recently into Manitoba (Wrigley et al. 1973), Montana, and western New York (Lampe et al. 1974; Hamilton 1957). Introductions of *S. n. rufiventer* have occurred in California, Colorado, Georgia, Idaho, Oregon, Texas, Washington, and Mexico (Golley 1966; Flyger and Gates 1982; Laerm 1981; Chapman and Feldhamer 1987).

Sciurus niger shermani Moore, first described in 1956, is found in longleaf pine (*P. palustris*) savanna and live oak (*Q. virginiana*) forest edges (Kantola and Humphrey 1990) from peninsular Florida to the Caloosahatchee River in Georgia (Fig. 1; Roe 1994). *S. n. shermani* has coloration is similar to *S. n. niger*; the former is slightly larger than *S. n. niger* and often has tan markings on the ears and feet (Moore 1956). Currently, *S. n. shermani* is listed as a species of special concern (Florida Game and Freshwater Fish Commission 1994).

Sciurus niger subauratus was first described in 1839 by Bachman. This subspecies inhabits lowland forests of bald cypress and tupelo gum (*Nyssa* spp.) in the Yazoo and Tenasas Basins and the Pine Flats of Louisiana (Roe 1994). They are smaller

than *S. n. rufiventer*, with a browner dorsum and a more reddish venter (Roe 1994). High levels of melanism have been observed (Lowery and Davis 1942).

The southeastern fox squirrel subspecies, *Sciurus niger vulpinus* Gmelin 1788 is based on a type specimen from White Sulphur Springs, West Virginia (Hall 1981). *S. n. vulpinus* is distinguished from other subspecies by its gray color phase with yellow or orange grizzling on the back and sides (Poole 1944). *S. n. vulpinus* is found in mature hardwood or pine-hardwood stands with little understory cover. This subspecies ranges from southern Pennsylvania through the mountainous portions of western Maryland into western Virginia and eastern parts of West Virginia. In Pennsylvania, *S. n. rufiventer* populations have expanded their range and may be displacing *S. n. vulpinus* populations, suggesting the latter may be threatened in the northern part of its range (Derge and Steele 1999). Populations in Maryland have been reduced to a few isolated colonies in Montgomery and Harford counties (Flyger and Lustig 1983). *S. n. vulpinus* may be expanding eastward in Virginia (Fies 1993) and into abandoned farmland areas in Pennsylvania (Derge 1997). Overall, changing land use in the mid-Atlantic region over the past 100 years has probably reduced the home range of *S. n. vulpinus* and led to population decline (Flyger and Lustig 1976; Loeb and Moncrief 1993). In Pennsylvania, Zegers (1985) recommended this subspecies for listing as "status undetermined". Derge and Steele (1999) recommended the status be changed to threatened.

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Curriculum Vitae

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