Journal of Mammalogy, 93(5):1247–1264, 2012

General lack of phylogeographic structure in two sympatric, forest obligate squirrels (*Sciurus niger* and *S. carolinensis*)

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We examined intraspecific relationships in the eastern fox squirrel (*Sciurus niger*) and the eastern gray squirrel (*S. carolinensis*) using sequence variation in a portion of the mitochondrial DNA cytochrome-*b* gene and part of the D-loop in the control region. These closely related species are codistributed temperate forest obligates that have similar generation time and population ecologies. For both species, we documented high haplotype diversity, low nucleotide variation, and several groups of divergent haplotypes. However, there is a general lack of spatial structure in maternal lineages within each species. For *S. carolinensis*, we observed a pattern of population genetic structure that suggests the presence of at least 2 distinct refugial populations that evolved in isolation during the Pleistocene (approximately 98.3–266.3 thousand years ago [kya]) and subsequently expanded to the species' current range following the last glacial maximum. For *S. niger*, the genetic structure was much less pronounced, with fewer strongly diverged haplotypes. This finding suggests that eastern fox squirrels persisted in either a single population in a glacial refugium or as several refugial populations that maintained gene flow throughout the Pleistocene. For both species, there is evidence that scattered populations were present in multiple, small refugia close to the Laurentide Ice Sheet, allowing rapid range expansion following glacial recession. Taken together, our results indicate that *S. niger* and *S. carolinensis* underwent multiple episodes of genetic divergence during isolation in glacial refugia, followed by range expansion and contact that resulted in admixture of divergent maternal lineages within each species during interglacials. Examination of our data further indicates that the most recent range expansion in both species occurred within the past 12–20 kya.

Key words: coalescent, eastern North America, fox squirrel, glacial refugia, gray squirrel, incomplete lineage sorting, mismatch distribution, mitochondrial DNA, phylogeography, Pleistocene

DOI: 10.1644/11-MAMM-A-383.1

Climate change, and the associated changes in size and position of ice sheets, dramatically shifted the distributions of temperate plants and animals in eastern North America during the Pleistocene (Pielou 1991). Evidence from fossil pollen and plant macrofossils indicates that many temperate tree species underwent episodes of being pushed into southern refugia during glacial advances, followed by northward expansion after the glaciers receded (Davis 1981; Delcourt and Delcourt 1981; Soltis et al. 2006). Phylogeographic data often are used to examine current patterns of genetic connectivity and differentiation to gain insight into historical factors, such as climate change, that have altered the distributions of plants and animals. Analyses of annual plants and forest trees indicate that Pleistocene glacial refugia of forest trees existed in central Texas, southern Florida, and the southern Appalachians (Jaramillo-Correa et al. 2009). Alternative scenarios for

postglacial spread of these forests suggest that temperate species were present (in "cryptic" refugia) in low densities across much of the continent, even during the most severe glacial periods (Bennett 1985; McLachlan et al. 2005).

Many tree species have low chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) mutation rates, thereby providing limited polymorphism for phylogeographic analyses (Hu et al. 2009). Hu et al. (2009) recently observed that the short generation times and rapidly evolving mtDNA of animals closely associated with forests can act as a "magnifying glass" to help illuminate the population history of trees, which evolve at a relatively slow rate. Hu et al. (2009) also noted that the increased level of mtDNA polymorphisms in many animals can be analyzed with sophisticated statistical techniques, which can be used to infer paleopopulation information such as the timing of divergence and estimates of refugial population sizes for tree species. Hu et al. (2009) further noted that the most reliable "magnifying glasses" of tree population histories are phylogeographic surveys of specialist species.

In the temperate forests of eastern North America, 2 of the most notable tree specialists are the eastern gray squirrel (*Sciurus carolinensis*) and the eastern fox squirrel (*S. niger*). Both of these species are obligates of mature forests that depend on mature trees for food, nests, and cover from predators (Koprowski 2005). Both species coevolved with seed, fruit, and nut trees in the temperate forests of eastern North America in their dual role as seed predators and seed dispersers (Steele 2008).

Sciurus carolinensis is one of the most familiar wild mammals in eastern North America, occurring naturally over most of eastern North America in temperate forests east of about 100°W and south of about 47°N (Hall 1981; Koprowski 1994b). The eastern gray squirrel also is a conspicuous (diurnal) and common resident of urban parks and suburban neighborhoods. Although *S. carolinensis* has been the subject of numerous studies of ecology and behavior (Steele and Koprowski 2001), few studies have examined geographic variation and population genetics in this species (Moncrief 1993, 1998).

The closely related eastern fox squirrel (*S. niger*) also occurs naturally in temperate forests over most of eastern North America south of about 49°N and east of about 105°W (Hall 1981; Koprowski 1994a). *S. carolinensis* and *S. niger* are sympatric, if not syntopic, over broad portions of their range (Edwards et al. 2003). A recent range-wide phylogeographic study of *S. niger* using 402 base pairs (bp) of the mtDNA cytochrome-*b* (*Cytb*) gene found evidence for a recent and rapid range expansion in this species following the last glacial maximum (LGM) of the Pleistocene and failed to detect regional differentiation (Moncrief et al. 2010). This is in sharp contrast to the well-documented regional differences the eastern fox squirrel exhibits in morphology (Moncrief 1993; Turner and Laerm 1993; Weigl et al. 1998) and ecology (Ditgen et al. 2007; Edwards et al. 1998; Jodice and Humphrey 1992; Kantola and Humphrey 1990; Perkins and Conner 2004; United States Fish and Wildlife Service 1993; Weigl et al. 1989).

Eastern fox squirrels and eastern gray squirrels have similar life histories (Wood et al. 2007). Females of both species typically do not reproduce until >1.25 years of age, and reproductive longevity in females may exceed 12 years (Koprowski 1994a, 1994b). Adult females (>1.0 year old) may produce 2 litters in the same year (early spring and late summer), although reproduction in both species is highly dependent on food availability (Edwards et al. 2003). Average litter size ranges from 1.7 to 3.0 in eastern fox squirrels and 2.3 to 2.9 in eastern gray squirrels (Koprowski 1994a, 1994b). Sex ratios in litters of both species approximate 1:1 (Edwards et al. 2003).

These species also have similar ecological requirements. Both species feed heavily on the acorns, nuts, flowers, and buds of oaks (*Quercus*), hickories and pecans (*Carya*), walnuts (*Juglans*), and beech (*Fagus*—Edwards et al. 2003; Koprowski 1994a, 1994b). Other foods for both species include fruits, seeds, buds, and flowers of a variety of other trees including maples (*Acer*) and Alleghany chinkapin (*Castanea pumila*—Edwards et al. 2003). Both species construct leaf nests and use tree cavities to escape from predators, for protection from inclement weather, and for rearing young (Edwards et al. 2003; Koprowski 1994a, 1994b).

Studies of codistributed, closely related species with similar generation time and population ecologies can provide insight into how ecological, geological, and historical processes have shaped regional communities (Arbogast and Kenagy 2001; Austin and Zamudio 2008). In this study we use variation in mtDNA sequences to investigate intraspecific relationships in *S. niger* and *S. carolinensis* to increase our understanding of the evolutionary histories of these 2 ecologically similar, closely related sciurids. We augment existing *Cytb* data for *S. niger* (Moncrief et al. 2010) with sequence data from the D-loop in the control region, which is the most rapidly evolving portion of the mitochondrial genome (Moritz et al. 1987). We also report and analyze comparable *Cytb* and D-loop data for *S. carolinensis* to address the following questions for each species: Does phylogeographic structure exist? What historical evolutionary processes (e.g., isolation in glacial refugia and range expansion) have led to the contemporary distribution of genetic diversity?

Materials and Methods

Sample collection.—We obtained tissue samples from 81 individuals of *S. niger* collected at 16 localities in Arkansas, Georgia, Indiana, Kansas, Louisiana, Maryland, Mississippi, South Dakota, Texas, and Virginia from 1984 through 1998 (Fig. 1). We also obtained tissue samples from 69 individuals of *S. carolinensis* collected at 14 localities in Alabama, Georgia, Indiana, Louisiana, Maryland, Mississippi, Tennessee, and Virginia from 1983 through 1993 (Fig. 1). Nine localities (in Georgia, Indiana, Louisiana, Maryland, Mississippi, and Virginia) yielded samples of both species (Fig.

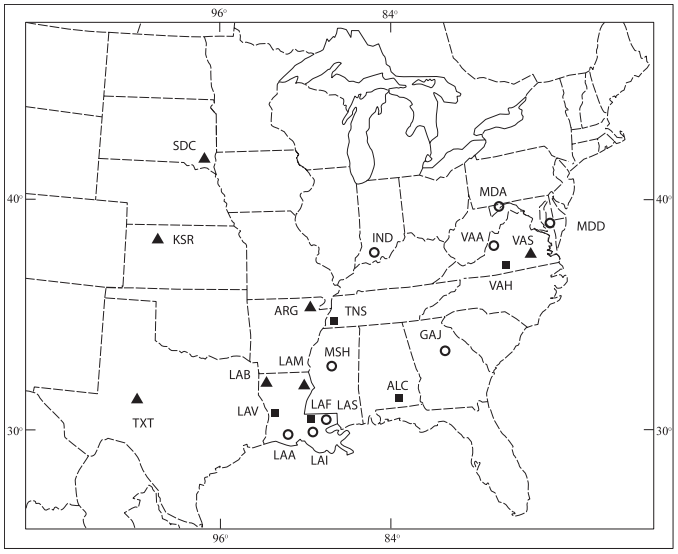

Fig. 1.—Sampling localities for the eastern fox squirrel (*Sciurus niger*) and the eastern gray squirrel (*S. carolinensis*). Codes for sampling localities (3 letters) correspond to those defined in Tables 1–3 and Appendices I and II. Samples of both species were obtained at localities shown as open circles; samples of only *S. niger* were obtained at localities shown as closed triangles; and samples of only *S. carolinensis* were obtained at localities shown as closed squares.

1). All samples were collected following guidelines approved by the American Society of Mammalogists (Sikes et al. 2011). All samples (0.5 g of liver) were obtained from the frozen tissue collections of the Virginia Museum of Natural History (VMNH) and Louisiana State University Museum of Natural Sciences (LSUMZ); voucher specimens are deposited in their respective mammal collections. Voucher specimens and tissues of *S. n. cinereus* were transported to and are housed at VMNH under Regional Blanket Permit 697823, issued to NDM.

