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Genetic evidence indicates ecological divergence rather than geographic barriers structure Florida fox squirrels

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For polytypic mammal species, biogeographic barriers including rivers have often been used to delineate taxonomic units under the assumption that barriers have structured their distribution. We tested the importance of major riverine systems as biogeographic barriers in fox squirrels (*Sciurus niger*) across the state of Florida, where 4 currently recognized subspecies are delineated at major rivers. We also explored whether phylogeographic structure may be limited to ecologically divergent subspecies, specifically between *S. n. avicennia* and *S. n. shermani*. Using a multilocus approach to examine diversity, we found that restricted gene flow was only present between *S. n. avicennia*, located south of the Caloosahatchee River in south Florida, and the rest of *S. niger*, which is widespread across the state. Mitochondrial DNA revealed that 2 divergent groups of haplotypes are present and widespread across Florida, thus supporting the hypothesis that fox squirrels persisted in multiple refugia during the Pleistocene, and that *S. n. avicennia* diverged ecologically from other populations of fox squirrels relatively recently. This was supported by isolation with migration models that indicated matrilineal isolation since the onset of divergence between *S. n. avicennia* and *S. n. shermani*, which corresponds to the onset of ecological divergence in south Florida during the early Holocene. Isolation by distance at 8 microsatellite loci from the western panhandle to the southern end of the peninsula was significant only when *S. n. avicennia* was included; however, this was due to the hierarchical genetic patterns identified between *S. n. avicennia* and the other subspecies as determined by Bayesian clustering, and not due to spatially restricted dispersal. We postulate that the demographic isolation of *S. n. avicennia* is the result of adaptation to the unique ecological conditions of south Florida.

Key words: ecological speciation, fox squirrel, isolation with migration, parapatric speciation, phylogeography, refugia, riverine barrier, *Sciurus niger*, subspecies

In North America and Europe, phylogeographic studies have identified putative barriers to gene flow such as rivers and mountain ranges, as well as refugial areas where species persisted during major glacial cycles (Soltis et al. 2006). The southeastern United States has been a key region for the development of many of the central tenets of phylogeographic theory (Avise et al. 1987; Avise 2000). This region saw major shifts in sea level and habitat during Plio-Pleistocene glacial-interglacial cycles, while much of the northern part of the continent was glaciated or otherwise inhospitable for many currently widespread taxa. These large-scale shifts had complex effects on population demographics that are reflected in the distribution of genetic lineages of mammals and many other vertebrate taxa (Williams et al. 2004; Soltis et al. 2006). Because the southeastern United States is relatively well-studied, several testable hypotheses pertaining to the location of refugia have been proposed. Refugial areas served as habitat reservoirs where warm-adapted species persisted during glacial periods (Haffer 1969) and from which allopatric populations subsequently expanded, often coming into secondary contact (e.g., Austin et al. 2002; Reid et al. 2010). Proposed refugial areas in eastern North America have been broadly defined and include the Florida peninsula and Texas coastal plain (reviewed in Swenson and Howard 2005). The idea that historical refugia or geographically restricted

plant communities, particularly during the Pleistocene, resulted in the diversification of small mammals has been supported in a number of systems (Stewart and Lister 2001; Nicolas et al. 2011; Demos et al. 2014; Morales-Jimenez et al. 2015), though the role of refugia as "species pumps" in tropical systems has been the subject of much debate (Knapp and Mallet 2003).