DNA extraction, amplification, and sequencing.—Total genomic DNA was isolated using standard phenol–chloroform protocols (Longmire et al. 1997). A 486-bp fragment of the D-loop was amplified using polymerase chain reaction with primers DFSloopF (5′CGCAATACTCGACCAATCC-3′) and DFSloopR (5′TGATGATTTCACGGAGGTAGG-3′—Lance et al. 2003). Also, for *S. carolinensis*, a fragment consisting of the first 402 bp of *Cytb* was amplified with primers MVZ05 and MVZ04 (Smith and Patton 1991). Double-stranded products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin), and both strands of the purified polymerase chain reaction products were sequenced using Big Dye chain terminators and a 3130 Genetic Analyzer (Applied Biosystems, Inc., Foster City, California). AssemblyLIGN 1.0.9 (1998—Oxford Molecular Group, Oxford, United Kingdom) was used to assemble contigs for each individual, and CLUSTAL X (Thompson et al. 1997) was used to generate multiple sequence alignments. MacClade 4.0 (Maddison and Maddison 2000) was used for visual inspection of the multiple sequence alignment and to determine unique haplotypes using the REDUNDANT TAXA option.

Data analysis.—We used Arlequin version 3.5.1.21 (Excoffier and Lischer 2010) to estimate measures of intraspecific genetic variation (haplotype diversity and nucleotide diversity) in D-loop sequence data for both *S. niger* and *S. carolinensis* and in *Cytb* sequence data for *S. carolinensis*. For the remainder of the analyses, we combined both fragments for *S. carolinensis* into a segment that totaled 888 bp. For *S. niger* we combined a 402-bp fragment of *Cytb* sequence published previously (18 haplotypes—Moncrief et al. 2010) with D-loop sequences for each individual, also yielding a segment that totaled 888 bp.

For each species, we used BEAST version 1.6.1 (Drummond and Rambaut 2007) to estimate divergence times and to generate Bayesian skyline plots (Drummond et al. 2005). The Bayesian skyline plot estimates effective sample size (Drummond et al. 2002) through time, providing a graphical representation of past population dynamics while simultaneously sampling phylogenies and node ages (Drummond et al. 2002). A likelihood ratio test was unable to reject the molecular clock hypothesis for concatenated *Cytb* and D-loop for both species (*S. niger*, $2\Delta L = 4.0284$, $P = 0.6728$; *S. carolinensis*, $2\Delta L = 82.4083$, $P = 0.0972$); therefore, to produce time estimates in years we used a strict molecular clock. For *Cytb*, we utilized a pairwise divergence of 7.5–12% per million years, as had been estimated previously for rodents (Arbogast et al. 2001). Past estimates of D-loop substitution rates have been extremely variable (Parsons et al. 1997), making it essentially impossible to identify in the literature a consistent substitution rate for the rodent D-loop. To overcome this, the substitution rate for the D-loop was left with an uninformative prior in all analyses and estimated relative to the *Cytb* rate. All BEAST analyses utilized the HKY + I + Γ model of nucleotide substitution, as was indicated to be most appropriate by MrModeltest version 2.3 (Nylander 2004). *Cytb* and D-loop sequences were concatenated for all BEAST analyses, but partitioned to allow a distinct substitution model for the entire D-loop, and for each codon position within the *Cytb* alignment (in addition to the substitution rate partitioning described above). Tree topologies were linked across the *Cytb* and D-loop loci, due to the permanent link among mtDNA loci, and utilized the Bayesian skyline plot tree prior (Drummond et al. 2005). For each species, BEAST analyses consisted of an initial run of 5×10^7 generations, following which operator values were adjusted to optimize search settings. Two final runs of 5×10^7 generations were run with optimized search settings, and the resulting log and tree files were combined to produce final estimates of demographic parameters, to generate Bayesian skyline plots, and to obtain time-calibrated phylogenies (following removal of 10% of samples as burn-in). All runs were checked for sufficient mixing, stable convergence on a unimodal posterior, and effective sample size >200 for all parameters using TRACER version 1.4 (Drummond and Rambaut 2003). One assumption of the Bayesian skyline plot analysis is the absence of strong population structure. Although this is a reasonable assumption for *S. niger* based on previous work (Moncrief et al. 2010) and analyses herein, network and

Table 1.—Geographic information and distribution of D-loop haplotypes among 16 sampling localities of the eastern fox squirrel (*Sciurus niger*). Locality codes (code) and haplotype designations correspond to those shown in Fig. 1 and Appendix I. For each locality, we provide the number of individuals sequenced (*n*), number of individuals with a haplotype shared by 2 or more localities (F, I, K, N, and O), private haplotypes (with number of individuals in parentheses), haplotype diversity (*h*, with standard deviation in parentheses), and nucleotide diversity (π, with standard deviation in parentheses).

Code	County(ies) or parish(es) and state	*n*	Haplotypes: Shared F	I	K	N	O	Private	*h*	π
ARG	Greene, Arkansas	1						u (1)	0.000	0.000
GAJ	Jasper, Georgia	10						e (3), g (1), p (2), rr (1), ss (1), tt (1), uu (1)	0.911 (0.077)	0.019 (0.011)
IND	Dubois, Indiana	1					1		0.000	0.000
KSR	Rooks and Ellis, Kansas	8		1		1		b (4), ggg (1), hhh (1)	0.786 (0.151)	0.011 (0.007)
LAA	Acadia, Louisiana	6						bb (1), d (3), h (2)	0.733 (0.155)	0.015 (0.010)
LAB	Bossier, Louisiana	3						w (1), x (1), y (1)	1.000 (0.272)	0.009 (0.008)
LAI	East Baton Rouge and Iberville, Louisiana	7						gg (1), q (2), r (1), s (1), v (1), z (1)	0.952 (0.096)	0.014 (0.008)
LAM	Madison, Louisiana	5						hh (1), j (2), l (2)	0.800 (0.164)	0.013 (0.009)
LAS	St. Tammany, Louisiana	3						ii (1), jj (1), kk (1)	1.000 (0.272)	0.029 (0.022)
MDA	Allegany, Maryland	8	1		1	1	1	c (2), ccc (1), zz (1)	0.964 (0.077)	0.017 (0.010)
MDD	Dorchester, Maryland	8	3		1			ll (1), mm (1), nn (1), pp (1)	0.893 (0.111)	0.015 (0.009)
MSH	Holmes, Mississippi	4		1				cc (1), ee (1), ff (1)	1.000 (0.177)	0.024 (0.017)
SDC	Clay, South Dakota	2						nnn (1), ooo (1)	1.000 (0.500)	0.021 (0.022)
TXT	Tom Green, Texas	10						a (7), kkk (1), lll (1), mmm (1)	0.533 (0.180)	0.006 (0.005)
VAA	Alleghany, Virginia	3						ww (1), xx (1), yy (1)	1.000 (0.272)	0.018 (0.014)
VAS	Sussex, Virginia	2						ppp (1), qqq (1)	1.000 (0.500)	0.008 (0.009)

phylogenetic analyses of *S. carolinensis* in this study recovered 2 highly divergent clades, with some structure within these clades. To minimize the structure in the Bayesian skyline plot analysis of *S. carolinensis*, we analyzed the 2 divergent clades separately with the same search parameters as described above.

We also constructed a minimum spanning network at a 95% confidence level using TCS version 2.1 (Clement et al. 2000). Finally, we used DNAsp version 4.20.2 to generate mismatch distributions, which plot the distribution of pairwise genetic differences between pairs of individuals (Rogers and Harpending 1992). The plots generated from the observed data set were compared to expected distributions under models of sudden expansion (Rogers and Harpending 1992). For each data set, we used Arlequin version 3.5.1.21 to calculate sum of squared deviation and Harpending's raggedness index (Harpending 1994) to assess the fit of the observed data to a model of sudden expansion.

Results

Sciurus niger.—Among the 81 individuals of *S. niger* examined, we detected 55 unique D-loop haplotypes. Representative sequences of these 55 haplotypes have been deposited in GenBank (accession numbers JX104344–JX104398; Appendix I). Five haplotypes (f, i, k, n, and o) were shared between 2 or more localities; the remaining 50 haplotypes were private, occurring at only a single locality (Table 1; Appendix I). Seven haplotypes (when reduced by approximately 200 bp) were identical to haplotypes reported by the only other study of *S. niger* that used D-loop sequences (Lance et al. 2003) as follows: haplotype f is the same as GenBank accession number AF533258, ll is AF533254, mm is AF533256, nn is AF533255, pp is AF533260, ooo is AF533268, and rr is AF533272. Five of these (f, ll, mm, nn, and pp) are restricted in both studies to samples from Maryland. Haplotype ooo in our study was from South Dakota; AF533268 in Lance et al. (2003) was from South Carolina. Haplotype rr in our study was from Georgia; AF 533272 in Lance et al. (2003) was from South Carolina.

Haplotype diversity for the 16 sampling localities of *S. niger* in our study ranged from 0 to 1.00, and nucleotide diversity ranged from 0 to 0.029 (Table 1). Overall haplotype diversity was high (0.985 ± 0.006), and overall nucleotide diversity was low (0.023 ± 0.012). This latter estimate was similar to values (0.019–0.031) reported by Lance et al. (2003).

Combining *Cytb* and D-loop sequences for the 81 individuals of *S. niger* from 16 localities yielded 55 unique combined haplotypes (Appendix I). The BEAST chronogram indicated all sampled haplotypes of *S. niger* coalesce on a common ancestor approximately 33.3–91 thousand years ago (kya; Fig. 2). Three major groups of haplotypes that occurred at 2 or more localities (consisting of 16, 6, and 9 haplotypes, respectively) were supported by posterior probability values $\geq$ 0.95. Haplotypes in group 1 were widely distributed across the species' range and occurred in 10 of 16 localities (Figs. 1 and 2). Haplotypes in group 2 were absent from the southwest and lower Mississippi River valley, whereas those in group 3 were restricted to the lower Mississippi River valley (Figs. 1 and 2). The remaining 24 haplotypes occurred throughout the species' range (Figs. 1 and 2). Each of these major groups of *S. niger* coalesce on a common ancestor approximately 9.9–48 kya (Fig. 2). The upper end of the estimated range of dates at which each of these major groups of *S. niger* coalesces on a common

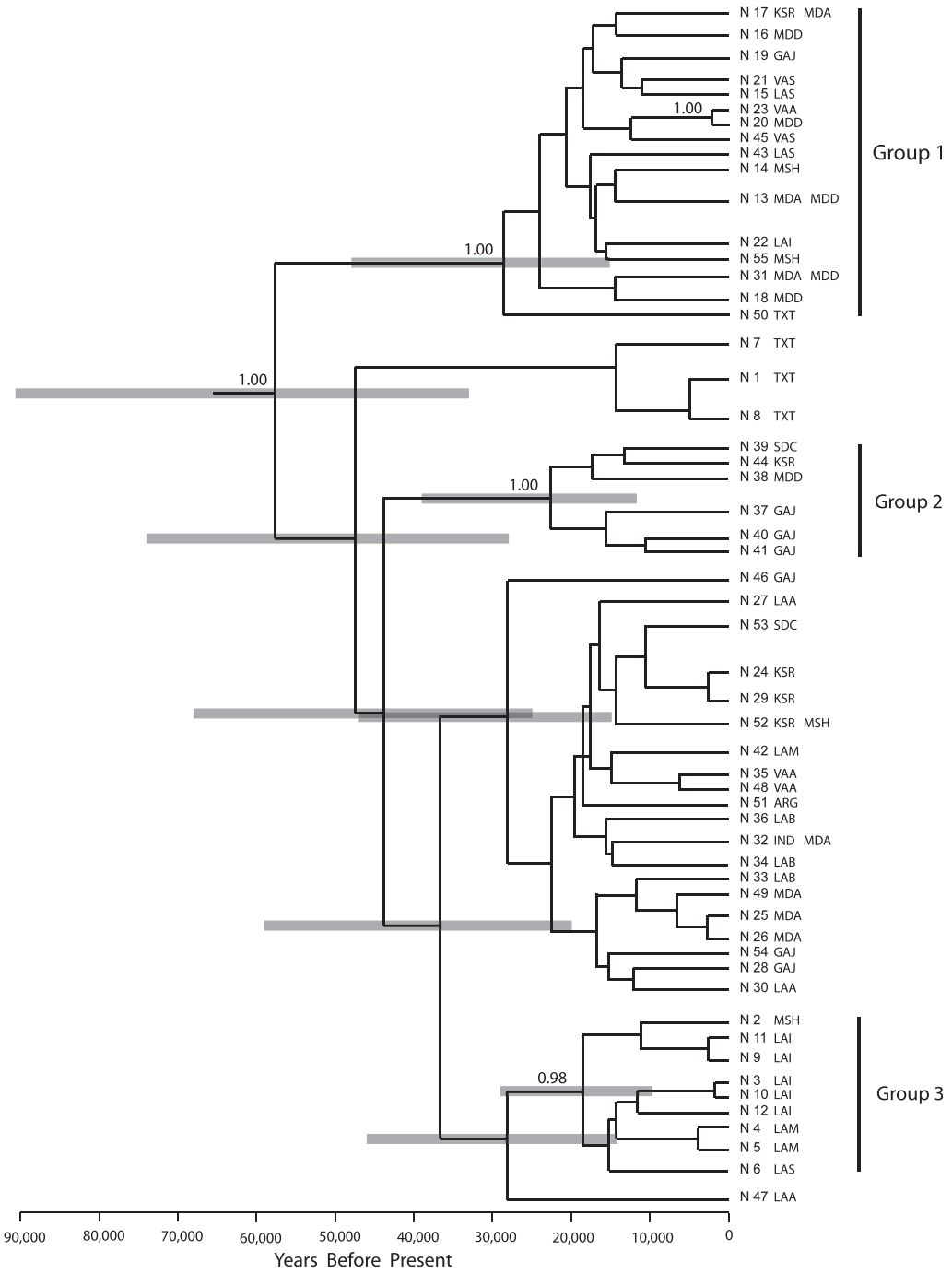

Fig. 2.—BEAST chronogram for the 55 combined cytochrome-*b* and D-loop mitochondrial DNA haplotypes of the eastern fox squirrel (*Sciurus niger*). Gray bars indicate 95% highest posterior density estimates for major nodes. Numbers designate nodes supported by posterior probabilities of greater than or equal to 95% for groups of haplotypes that occur at 2 or more localities. Heavy vertical lines to the right of the tree indicate 3 groups of haplotypes. Haplotype labels and codes for sampling localities correspond to those shown in Appendix I and Fig. 1.

ancestor (48 kya) overlaps the lower end of the range of dates for the root of the chronogram (33.3 kya; Fig. 2).

The minimum spanning network (Fig. 3) revealed several divergent clusters of haplotypes, but these clusters did not correspond to any geographic grouping of localities. Clusters of 16, 6, and 9 haplotypes correspond to groups 1, 2, and 3, respectively, of the phylogenetic tree generated using BEAST (Fig. 2). Five haplotypes were shared between localities: N52 (KSR and MSH), N32 (IND and MDA), N17 (KSR and MDA), N31 (MDA and MDD), and N13 (MDA and MDD).

Mismatch analysis exhibited a unimodal distribution that did not differ significantly from the distribution expected under population expansion (Fig. 4). The Bayesian skyline plot analysis also indicated a significant population expansion,

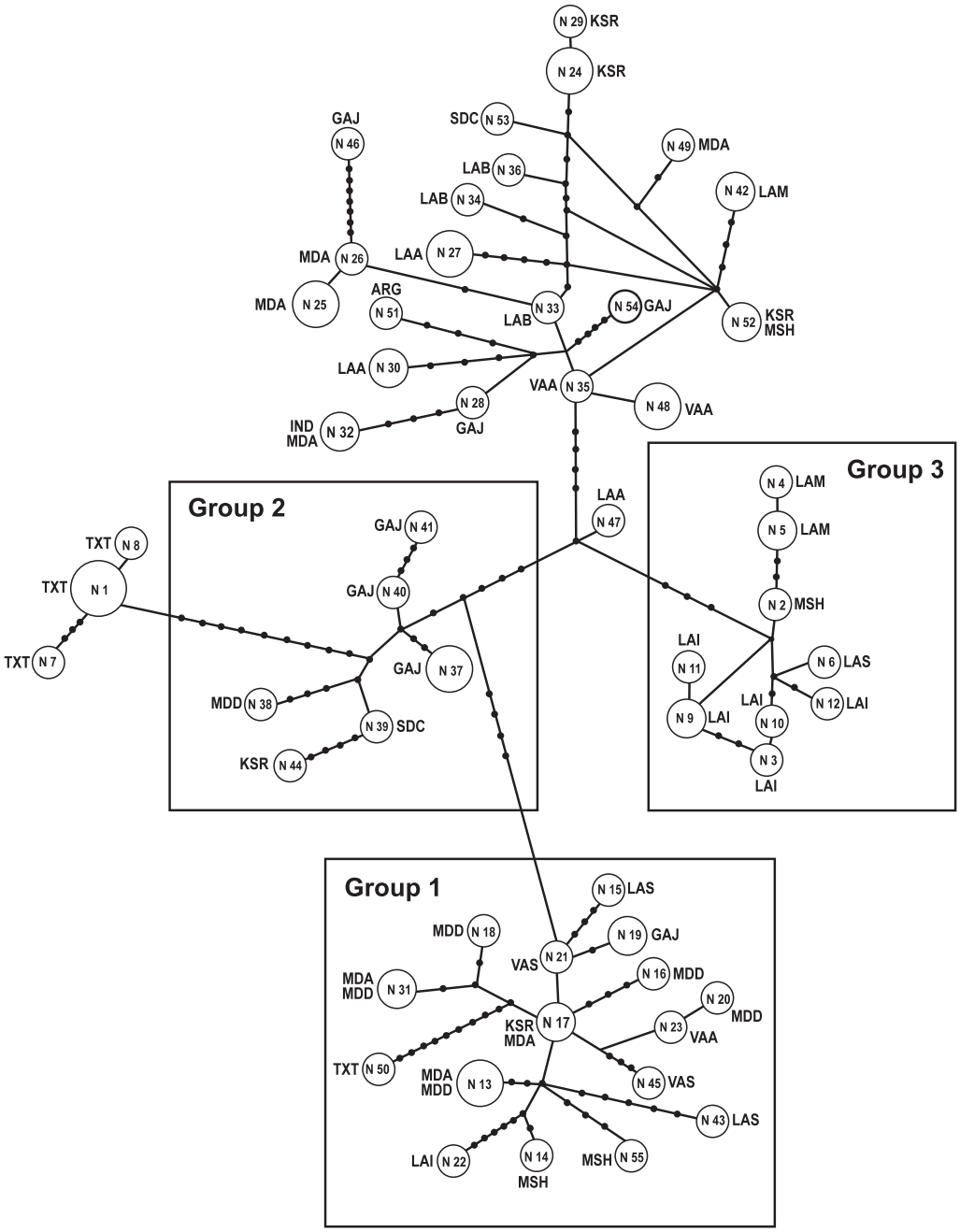

Fig. 3.—Parsimony network showing phylogenetic relationships among 55 combined cytochrome-*b* and D-loop mitochondrial DNA haplotypes of the eastern fox squirrel (*Sciurus niger*). Haplotype labels and codes for sampling localities correspond to those shown in Appendix I and Fig. 1. The small, filled circles represent unsampled or extinct haplotypes. Each line between circles, regardless of length, represents a single mutational change. Size of the labeled circles is proportionate to the number of individuals possessing that haplotype. Groups of haplotypes labeled as 1, 2, and 3 correspond to those shown in Fig. 2.

which began approximately 15 kya (Fig. 5a). This is in agreement with the expansion date previously reported by Moncrief et al. (2010).

Sciurus carolinensis.—We detected 16 *Cytb* haplotypes (Table 2) among the 69 individuals of *S. carolinensis* examined. Representative sequences have been deposited in GenBank (accession numbers JX104399–JX104414; Appendix II). Haplotype diversity for 14 sampling localities of *S. carolinensis* ranged from 0.00 to 0.810, and nucleotide diversity ranged from 0.00 to 0.020 (Table 2). Overall haplotype diversity was high (0.853 ± 0.025), and nucleotide diversity was low (0.017 ± 0.009). Two *Cytb* haplotypes (A and B) were widely distributed; each occurred at 7 of 14 localities, although they occurred together at only 4 of 14 localities (Table 2). Those 2 haplotypes also predominated in the entire data set, occurring in 18 and 17 animals, respectively (Table 2). Eleven *Cytb* haplotypes (in a total of 19 individuals) were restricted to a single locality (Table 2).

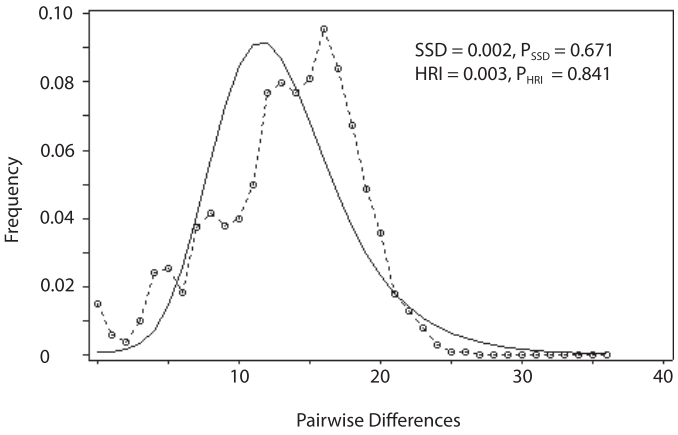

Fig. 4.—Mismatch distribution for 55 combined cytochrome-*b* and D-loop mitochondrial DNA haplotypes of the eastern fox squirrel (*Sciurus niger*). The observed distribution is represented by a dotted line, and the expected distribution based on a model of exponential population growth is represented by a solid line. The sum of squared deviation (SSD) and Harpending's raggedness index (HRI), with the respective *P*-values, are provided.