Additionally, such rivers have been postulated as putative barriers, corresponding to patterns of genetic divergence in several taxa including mammals, plants, snakes, and amphibians (Soltis et al. 2006). Major rivers have long been considered important barriers to the movement of animals (Wallace 1852; Grinnell 1914) and a potential driver of speciation in the tropics (Gascon et al. 1996; Boubli et al. 2015). However, more recent examinations of the riverine barrier hypothesis have been equivocal (Gascon et al. 2000; Nicolas et al. 2011; Roratto et al. 2015) and distinguishing between processes associated with long-term stable riverine barriers versus ecological transitions (e.g., Mississippi River) has been rarely addressed (McKelvy and Burbrink 2017). For some small terrestrial mammals, rivers appear to be a clear barrier to movement and gene flow (Smith and Patton 1980; Nicolas et al. 2011; Soto-Centeno et al. 2013), but for many other small mammal species there is little evidence that rivers restrict movement on an evolutionary timescale (Patton et al. 1994; Yu 1995; Gascon et al. 2000; Vignieri 2005; Roratto et al. 2015).

Many common North American small mammals (e.g., *Sciurus* spp., *Microtus* spp., *Peromyscus* spp.) have subspecific boundaries delineated at rivers (Hall 1981; Steele and Koprowski 2001), leading to the assumption that rivers at least serve as partial barriers to dispersal. The latter argument assumes that subspecies distinction reflects, at least in part, restricted gene flow. Alternatively, using such biogeographic features as boundaries reflects a matter of convenience, particularly in instances where subspecific taxonomy is based on phenotypic variables that are properly recognized as having a clinal distribution. Whitaker (1970) argued that subspecies recognition should be limited to instances where gene flow is restricted between subspecies, relative to within subspecies, thus representing a "biological" definition of subspecies. Similar arguments have been applied to the delineation of evolutionary significant units (Crandall et al. 2000).

In contrast to examples where subspecies transition at natural biogeographic boundaries such as rivers, species with high phenotypic polymorphism often display more gradual changes across broad geographic areas. This dichotomy is problematic when there is a lack of easily diagnosable characteristics and a reliance on subspecific taxonomy in conservation and management (Avise 1989). Ideally, phenotypic variation that informs taxonomy will reflect ecological differences and show more abrupt changes from one subspecies to another. Where gene flow can be determined to have been historically limited, relative to within a subspecies range, this would be ideal information for supporting management decisions (Whitaker and Hamilton 1979; Crandall et al. 2000).

The distribution of the fox squirrel (*Sciurus niger*) in Florida affords a unique opportunity to explore patterns of gene flow and to specifically contrast the evidence for the "riverine barrier" hypothesis versus alternatives (refugial-allopatric divergence, parapatric divergence) in shaping fox squirrel phylogeographic structure in Florida. For example, fox squirrels from the southern part of the Florida peninsula are smaller and have different cranial morphology from squirrels north of the Caloosahatchee River, but there appears to be negligible difference in the morphology among southeastern fox squirrels north of the Caloosahatchee River (Turner and Laerm 1993). Coupled with the lack of any distinct morphometric differences, there is considerable uncertainty about whether geological features (rivers in particular) have isolated or restricted the movement of fox squirrels in both Florida and throughout the southeastern United States. Consistent with the range map of Koprowski (1994), the Florida Fish and Wildlife Conservation Commission (FWC) recognizes geographic separation between *S. n. bachmani* and *S. n. niger* at the Escambia-Blackwater rivers, located in the far western panhandle (Fig. 1A). In contrast, others place the boundary further east at the Apalachicola River (Steele and Koprowski 2001). *Sciurus n. shermani* is the most broadly distributed subspecies in Florida, and its distribution interfaces with 2 other subspecies, *S. n. niger* and *S. n. avicennia*. The distributional limits characterizing *S. n. shermani* and *S. n. niger* are complicated, and previous authors differ as to which rivers define these boundaries. For instance, some distribution maps suggest that the Suwannee River separates *S. n. niger* from *S. n. shermani* (Humphrey 1992; Koprowski 1994), whereas others postulate that the area between the Aucilla and Suwannee rivers represent an intergrade zone between *S. n. niger* and *S. n. shermani* (Moore 1956; Steele and Koprowski 2001). This intergradation has now been extended to an area between the Apalachicola and the Suwannee rivers, due to the difficulty associated with distinguishing among recognized subspecies (Fig. 1A). The southern distribution of *S. n. shermani* extends to the Caloosahatchee River, where it is distinguished from *S. n. avicennia* (Fig. 1; Howell 1919; Williams and Humphrey 1979; Koprowski 1994).

Given the difficulty in delineating subspecific distributions and the permeability of many river systems to small mammals including fox squirrels (Applegate and McCord 1974; Trauth and Jamieson 1997), it is surprising that this riverine barrier assumption of small mammal biogeography in North America has rarely been tested. Because the potential for alternative biogeographic explanations for regional patterns of genetic variation in fox squirrels are possible, our objective was to formally explore alternative hypotheses, in particular: 1) Riverine barriers shape genetic structure among recognized fox squirrels in Florida. 2) Fox squirrels fit a pattern of isolation by distance, whereby a lack of clear geographic structure precludes the recognition of geographic or ecological barriers. 3) Genetic differentiation among fox squirrels reflects major changes in habitat, which is also correlated with recognized morphological differentiation in south Florida.

Although fox squirrels in Florida have been differentiated based on major rivers in the region, the ecological features on both sides of the Apalachicola and Suwannee rivers have

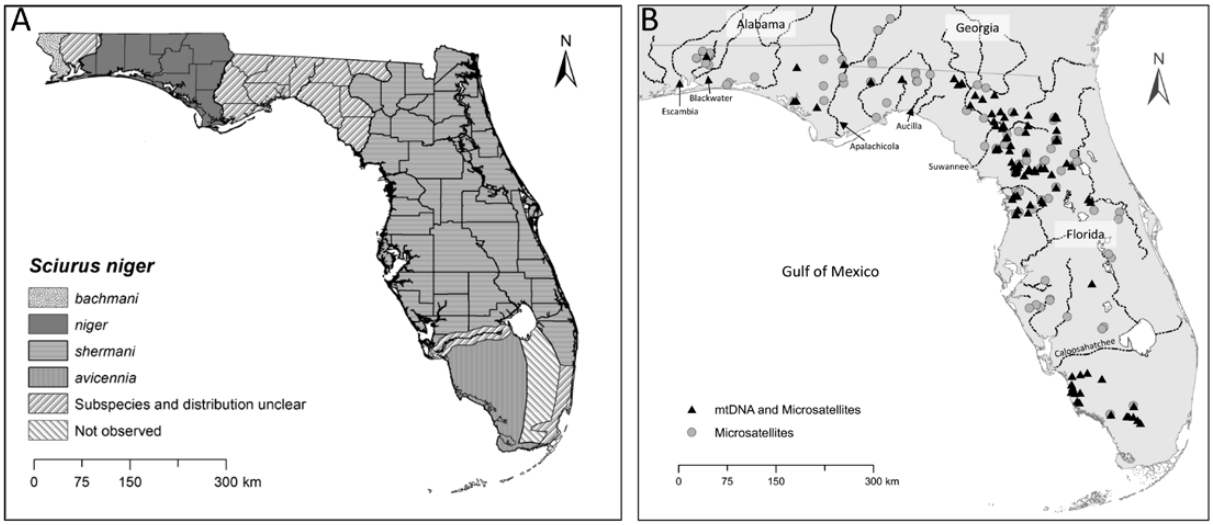

Fig. 1.—A) Compilation of previously published boundaries of Florida's fox squirrel subspecies (*Sciurus niger* ssp.) Data sources include Moore (1956), Williams and Humphrey (1979), Humphrey and Jodice (1992), Turner and Laerm (1993), Koprowski (1994), and unpublished sources. Areas designated as "Subspecies distributions unclear" indicate conflicting river boundaries between sources and putative intergrade zones. B) Distribution of genetic samples collected from fox squirrels in Florida and southern Georgia from 2012 to 2015. Major rivers are represented by dashed lines, and rivers mentioned in the text are labeled.