Among the 69 individuals of *S. carolinensis* examined, we detected 51 D-loop haplotypes (Table 3). Representative sequences have been deposited in GenBank (accession numbers JX104415–JX104465; Appendix II). Haplotype diversity for the 14 sampling localities of *S. carolinensis* ranged from 0.00 to 1.0, and nucleotide diversity ranged from 0.00 to 0.039 (Table 3). Overall haplotype diversity was high (0.984 ± 0.007), and nucleotide diversity was low (0.032 ± 0.016). Only 1 of 51 D-loop haplotypes (f) occurred at more than 1 locality (Table 3).

Combining *Cytb* and D-loop sequences for the 69 individuals of *S. carolinensis* from 14 localities yielded 51 unique combined haplotypes (Appendix II). The minimum spanning network (Fig. 6) revealed 3 unlinked networks, where the 95% confidence interval for parsimony analysis allowed up to 9 mutational steps to link haplotypes, but there is an overall lack of spatial structure in these data. Network 1 consisted of 15 haplotypes, from all localities except those in Louisiana, Mississippi, and Tennessee. Networks 2 and 3 consisted of 11 and 10 haplotypes, respectively. The remaining 15 haplotypes were either singletons or linked together in pairs or triplets. Only 1 haplotype (C42) was shared by 2 localities (MDD and VAH); it was not part of a network.

The BEAST chronogram indicated that all sampled haplotypes of *S. carolinensis* coalesce on a common ancestor approximately 98.3–266.3 kya (Fig. 7). Two major subsets of haplotypes (consisting of 23 and 28 haplotypes, respectively) were supported by posterior probability values ≥0.99. Haplotypes in subset 1 were not present at any locality in Louisiana, Mississippi, or Tennessee; subset 2 included haplotypes from all 14 localities (Figs. 1 and 7). Group 1 includes 17 haplotypes, which are the 15 haplotypes in network 1 in Fig. 6, plus C42 and C18. Groups 2 and 3 correspond exactly to networks 2 and 3, respectively, in Fig. 6. Each of

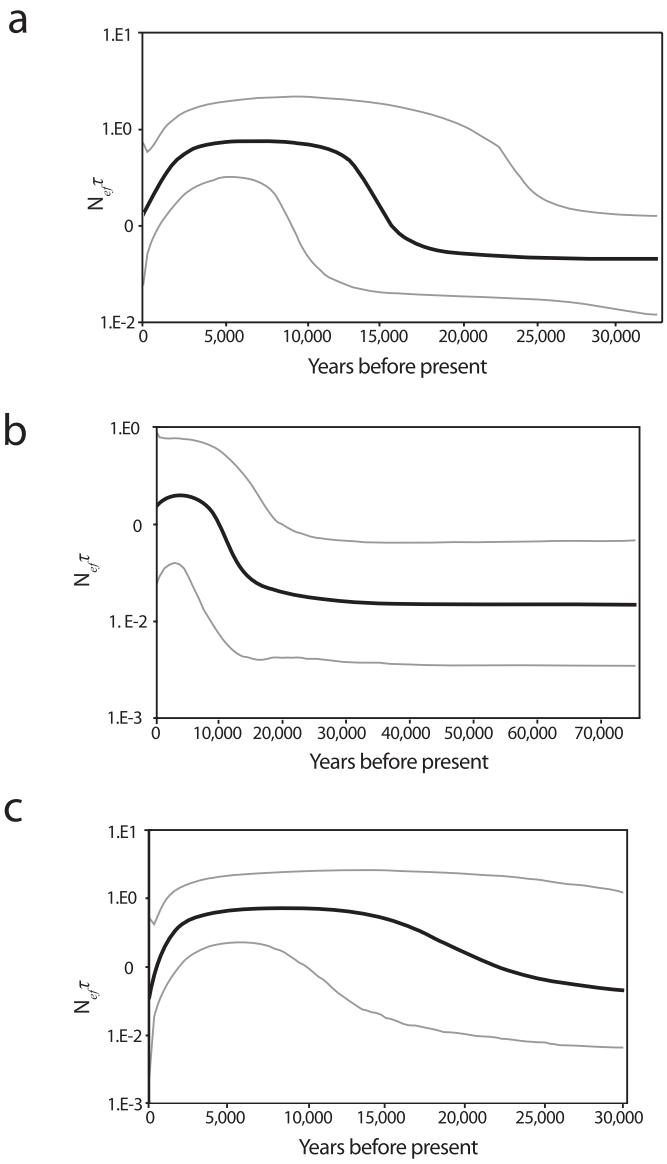

Fig. 5.—Bayesian skyline plots of effective female population size × generation time, $N_{ef}\tau$ (logarithmic scale), based on combined cytochrome-*b* and D-loop mitochondrial DNA haplotypes. a) Fifty-five haplotypes of the eastern fox squirrel (*Sciurus niger*); b) 23 haplotypes of the eastern gray squirrel (*Sciurus carolinensis*) identified as subset 1 in Fig. 7; and c) 28 haplotypes of *S. carolinensis* identified as subset 2 in Fig. 7. In each plot, the thick black line is the median estimate, and the thin gray lines correspond to the 95% highest posterior density estimate. Note that the scales on the y-axes are different.

these major groups of *S. carolinensis* coalesce on a common ancestor approximately 26.1–79.7 kya (Fig. 7). The upper end of the estimated range of dates at which each of these major groups of *S. carolinensis* coalesces on a common ancestor (79.7 kya) does not overlap the lower end of the range of dates for the root of the chronogram (98.3 kya; Fig. 7).

Mismatch analyses of the entire data set (Fig. 8a) and subsets 1 and 2 shown in Fig. 7 (Figs. 8b and 8c) exhibited distributions that did not differ significantly from the distribution expected under population expansion. Mismatch analysis of each of the 3

Table 2.—Geographic information and distribution of cytochrome-*b* haplotypes among 14 sampling localities of the eastern gray squirrel (*Sciurus carolinensis*). Locality codes (code) and haplotype designations correspond to those shown in Fig. 1 and Appendix II. For each locality, we provide the number of individuals sequenced (*n*), number of individuals with a haplotype shared by 2 or more localites (A, B, C, J, and M), private haplotypes (with number of individuals in parentheses), haplotype diversity (*h*, with standard deviation in parentheses), and nucleotide diversity (π, with standard deviation in parentheses).

Code	County(ies) or parish(es) and state	*n*	Haplotypes						*h*	π
			Shared							
			A	B	C	J	M	Private		
ALC	Covington, Alabama	6	1	1				E (4)	0.600 (0.215)	0.011 (0.007)
GAJ	Jasper, Georgia	7		2			1	K (3), L (1)	0.810 (0.130)	0.011 (0.007)
IND	Dubois, Indiana	9		2			7		0.389 (0.164)	0.012 (0.007)
LAA	Acadia, Louisiana	1						I (1)	0.000 (0.000)	0.000 (0.000)
LAF	West Feliciana, Louisiana	3	3						0.000 (0.000)	0.000 (0.000)
LAI	East Baton Rouge, Louisiana	6	5		1				0.333 (0.215)	0.001 (0.001)
LAS	St. Tammany, Louisiana	3			2			O (1)	0.667 (0.314)	0.003 (0.003)
LAV	Vernon, Louisiana	2						F (2)	0.000 (0.000)	0.000 (0.000)
MDA	Allegany, Maryland	8	4	3				P (1)	0.679 (0.122)	0.017 (0.010)
MDD	Dorchester, Maryland	7	1	4		2			0.667 (0.160)	0.011 (0.007)
MSH	Holmes, Mississippi	6	1					D (3), H (1), N (1)	0.800 (0.170)	0.005 (0.004)
TNS	Shelby, Tennessee	1						G (1)	0.000 (0.000)	0.000 (0.000)
VAA	Alleghany and Augusta, Virginia	4		3			1		0.500 (0.265)	0.015 (0.011)
VAH	Henry, Virginia	6	3	2		1			0.733 (0.155)	0.020 (0.012)

networks in Fig. 6 (Figs. 8d–f) exhibited a unimodal distribution that did not differ significantly from the distribution expected under population expansion. All 6 plots show peaks at 7 pairwise differences, suggesting that these data sets share the same expansion event. The Bayesian skyline plot analysis for each of the 2 subsets in Fig. 7 indicated that a significant population expansion occurred in *S. carolinensis* between 12 and 25 kya (Figs. 5b and 5c).

Discussion

There is a general lack of spatial structure in the maternal lineages of both *S. niger* and *S. carolinensis*, even though we observed several clusters of divergent haplotypes in each species (Figs. 3 and 6). Examination of our data revealed high haplotype diversity and low nucleotide diversity in the D-loop and *Cytb* sequences of both *S. niger* and *S. carolinensis* (Tables 1–3; Moncrief et al. 2010). We also observed in *S. carolinensis* 2 *Cytb* haplotypes that are present at high frequency in the overall data set and are shared by populations separated by large geographic distances (Table 2). These 2 haplotypes were internal nodes for 2 separate parsimony networks (results not shown), suggesting that they represent divergent ancestral maternal lineages resulting from distinct glacial refugia during the Pleistocene.

Table 3.—Geographic information and distribution of D-loop haplotypes among 14 sampling localities of the eastern gray squirrel (*Sciurus carolinensis*). Locality codes (code) and haplotype designations correspond to those shown in Fig. 1 and Appendix II. For each locality, we provide the number of individuals sequenced (*n*), number of individuals with a haplotype shared by 2 or more localites (F), private haplotypes (with number of individuals in parentheses), haplotype diversity (*h*, with standard deviation in parentheses), and nucleotide diversity (π, with standard deviation in parentheses).