remained the same since the early Holocene. More recently (5000 BP to present), this region of Florida has been characterized by an extensive pine savanna (Myers and Ewel 1990) that was distributed throughout the southeastern United States, southward in Florida to the Caloosahatchee River. In contrast, the area south of the river represents a heterogeneous mix of sloughs, cypress swamps and cypress domes, open marshes, and cypress prairies (Williams and Humphrey 1979) beginning at the Holocene-Pleistocene boundary, with the Everglades reaching its maximal extent only a few thousand years ago, as a result of rising sea levels forming the swamps and wetlands that characterize much of the areas south of the Caloosahatchee River, including Big Cypress Swamp (Lodge 2010).

Our goal for this study was to understand the extent of recent and historic barriers to gene flow of Florida fox squirrels. Specifically, we wanted to understand whether rivers play a major role in substructuring fox squirrels (i.e., following taxonomic boundaries or otherwise), and whether any substructuring was geographically isolated. Accordingly, genetic structuring should be associated with major rivers (Fig. 1) delineating recognized fox squirrel taxonomic boundaries. If rivers are only weakly associated with taxonomy, then we predict that evidence of introgression will be detected across putative intergrade zones (Fig. 1). In the absence of partial or complete barriers to dispersal and gene flow, we predict that fox squirrel genotypes across Florida will reflect a pattern of isolation by distance (Wright 1943), where genetic similarity will decrease with geographic distance irrespective of putative barriers. Alternatively, phylogeographic structure may be limited to ecologically divergent populations, specifically in this case between *S. n. avicennia* and *S. n. shermani*, where the 2 subspecies inhabit distinct biogeographic provinces. We predicted that distinct habitat, rather than rivers alone, allowed for the selection of traits favorable for local environments and helped shape gene flow. To test this, we examined patterns of gene flow and timing of divergence between the 2 subspecies, *S. n. shermani* and *S. n. avicennia*, given evidence of substantial genetic substructuring. Lastly, our null hypothesis was that there may be no phylogeographic structure across Florida, reflecting previous phylogeographic studies on fox squirrels (Moncrief et al. 2010, 2012).

Materials and Methods

Sample collection.—Between 2009 and 2015, we collected DNA samples from ear clippings of live-captured individuals and muscle or liver opportunistically from road-killed squirrels (Supplementary Data SD1). The geographic coverage of sampling included 232 individuals from Florida and 5 individuals from southern Georgia (Fig. 1A).

When livetrapping, ear clippings were collected following guidelines approved by the American Society of Mammalogists (Sikes et al. 2016). Livetrapping and handling were conducted under the Florida Fish and Wildlife Conservation Commission's Scientific Collecting Permit (Permit Number: LSSC-11-00026) and approved by the University of Florida's Non-Regulatory Animal Research Committee (021-10WEC). All samples were stored in 95% ethanol and held at −10°C. We used a QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Valencia, California) to extract and purify total genomic DNA from approximately 25 mg of tissue.

Microsatellite genotyping.—We genotyped 226 fox squirrels at 8 unlinked microsatellite loci, originally characterized for *S. niger* by Fike and Rhodes (2009), representing 4 putative subspecies: *S. n. avicennia* (n = 25), *S. n. shermani* (n = 155), *S. n. niger* (n = 20), and 26 additional samples from within the putative *S. n. shermani-niger* intergrade zone (Fig. 1B). The forward primer of each set of loci was labeled with either