Code	County(ies) or parish(es) and state	*n*	Haplotypes		*h*	π
			Shared	Private		
ALC	Covington, Alabama	6		eee (1), ff (1), lll (1), nnn (1), ss (1), yy (1)	1.000 (0.096)	0.032 (0.019)
GAJ	Jasper, Georgia	7		aa (1), bb (1), dd (1), ddd (1), ee (2), iii (1)	0.952 (0.096)	0.023 (0.014)
IND	Dubois, Indiana	9		a (7), ggg (1), t (1)	0.417 (0.191)	0.023 (0.013)
LAA	Acadia, Louisiana	1		pp (1)	0.000 (0.000)	0.000 (0.000)
LAF	West Feliciana, Louisiana	3		c (2), l (1)	0.667 (0.314)	0.001 (0.002)
LAI	East Baton Rouge, Louisiana	6		b (4), hh (1), i (1)	0.600 (0.215)	0.023 (0.014)
LAS	St. Tammany, Louisiana	3		kkk (2), m (1)	0.667 (0.314)	0.032 (0.025)
LAV	Vernon, Louisiana	2		ii (1), mmm (1)	1.000 (0.500)	0.039 (0.040)
MDA	Allegany, Maryland	8		e (2), fff (1), u (1), v (2), w (1), y (1)	0.929 (0.084)	0.031 (0.018)
MDD	Dorchester, Maryland	7	F (2)	gg (1), n (2), o (1), vv (1)	0.905 (0.103)	0.007 (0.004)
MSH	Holmes, Mississippi	6		jj (1), ll (1), mm (1), nn (1), oo (1), rr (1)	1.000 (0.096)	0.026 (0.016)
TNS	Shelby, Tennessee	1		j (1)	0.000 (0.000)	0.000 (0.000)
VAA	Alleghany and Augusta, Virginia	4		hhh (1), p (1), q (1), zz (1)	1.000 (0.177)	0.039 (0.026)
VAH	Henry, Virginia	6	F (1)	bbb (1), d (2), r (1), ww (1)	0.933 (0.122)	0.025 (0.015)

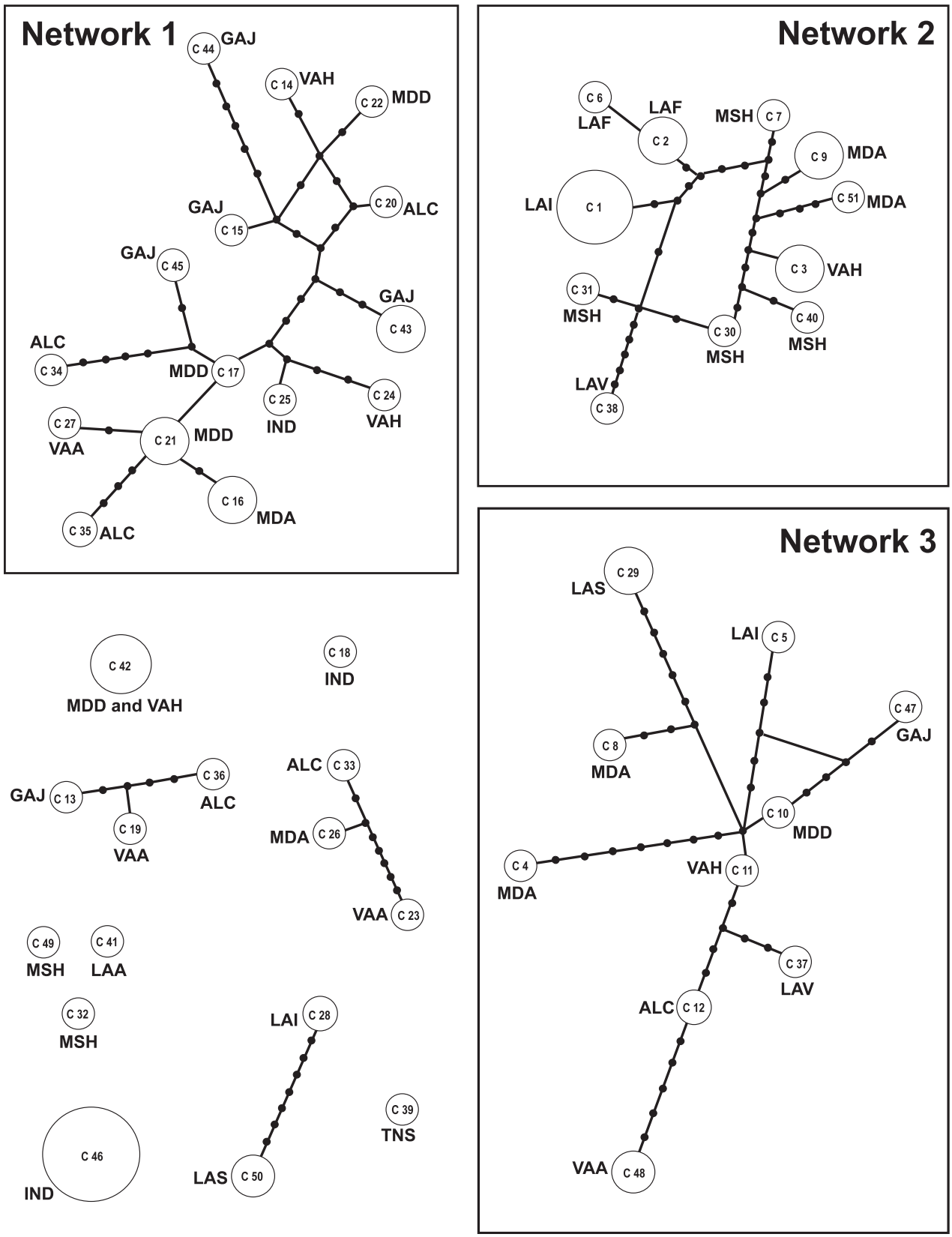

Fig. 6.—Parsimony networks showing phylogenetic relationships among 51 combined cytochrome-*b* and D-loop mitochondrial DNA haplotypes of the eastern gray squirrel (*Sciurus carolinensis*). Haplotype labels and codes for sampling localities correspond to those shown in Appendix II and Fig. 1. The small, filled circles represent unsampled or extinct haplotypes. Each line between circles, regardless of length, represents a single mutational change. Size of the labeled circles is proportionate to the number of individuals possessing that haplotype. Networks of more than 3 haplotypes are labeled 1, 2, and 3.

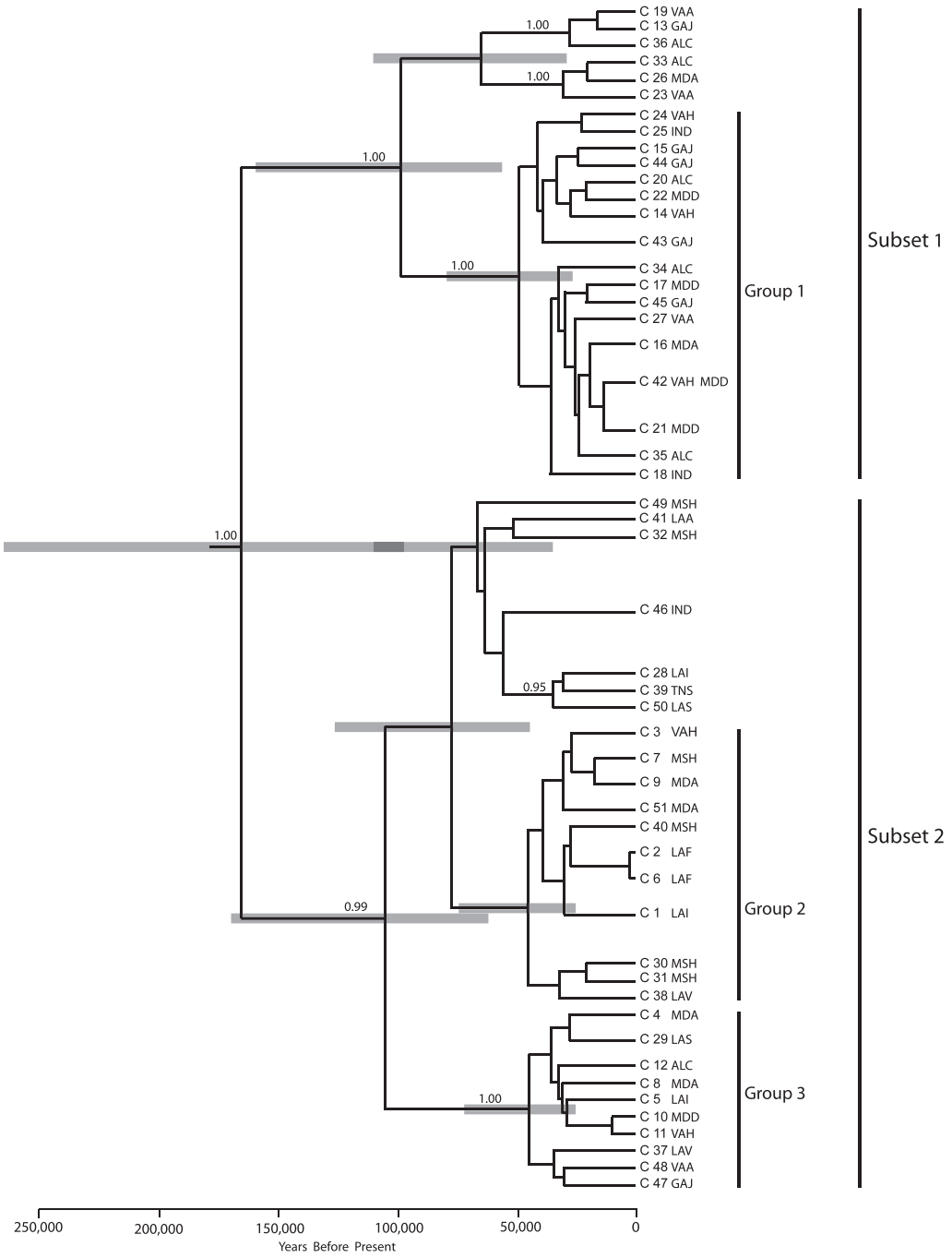

Fig. 7.—BEAST chronogram for the 51 combined cytochrome-*b* and D-loop mitochondrial DNA haplotypes of the eastern gray squirrel (*Sciurus carolinensis*). Gray bars indicate 95% highest posterior density estimates for major nodes. Numbers designate nodes supported by posterior probabilities of greater than or equal to 95% for groups of haplotypes that occur at 2 or more localities. Heavy vertical lines to the right of the tree indicate 2 major subsets of haplotypes and 3 groups of haplotypes. Group 1 includes the 15 haplotypes labeled as network 1 in Fig. 6 plus 2 additional haplotypes (C42 and C18). Groups 2 and 3 correspond to networks 2 and 3, respectively, in Fig. 6. Haplotype labels and codes for sampling localities correspond to those shown in Appendix II and Fig. 1.

We also documented evidence for rapid, recent population expansion in each species. Bayesian skyline plot estimates indicate expansion in both species within the past 12–20 kya (Fig. 5). Mismatch distribution of pairwise differences in the combined *Cytb* and D-loop sequences of *S. niger* (Fig. 4) and *S. carolinensis* (Figs. 8d–f) were unimodal. All of these results are in agreement with the findings of a previous study of *Cytb* sequences of *S. niger* (Moncrief et al. 2010) and all are indicative of a rapid population expansion (Avise 2000; Rogers and Harpending 1992). Similarly, Moncrief (1993, 1998) reported relatively low levels of allozymic variation in both species, and Lance et al. (2003) reported low levels of

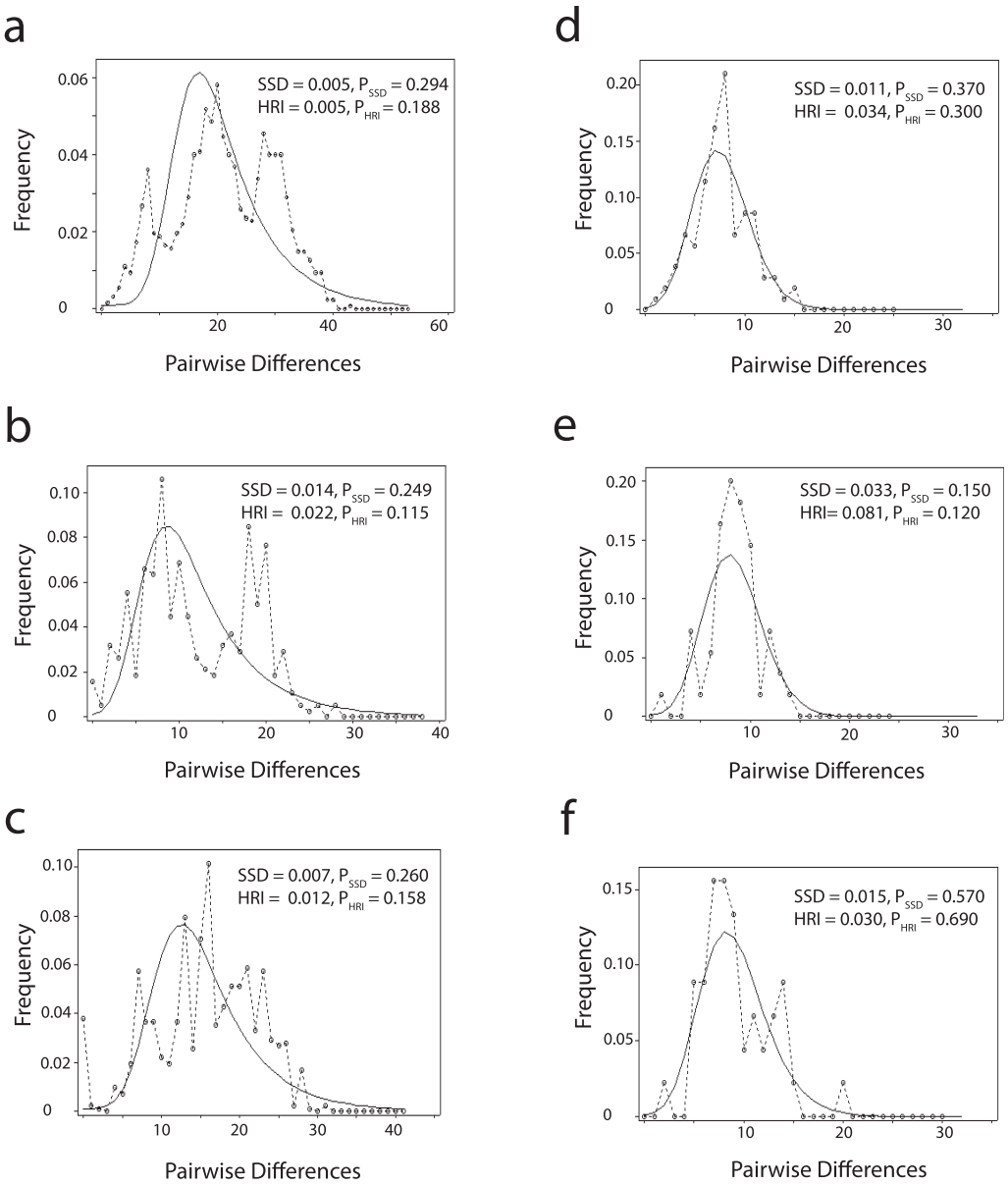

Fig. 8.—Mismatch distribution for combined cytochrome-*b* and D-loop haplotypes of the eastern gray squirrel (*Sciurus carolinensis*): a) 51 haplotypes; b) 23 haplotypes identified as subset 1 in Fig. 7; c) 28 haplotypes identified as subset 2 in Fig. 7; d) 15 haplotypes clustered in network 1 in Fig. 6; e) 11 haplotypes clustered in network 2 in Fig. 6; and f) 10 haplotypes clustered in network 3 in Fig. 6. In each plot, the observed distribution is represented by a dotted line, and the expected distribution based on a model of exponential population growth is represented by a solid line. The sum of squared deviation (SSD) and Harpending's raggedness index (HRI), with the respective *P*-values, are provided for each plot.

microsatellite polymorphism in *S. niger*, providing evidence from unlinked nuclear markers that these species have undergone recent population bottlenecks or range expansion, or both. The high haplotype diversity observed here and the low nuclear diversity presented in previous studies suggest that *S. niger* and *S. carolinensis* underwent a recent population expansion from glacial refugia, and mtDNA diversity has recovered whereas nuclear diversity has not.

This rapid expansion in populations of eastern fox squirrels and eastern gray squirrels is consistent with a scenario of rapid postglacial range expansion of deciduous tree species following the LGM in eastern North America (Williams 2003). These results are not surprising: both *S. niger* and *S. carolinensis* are temperate forest obligates that coevolved with seed, fruit, and nut trees (Koprowski 2005; Steele 2008). Because eastern fox squirrels and eastern gray squirrels do not hibernate, the presence and persistence of populations of these animals are especially dependent on the presence of mature trees that produce winter-storable foods (acorns and nuts), including *Quercus* and *Juglans* (Edwards et al. 2003; Koprowski 1994a, 1994b). The American chestnut (*Castanea dentata*) also was an important cacheable food source until the early 1900s, when

it was almost eliminated by a fungus (Dane 2009). Other important foods for *S. niger* and *S. carolinensis* include the seeds and nuts of *Fagus*, *Acer*, and *C. pumila* (Edwards et al. 2003; Koprowski 1994a, 1994b).

Molecular analyses of many animals and plants have supported hypotheses that southern glacial refugia existed along the Gulf and Atlantic coasts in eastern North America during the Pleistocene (Soltis et al. 2006). Our data provide tentative evidence for a refugium on the Gulf Coast for *S. niger*: all haplotypes of the eastern fox squirrel in group 3 are restricted to the lower Mississippi River valley (localities LAI, LAM, LAS, and MSH; Figs. 1–3), which corresponds to glacial refugial area "G" in Swenson and Howard (2005). Additionally, haplotype N52 of *S. niger*, which is shared by localities MSH and KSR (Figs. 1–3), provides some evidence of postglacial expansion from a refugium in the Gulf Coast state of Mississippi (MSH) to areas north and west, including Kansas (KSR). Recent molecular support for a Pleistocene refugium for trees in the lower Mississippi River valley includes studies of *J. nigra* (Victory et al. 2006), *Acer* (Saeki et al. 2011), *F. grandifolia* (Morris et al. 2010), and *Q. rubra* (Magni et al. 2005).

Several recent studies of cpDNA variation in trees also have provided evidence supporting the existence of interior glacial refugia in the southern Appalachian Mountains and on the interior plateaus to the west of those mountains. These include analyses of *Q. rubra* (Birchenko et al. 2009; Magni et al. 2005), *Acer* (McLachlan et al. 2005; Saeki et al. 2011), *F. grandifolia* (McLachlan et al. 2005), and *C. dentata* and *C. pumila* (Dane 2009). In one of the 1st studies to use cpDNA for phylogeographic analyses of trees, McLachlan et al. (2005) concluded that *F. grandifolia* and *A. rubrum* persisted during the LGM as low-density populations in the Appalachians and on interior plateaus, much farther north (and much closer to modern range limits) than previously hypothesized. Similarly, Magni et al. (2005) suggested that oak stands grew close to the Laurentide Ice Sheet (which extended south to about 39°N—Soltis et al. 2006) shortly after the LGM and that northward recolonization was limited to a few hundred kilometers. Magni et al. (2005) concluded that their results are consistent with palynological evidence that *Quercus* was abundant during the LGM in the lower Mississippi River valley and Florida, but that scattered populations also were present farther north, between these 2 regions and the ice sheet (Jackson et al. 2000; Williams 2003).

For both species of *Sciurus* in our study, we found tentative evidence for 1 or several interior glacial refugia. In eastern fox squirrels, group 2 consists of haplotypes that are present only in localities KSR, SDC, GAJ, and MDD (Figs. 1–3). Also, haplotype N32 is shared by localities in Indiana (IND) and Maryland (MDA; Figs. 1–3), and haplotype N17 is an internal node in group 1 that is shared by localities in Kansas (KSR) and Maryland (MDA; Figs. 1 and 3). In each case, we suggest that these haplotypes of *S. niger* originated in 1 or more interior refugia and that the current distribution of haplotypes is the result of postglacial range expansion. Similarly, haplotypes that comprise subset 1 in eastern gray squirrels are restricted to localities IND, ALC, GAJ, VAA, VAH, MDA, and MDD (Figs. 1 and 7), providing evidence for an interior refugium west of the southern Appalachian Mountains.

Scenarios for *Sciurus* that include expansion from scattered populations in interior refugia presume survival of populations of squirrels in numerous small, low-density fragments of thermophilous forests. The ecology of modern populations of eastern fox squirrels and eastern gray squirrels suggests that ancestral populations of these animals could have persisted in relatively small forest fragments (<40 ha—Koprowski 2005) during the LGM. Koprowski (2005) reported that, in both *S. niger* and *S. carolinensis*, density is negatively related to fragment size, and the size of home ranges is positively related to forest fragment size. Therefore, compaction of home ranges can provide a mechanism by which population densities may increase (or hold steady) in small forest fragments.

Wood et al. (2007) conducted population viability analyses for *S. niger* and *S. carolinensis* and found that populations of both species could be successfully established by as few as 35 individuals. Wood et al. (2007) concluded that the high biotic potential of tree squirrels and lack of density-dependent reproduction at low population densities allows even a small population to increase during a year of good or modest food availability. Wood et al. (2007) also noted that tree squirrels such as *S. niger* and *S. carolinensis* possess good dispersal capability, can colonize and use novel habitats, and can make their own nests. Furthermore, these animals eat many different foods, which permits persistence in a variety of forest types and allows them to survive during years of low seed production.

Our study revealed similar timing of recent population expansion in these 2 species (Fig. 5), but we documented more genetic structure in maternal lineages of eastern gray squirrels than in eastern fox squirrels (Figs. 2, 3, 6, and 7). This may reflect differences in the ecology of these animals. Female eastern gray squirrels are more philopatric than female eastern fox squirrels (Koprowski 2005). Female eastern gray squirrels tend to remain in their natal areas to form overlapping generations of kin (Koprowski 2005). Additionally, dispersal distances in the eastern gray squirrel tend to be shorter than in the eastern fox squirrel; dispersal distances in eastern gray squirrels rarely exceed about 3.5 km, whereas eastern fox squirrels have dispersed more than 60 km (Edwards et al. 2003).