6-Fam or Hex. We conducted polymerase chain reactions (PCRs) in 25 µl volumes containing the following: 1 µl DNA template, 2 µl $MgCl_2$ (25 mg/ml), 2.5 µl buffer, 2 µl dNTPs, 1 µl each of forward and reverse primers, 0.25 µl Takara *Taq* polymerse (Thermo Fisher Scientific, Waltham, Massachusetts), and 15.25 µl of dH_2O. PCR amplification was conducted using a thermal cycler (Bio Rad T100 and C1000, Bio Rad Laboratories, Foster City, California) with the following conditions: initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, annealing (temperature per locus same as those listed by Fike and Rhodes 2009) for 30 s, and 72°C for 30 s, followed by an extension at 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Reactions for fragment analysis contained 8.8 µl Hi-Di Formamide (Applied Biosystems, Carlsbad, California) and 0.2 µl of GeneScan 400HD Rox size standard (Applied Biosystems). Genotyping was conducted on an ABI 3130 automated sequencer (Applied Biosystems), and alleles were scored using GeneMapper (version 3.7; Applied Biosystems).

Nucleotide sequencing.—Primers H15915 and L14723 (Kocher et al. 1989) were used to PCR amplify and sequence an approximately 1,200-bp fragment of the mitochondrial cytochrome *b* (*Cytb*) gene from 135 fox squirrels representing parts of the range, or putative intergrade zones between, *S. n. bachmani*, *S. n. niger*, *S. n. shermani*, and *S. n. avicennia* (Fig. 1). PCR amplifications contained the following: 1 µl DNA template, 1 µl of each primer (20 µM), 2 µl 10× buffer, 2 µl $MgCl_2$, 2 µl dNTPs, 0.25 µl Takara *Taq*, and 10.75 µl of dH_2O. PCR conditions included denaturation at 94°C for 5 min, 35 cycles of 94°C for 40 s, 54°C for 45 s, 72°C for 40 s, followed by an extension at 72°C for 10 min. PCR products were purified using EXoSAP-IT (Affymetrix, Inc., Santa Clara, California), and both strands were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Excess dye terminators were removed using DyeEx 2.0 spin columns (Qiagen). Sequencing was performed on an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific), and Sequencher version 4.1.1 (Gene Codes, Ann Arbor, Michigan) was used to create contigs derived from both sequenced strands.

Microsatellite analysis.—For microsatellite loci, we summarized the number of private alleles among subspecies categories, allelic diversity (*A*), and observed (H_O) and expected (H_E) heterozygosity using Arlequin 3.5.1.3 (Excoffier and Lischer 2010). Allelic richness was estimated based on a sample size of 20 (smallest sample from a subspecies, *S. n. niger*) using SPAGeDi vers. 1.4 (Hardy and Vekemans 2002).

Prior to estimating differentiation (F_{ST}), we tested for evidence of null alleles and genotyping error using Microchecker vers. 2.2.3 (Van Oosterhout et al. 2004). We examined microsatellite loci for deviations from Hardy–Weinberg proportions using the exact test in Genepop vers. 4.2 (Raymond and Rousset 1995; Rousset 2008) using 5,000 demarcations, 1,000 batches, and 5,000 iterations per batch for the Markov chain mixing, to ensure that *SE*s were below 0.01 and increase the accuracy of estimated *P*-values (Raymond and Rousset 1995). We examined patterns of significance at $\alpha \leq 0.05$ adjusted for multiple tests using the B-H method (Narum 2006). Because we focused on individual-based sampling over populations, all population-based tests were performed at the level of subspecies (and intergrade zones). We used Arlequin to estimate pairwise F_{ST} between subspecies classifications and for hierarchical analysis of molecular variance (AMOVA—Excoffier et al. 1992) to assess the proportion of genetic variation within and among subspecies.