Both *S. niger* and *S. carolinensis* exhibited genetic structure that predates the LGM (Figs. 2 and 7). The timing of divergence of major subsets of eastern gray squirrels (approximately 57.5–170.2 kya; Fig. 7) includes the Sangamonian interglacial (approximately 130–100 kya—Gibbard and Van Kolfschoten 2004). The timing of divergence within subsets of *S. carolinensis* (26.1–79.7 kya; Fig. 7) is roughly coincident with timing of coalescence of *S. niger* (33.3–91 kya; Fig. 2). This may reflect fragmentation of populations during glacial advances in the Wisconsinan (Gradstein 2004), which would cause habitat fragmentation into refugia. Expansion events for both species (15–20 kya; Fig. 5) are coincident with the recession of glaciers that followed the LGM.

Clearly, cycles of Pleistocene glaciation were major determinants of historical range contractions and expansions, which shaped current patterns of genetic diversity in eastern fox squirrels and eastern gray squirrels. These cycles also undoubtedly affected the demographic histories of other organisms in eastern North America. Indeed, phylogeographic analyses of several other codistributed, nonvolant mammals, including the common gray fox (*Urocyon cinereoargenteus*—Bozarth et al. 2011), the red fox (*Vulpes vulpes*—Aubry et al. 2009), the northern raccoon (*Procyon lotor*—Cullingham et al. 2008), the American black bear (*Ursus americanus*—Wooding and Ward 1997), the northern short-tailed shrew (*Blarina brevicauda*—Brant and Orti 2003), the northern flying squirrel (*Glaucomys sabrinus*—Arbogast 1999), the white-footed mouse (*Peromyscus leucopus*—Rowe et al. 2006), the eastern chipmunk (*Tamias striatus*—Rowe et al. 2006), and the eastern woodrat (*Neotoma floridana*—Hayes and Harrison 1992), all reported weak genetic structure in these species in eastern North America. Moreover, studies of the southern flying squirrel (*G. volans*), another nonhibernating sciurid that is considered to be a temperate forest obligate, reported that this species displays very little genetic variation, despite broad geographic sampling over much of eastern North America (Arbogast 1999; Arbogast et al. 2005; Petersen and Stewart 2006).

Taken together, examination of our data indicates that *S. niger* and *S. carolinensis* underwent multiple episodes of genetic divergence during isolation in glacial refugia, followed by range expansion and contact that resulted in admixture of divergent maternal lineages within each species during interglacials. The location of refugia for both species of these squirrels probably shifted geographically through time, and animals that comprised populations in successive iterations of refugia were descendants of different combinations of source populations. Examination of our data further indicates that the most recent range expansion in both species occurred within the past 12–20 kya. This estimate is consistent with analyses of historical tree-cover density. Williams (2003) found that density of *Quercus* increased in southeastern North America between 21 and 16 kya and further increased after 14 kya, followed by a northward range expansion from 13 to 9 kya.

For both species, examination of our data revealed several clusters of divergent haplotypes, but a general lack of spatial structure (Figs. 3 and 6). For *S. carolinensis*, we observed a pattern that suggests the presence of at least 2 distinct refugial populations that evolved in isolation during the Pleistocene (approximately 98.3–266.3 kya; Figs. 6 and 7) and expanded to the species' current range following the LGM. For *S. niger*, structure was much less pronounced, with fewer strongly diverged groups of haplotypes (Fig. 3). Additionally, there was overlap between the estimated dates of coalescence for major groups and the root of the chronogram in *S. niger*, but not *S. carolinensis* (Figs. 2 and 7). These findings all suggest that eastern fox squirrels persisted in either a single population in a glacial refugium or as several refugial populations that maintained gene flow throughout the Pleistocene. The highly dispersed haplotypes of *S. niger* relative to the stronger geographic structure of haplotypes of *S. carolinensis* suggests that dispersal and gene flow following glacial recession and range expansion has occurred to a greater extent in eastern fox squirrels than in eastern gray squirrels.

The relatively frequent climatic oscillations throughout the Pleistocene likely resulted in numerous cycles of range expansions and contractions, overlaying multiple evolutionary signals on phylogeographic structure in these squirrels and temperate tree species (Morris et al. 2008). We suggest a scenario by which temperate forests (and the eastern fox squirrels and eastern gray squirrels that used them) were concentrated at various times in coastal refugia (including the lower Mississippi River valley) and interior refugia (including areas west of the southern Appalachian Mountains), but that scattered populations of trees and squirrels also were present in multiple, small refugia between these regions and the Laurentide Ice Sheet. Because populations of trees and squirrels persisted so close to the ice sheet, the most recent recolonization (following the LGM) occurred rapidly.

Our data support evidence presented by McLachlan et al. (2005) and Morris et al. (2010) that thermophilous trees persisted close to the ice sheets during the LGM. This evidence, accumulated from molecular and fossil data for trees, and now augmented by data from this phylogeographic study of 2 closely related sympatric species that are nonhibernating temperate forest obligates, points to potential losses of dominant tree species over much or all of their ranges (as a result of rapid climate change that has been predicted due to 21st century warming [McLachlan et al. 2005]). Together with recent findings from studies of other nonvolant mammals and temperate tree species in eastern North America (Aubry et al. 2009; Birchenko et al. 2009; Bozarth et al. 2011; Cullingham et al. 2008; Dane 2009; Morris et al. 2008, 2010; Rowe et al. 2006; Saeki et al. 2011; Soltis et al. 2006; Victory et al. 2006), our study indicates that demographic histories of species in glaciated landscapes are often more complex and variable than previously suggested. Additional studies of temperate forest obligates and analyses that include finer-scale genetic data are necessary to more confidently infer the locations of glacial refugia for the trees that compose the temperate forests of eastern North America and the animals that inhabit those forests.

Acknowledgments

We thank M. Hafner for allowing access to tissue samples from Arkansas, Louisiana, Mississippi, and Tennessee that are housed at LSUMZ. S. Donnelson, T. Henry, D. Onorato, and J. Streicher provided assistance in the laboratory. We thank J. Davenport and M. Cartwright for valuable advice and assistance in producing the figures. We thank P. Weigl and A. Dooley, Jr., for helpful discussions about the ecology of tree squirrels and Pleistocene glaciations.

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Submitted 1 November 2011. Accepted 15 March 2012.

Associate Editor was Ryan W. Norris.

Appendix I

Specimens examined.—Locality code, state, county or parish, haplotype designations, and voucher number for the 81 eastern fox squirrels (*Sciurus niger*) included in this study. Samples without vouchers are marked with an asterisk (*). GenBank accession numbers are provided for each of the 55 D-loop haplotypes; Moncrief et al. (2010) reported GenBank accession numbers for the cytochrome-*b* haplotypes. Vouchers are housed in the Virginia Museum of Natural History (VMNH) and Louisiana State University Museum of Natural Sciences (LSUMZ).

Locality code	State	County or parish	Combined haplotype	Cytochrome-*b* haplotype	D-loop haplotype	GenBank accession no. for D-loop haplotype	Voucher no.
ARG	Arkansas	Greene	N51	O	u	JX104388	JAH 0467*
GAJ	Georgia	Jasper	N19	B	p	JX104378	VMNH 1261
GAJ	Georgia	Jasper	N19	B	p	JX104378	VMNH 1263
GAJ	Georgia	Jasper	N28	C	g	JX104355	VMNH 1253
GAJ	Georgia	Jasper	N37	D	e	JX104351	VMNH 1254
GAJ	Georgia	Jasper	N37	D	e	JX104351	VMNH 1260
GAJ	Georgia	Jasper	N37	D	e	JX104351	VMNH 1264
GAJ	Georgia	Jasper	N40	D	ss	JX104386	VMNH 1258
GAJ	Georgia	Jasper	N41	D	uu	JX104389	VMNH 1255
GAJ	Georgia	Jasper	N46	J	rr	JX104384	VMNH 1257
GAJ	Georgia	Jasper	N54	S	tt	JX104387	VMNH 1262
IND	Indiana	Dubois	N32	C	o	JX104376	VMNH 0326
KSR	Kansas	Rooks	N17	B	n	JX104373	VMNH 0296
KSR	Kansas	Ellis	N24	C	b	JX104345	VMNH 0289
KSR	Kansas	Rooks	N24	C	b	JX104345	VMNH 0300
KSR	Kansas	Rooks	N24	C	b	JX104345	VMNH 0301
KSR	Kansas	Rooks	N24	C	b	JX104345	VMNH 0302
KSR	Kansas	Rooks	N29	C	ggg	JX104357	VMNH 0297
KSR	Kansas	Rooks	N44	H	hhh	JX104360	VMNH 0298
KSR	Kansas	Ellis	N52	P	i	JX104361	VMNH 0290
LAA	Louisiana	Acadia	N27	C	d	JX104350	LSU M-7487
LAA	Louisiana	Acadia	N27	C	d	JX104350	LSU M-7488
LAA	Louisiana	Acadia	N27	C	d	JX104350	LSU M-7491
LAA	Louisiana	Acadia	N30	C	h	JX104358	LSU M-5914
LAA	Louisiana	Acadia	N30	C	h	JX104358	LSU M-7492
LAA	Louisiana	Acadia	N47	K	bb	JX104346	LSU M-7489
LAB	Louisiana	Bossier	N33	C	w	JX104391	LSU M-2093
LAB	Louisiana	Bossier	N34	C	x	JX104393	LSU M-7494
LAB	Louisiana	Bossier	N36	C	y	JX104395	LSU M-2313
LAI	Louisiana	Iberville	N3	A	gg	JX104356	LSU M-7498
LAI	Louisiana	Iberville	N9	A	q	JX104381	LSU M-3454
LAI	Louisiana	Iberville	N9	A	q	JX104381	LSU M-3462
LAI	Louisiana	Iberville	N10	A	r	JX104383	LSU M-3455
LAI	Louisiana	East Baton Rouge	N11	A	v	JX104390	LSU M-7495
LAI	Louisiana	Iberville	N12	A	z	JX104397	LSU M-2316
LAI	Louisiana	Iberville	N22	B	s	JX104385	LSU M-3461
LAM	Louisiana	Madison	N4	A	hh	JX104359	LSU M-2363
LAM	Louisiana	Madison	N5	A	j	JX104363	LSU M-2362
LAM	Louisiana	Madison	N5	A	j	JX104363	LSU M-2364
LAM	Louisiana	Madison	N42	E	l	JX104368	LSU M-2366
LAM	Louisiana	Madison	N42	E	l	JX104368	LSU M-7499
LAS	Louisiana	St. Tammany	N6	A	jj	JX104364	LSU M-2430
LAS	Louisiana	St. Tammany	N15	B	ii	JX104362	LSU M-2429
LAS	Louisiana	St. Tammany	N43	G	kk	JX104366	LSU M-2431
MDA	Maryland	Allegany	N13	B	f	JX104353	VMNH 0253
MDA	Maryland	Allegany	N17	B	n	JX104373	VMNH 0247
MDA	Maryland	Allegany	N25	C	c	JX104347	VMNH 0249
MDA	Maryland	Allegany	N25	C	c	JX104347	VMNH 0255
MDA	Maryland	Allegany	N26	C	ccc	JX104349	VMNH 0252
MDA	Maryland	Allegany	N31	C	k	JX104365	VMNH 0254
MDA	Maryland	Allegany	N32	C	o	JX104376	VMNH 0256
MDA	Maryland	Allegany	N49	M	zz	JX104398	VMNH 0248
MDD	Maryland	Dorchester	N13	B	f	JX104353	VMNH 1116
MDD	Maryland	Dorchester	N13	B	f	JX104353	VMNH 1121
MDD	Maryland	Dorchester	N13	B	f	JX104353	VMNH 1124
MDD	Maryland	Dorchester	N16	B	ll	JX104369	VMNH 1115
MDD	Maryland	Dorchester	N18	B	nn	JX104374	VMNH 1119
MDD	Maryland	Dorchester	N20	B	pp	JX104379	VMNH 1122

Appendix I.—Continued.