We used the Bayesian clustering algorithm implemented in Structure 2.3.4 (Pritchard et al. 2000; Falush et al. 2003) that employs reversible MCMC searches to identify genetic clusters without a priori geographic information bias. We ran the admixture model using default settings for the alpha admixture and correlated allele frequencies. We ran 30 replicates at each *K*-value, ranging from 1 to 10, a range chosen to capture the number of putative subspecies, intergrade areas, and to allow for the detection of additional substructure. Each run consisted of 2.0×10^5 burn-in steps followed by 1.0×10^6 post-burn-in replicates. Because likelihood values can produce ambiguous patterns (e.g., likelihood values plateau with increasing *K*), we also examined the ad hoc ΔK value proposed by Evanno et al. (2005). We calculated ΔK values using STRUCTURE HARVESTER (Earl and Von Holdt 2011). Cluster matching and plotting of replicate runs for relevant *K*-values was conducted using CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and DISTRUCT v1.1 (Rosenberg 2004).

We performed a Mantel's test (Mantel 1967) to evaluate an association between Euclidian geographic and squared genetic distance (Peakall et al. 1995; Smouse and Peakall 1999) from all microsatellite genotypes included in this study. The squared genetic distance approach is multivariate, thus does not depend on assumptions related to Hardy–Weinberg equilibrium (see Peakall et al. 2003). Significance was determined with 9,999 permutations. Given that the presence of hierarchical population structure can bias tests for isolation by distance (Meirmans 2012), we also conducted a stratified Mantel's test, permuting distance matrices within subspecies only (rather than across the entire matrix) to examine the effect of hierarchical genetic patterns detected by STRUCTURE. The stratified Mantel's test permuted genotypes within *S. n. niger*, *S. n. shermani*, *S. n. avicennia*, and the squirrels sampled within the putative intergrade zone between *S. n. niger* and *S. n. shermani* (Fig. 1). We repeated both stratified and unstratified Mantel's tests excluding *S. n. avicennia* to determine the impact of the observed structure between *S. n. avicennia* and other subspecies on the overall pattern of isolation by distance. Mantel's tests were performed using GENODIVE 2.0 (Meirmans and van Tienderen 2004).

mtDNA analyses.—We used Arlequin to quantify *Cytb* variation among subspecies and Florida overall. For *Cytb*, molecular diversity indices included haplotype diversity (N_H), private haplotypes among subspecies N_{PH}, polymorphic sites (*P*), nucleotide diversity (π), and mean number of pairwise differences (*h*).

To investigate genetic relationships among haplotypes, we generated a Bayesian phylogenetic tree in MrBayes v. 3.1 (Ronquist et al. 2012). A TRN+I+Γ model was selected as the most appropriate substitution model based on Akaike Information Criteria (AIC) estimated in jModelTest2 (Guindon

and Gascuel 2003; Darriba et al. 2012). The analysis ran for 40 million generations sampling every 500 generations and consisted of 2 simultaneous runs of 4 chains each with a temperature for heated chains of 0.175. The *SD* of split frequencies was 0.001 at the end of this analysis, and we discarded the first 10% of trees as burn-in. GenBank sequences from *Sciurus lis* (AB192923), *Sciurus aberti* (U10181), *S. carolinensis* (FJ200744), *S. deppei* (KC777298), and 2 *S. n. rufiventer* sequences from Iowa (FJ200745, U10180) and 1 from Wyoming (FJ200745) were included as outgroups.

In addition, we calculated TCS networks (Templeton et al. 1992; Clement et al. 2002) on ingroup haplotypes implemented using Popart (Leigh and Bryant 2015). This method uses haplotype frequency data to infer haplotype age, and the probability of parsimony (connection between haplotypes) is calculated among pairwise relationships until the probability exceeds 0.95 (Templeton et al. 1987).