Locality code	State	County or parish	Combined haplotype	Cytochrome-*b* haplotype	D-loop haplotype	GenBank accession no. for D-loop haplotype	Voucher no.
MDD	Maryland	Dorchester	N31	C	k	JX104365	VMNH 1118
MDD	Maryland	Dorchester	N38	D	mm	JX104371	VMNH 1117
MSH	Mississippi	Holmes	N2	A	ee	JX104352	LSU M-2327
MSH	Mississippi	Holmes	N14	B	ff	JX104354	LSU M-2330
MSH	Mississippi	Holmes	N52	P	i	JX104361	LSU M-2322
MSH	Mississippi	Holmes	N55	T	cc	JX104348	LSU M-2325
SDC	South Dakota	Clay	N39	D	ooo	JX104377	VMNH 2385
SDC	South Dakota	Clay	N53	R	nnn	JX104375	VMNH 2384
TXT	Texas	Tom Green	N1	A	a	JX104344	VMNH 0266
TXT	Texas	Tom Green	N1	A	a	JX104344	VMNH 0276
TXT	Texas	Tom Green	N1	A	a	JX104344	VMNH 0277
TXT	Texas	Tom Green	N1	A	a	JX104344	VMNH 0279
TXT	Texas	Tom Green	N1	A	a	JX104344	VMNH 0281
TXT	Texas	Tom Green	N1	A	a	JX104344	VMNH 0282
TXT	Texas	Tom Green	N1	A	a	JX104344	VMNH 0284
TXT	Texas	Tom Green	N7	A	kkk	JX104367	VMNH 0278
TXT	Texas	Tom Green	N8	A	lll	JX104370	VMNH 0280
TXT	Texas	Tom Green	N50	N	mmm	JX104372	VMNH 0283
VAA	Virginia	Alleghany	N23	B	yy	JX104396	VMNH 0450
VAA	Virginia	Alleghany	N35	C	xx	JX104394	VMNH 0454
VAA	Virginia	Alleghany	N48	L	ww	JX104392	VMNH 0449
VAS	Virginia	Sussex	N21	B	ppp	JX104380	VMNH 2275
VAS	Virginia	Sussex	N45	I	qqq	JX104382	VMNH 2276

Appendix II

Specimens examined.—Locality code, state, county or parish, haplotype designations, and voucher number for the 69 eastern gray squirrels (*Sciurus carolinensis*) included in this study. Samples without vouchers are marked with an asterisk (*). GenBank accession numbers are provided for each of the 16 cytochrome-*b* haplotypes and each of the 51 D-loop haplotypes. Vouchers are housed in the Virginia Museum of Natural History (VMNH) and Louisiana State University Museum of Natural Sciences (LSUMZ).

Locality code	State	County or parish	Combined haplotype	Cytochrome-*b* haplotype	GenBank accession no. for cytochrome-*b* haplotype	D-loop haplotype	GenBank accession no. for D-loop haplotype	Voucher no.
ALC	Alabama	Covington	C12	A	JX104399	yy	JX104464	VMNH 2251
ALC	Alabama	Covington	C20	B	JX104400	lll	JX104442	VMNH 2249
ALC	Alabama	Covington	C33	E	JX104403	eee	JX104426	VMNH 2248
ALC	Alabama	Covington	C34	E	JX104403	ff	JX104428	VMNH 2246
ALC	Alabama	Covington	C35	E	JX104403	nnn	JX104448	VMNH 2250
ALC	Alabama	Covington	C36	E	JX104403	ss	JX104456	VMNH 2247
GAJ	Georgia	Jasper	C13	B	JX104400	bb	JX104418	VMNH 1190
GAJ	Georgia	Jasper	C15	B	JX104400	ddd	JX104423	VMNH 1184
GAJ	Georgia	Jasper	C43	K	JX104409	ee	JX104425	VMNH 1180
GAJ	Georgia	Jasper	C43	K	JX104409	ee	JX104425	VMNH 1181
GAJ	Georgia	Jasper	C44	K	JX104409	iii	JX104436	VMNH 1183
GAJ	Georgia	Jasper	C45	L	JX104410	dd	JX104422	VMNH 1189
GAJ	Georgia	Jasper	C47	M	JX104411	aa	JX104416	VMNH 1187
IND	Indiana	Dubois	C18	B	JX104400	ggg	JX104431	VMNH 0315
IND	Indiana	Dubois	C25	B	JX104400	t	JX104457	VMNH 0314
IND	Indiana	Dubois	C46	M	JX104411	a	JX104415	VMNH 0307
IND	Indiana	Dubois	C46	M	JX104411	a	JX104415	VMNH 0308
IND	Indiana	Dubois	C46	M	JX104411	a	JX104415	VMNH 0309
IND	Indiana	Dubois	C46	M	JX104411	a	JX104415	VMNH 0310
IND	Indiana	Dubois	C46	M	JX104411	a	JX104415	VMNH 0311
IND	Indiana	Dubois	C46	M	JX104411	a	JX104415	VMNH 0312
IND	Indiana	Dubois	C46	M	JX104411	a	JX104415	VMNH 0313
LAA	Louisiana	Acadia	C41	I	JX104407	pp	JX104452	NDM 1057*
LAF	Louisiana	West Feliciana	C2	A	JX104399	c	JX104420	LSU M-1968
LAF	Louisiana	West Feliciana	C2	A	JX104399	c	JX104420	LSU M-2079
LAF	Louisiana	West Feliciana	C6	A	JX104399	l	JX104440	LSU M-1967
LAI	Louisiana	East Baton Rouge	C1	A	JX104399	b	JX104417	LSU M-2412

Appendix II.—Continued.

Locality code	State	County or parish	Combined haplotype	Cytochrome-*b* haplotype	GenBank accession no. for cytochrome-*b* haplotype	D-loop haplotype	GenBank accession no. for D-loop haplotype	Voucher no.
LAI	Louisiana	East Baton Rouge	C1	A	JX104399	b	JX104417	LSU M-2413
LAI	Louisiana	East Baton Rouge	C1	A	JX104399	b	JX104417	LSU M-2414
LAI	Louisiana	East Baton Rouge	C1	A	JX104399	b	JX104417	LSU M-2416
LAI	Louisiana	East Baton Rouge	C5	A	JX104399	hh	JX104432	LSU M-2415
LAI	Louisiana	East Baton Rouge	C28	C	JX104401	i	JX104434	LSU M-1960
LAS	Louisiana	St. Tammany	C29	C	JX104401	kkk	JX104439	LSU M-1964
LAS	Louisiana	St. Tammany	C29	C	JX104401	kkk	JX104439	LSU M-1966
LAS	Louisiana	St. Tammany	C50	O	JX104413	m	JX104443	LSU M-1135
LAV	Louisiana	Vernon	C37	F	JX104404	ii	JX104435	LSU M-2395
LAV	Louisiana	Vernon	C38	F	JX104404	mmm	JX104445	LSU M-2393
MDA	Maryland	Allegany	C4	A	JX104399	fff	JX104429	VMNH 0243
MDA	Maryland	Allegany	C8	A	JX104399	u	JX104458	VMNH 0244
MDA	Maryland	Allegany	C9	A	JX104399	v	JX104459	VMNH 0241
MDA	Maryland	Allegany	C9	A	JX104399	v	JX104459	VMNH 0242
MDA	Maryland	Allegany	C16	B	JX104400	e	JX104424	VMNH 0236
MDA	Maryland	Allegany	C16	B	JX104400	e	JX104424	VMNH 0238
MDA	Maryland	Allegany	C26	B	JX104400	y	JX104463	VMNH 0237
MDA	Maryland	Allegany	C51	P	JX104414	w	JX104461	VMNH 0240
MDD	Maryland	Dorchester	C10	A	JX104399	vv	JX104460	VMNH 0411
MDD	Maryland	Dorchester	C17	B	JX104400	gg	JX104430	VMNH 1114
MDD	Maryland	Dorchester	C21	B	JX104400	n	JX104446	VMNH 0365
MDD	Maryland	Dorchester	C21	B	JX104400	n	JX104446	VMNH 0410
MDD	Maryland	Dorchester	C22	B	JX104400	o	JX104449	VMNH 0408
MDD	Maryland	Dorchester	C42	J	JX104408	f	JX104427	VMNH 0412
MDD	Maryland	Dorchester	C42	J	JX104408	f	JX104427	VMNH 0413
MSH	Mississippi	Holmes	C7	A	JX104399	ll	JX104441	LSU M-2343
MSH	Mississippi	Holmes	C30	D	JX104402	mm	JX104444	LSU M-2336
MSH	Mississippi	Holmes	C31	D	JX104402	nn	JX104447	LSU M-2333
MSH	Mississippi	Holmes	C32	D	JX104402	oo	JX104450	LSU M-2331
MSH	Mississippi	Holmes	C40	H	JX104406	jj	JX104438	LSU M-2348
MSH	Mississippi	Holmes	C49	N	JX104412	rr	JX104455	LSU M-2341
TNS	Tennessee	Shelby	C39	G	JX104405	j	JX104437	LSU M-2091
VAA	Virginia	Augusta	C19	B	JX104400	hhh	JX104433	VMNH 0528
VAA	Virginia	Alleghany	C23	B	JX104400	q	JX104453	VMNH 0526
VAA	Virginia	Alleghany	C27	B	JX104400	zz	JX104465	VMNH 0490
VAA	Virginia	Augusta	C48	M	JX104411	p	JX104451	VMNH 0529
VAH	Virginia	Henry	C3	A	JX104399	d	JX104421	VMNH 1557
VAH	Virginia	Henry	C3	A	JX104399	d	JX104421	VMNH 1559
VAH	Virginia	Henry	C11	A	JX104399	ww	JX104462	VMNH 1551
VAH	Virginia	Henry	C14	B	JX104400	bbb	JX104419	VMNH 1554
VAH	Virginia	Henry	C24	B	JX104400	r	JX104454	VMNH 1558
VAH	Virginia	Henry	C42	J	JX104408	f	JX104427	VMNH 2243