Coalescent inferences of gene flow and divergence timing.—Our data revealed substantial geographic structuring between, but not within *S. n. shermani* and *S. n. avicennia*, suggestive of demographic isolation between these 2 subspecies. Isolation with migration (IM) models can be used to address questions about the role that both time and gene flow have played in the divergence of populations (Wakeley and Hey 1998; Nielsen and Wakeley 2001). We implemented an IM approach using IMa2 version 8.27.12 (Hey and Nielsen 2007; Hey 2010a) to quantify gene flow and divergence time between *S. n. shermani* and *S. n. avicennia*. The program IMa2 uses Metropolis-coupled Markov chain Monte Carlo (MC^3) simulations to estimate the posterior densities of the respective effective population sizes, their time of divergence from an ancestral population, and the scale and direction of gene flow since population divergence (Nielsen and Wakeley 2001; Hey and Nielsen 2007; Hey 2010b). The model assumes random sampling, population size and gene flow have remained consistent since divergence, no substructure within populations, a neutral mutation model (Kimura 1983), and that nuclear markers are freely recombining and follow a stepwise mutation pattern. These assumptions are not necessarily ideal for scenarios where sea level rise and changes in habitat are known; however, modeling demographic patterns under the IM model requires simplifying assumptions (Runemark et al. 2011).

We examined nuclear and mtDNA data sets combined and independently, as the 2 genomes represent distinct demographic processes (Ballard and Whitlock 2004). By combining data sets, we assumed that discrepancies reflect differential gene flow between genomes. We ran initial exploratory runs to identify appropriate parameters and prior limits for both data sets before initiating the final analyses. We reduced the data sets to include only fox squirrel samples south of the Suwannee River primarily to reduce the sample disparity between *S. n. shermani* and *S. n. avicennia*, which can cause problems for parameter convergence, and this was justified based on the lack of evidence for strong population structure north of the Caloosahatchee River (see "Results"). Generation time was set to 2 years, reflecting the minimum age at which > 50% of females reproduce (Harnishfeger et al. 1978). For *Cytb*, we applied a geometric heating scheme with 20 concurrent chains and applied heating parameter values of 0.6 and 0.97. Following a burn-in of 3.0×10^{-4} steps, we sampled 1.4×10^{-7} steps sampling genealogies every 100th iteration to avoid autocorrelation. Mutation-scale migration rate and population size uniform priors were restricted based on preliminary runs that produced well-behaved posterior distributions for the ancestral ($4N\mu = 250$) and derived populations ($4N\mu_{shermani} = 450$; $4N\mu_{avicennia} = 150$). The upper bound for the migration prior was set to $m = 5$. Because parameters are scaled by mutation rate, a mutation rate must be provided in order to convert estimates into more easily interpretable demographic units. Mutation rate for *Cytb* was assumed to be 0.083 substitutions/site/MYR (the average estimated for rodents according to Nabholz et al. 2008) or 9.997×10^{-5} substitutions/gene/year for estimating divergence in years. This represents a typical rodent-like *Cytb* mutation rate.

A similar approach was used to evaluate conditions for the combined mtDNA+nDNA and nDNA data set alone, where we assumed a stepwise microsatellite mutation rate per year of 4.0×10^{-4}, which represents an average across studies and loci (Ellegren 2000, 2004; Whittaker et al. 2003; Gryseels et al. 2016). We restricted the microsatellite data to 6 loci that contained allele identities that followed a stepwise pattern (omitting FO67 and FO14). For microsatellites, we applied a geometric heating scheme with 80 concurrent chains and applied heating parameter values of 0.80 and 0.96. Following a burn-in of 3.0×10^{-4} steps, we sampled 1.0×10^{-6} steps sampling genealogies every 100 to avoid autocorrelation. Uniform priors were 150 for all population estimates. The upper bound for the migration prior was set to $m = 200$. The combined data was analyzed using the same number of chains and parameter conditions as for the nDNA-only runs.

In IMa2, the rate at which one population received migrants from another, $2Nm$, is derived from integrating over the joint posterior density for the population size and migration parameters (Hey 2010a). The significance of migration (i.e., nonzero) was assessed using the Nielsen and Wakeley (2001) test implemented in IMa2. The divergence time parameter, $t = T\mu$, was converted into years by dividing with the assumed mutation rate and multiplying with the generation time.

Results

Microsatellite analysis.—Microsatellite variation was lowest in *S. n. avicennia*, with heterozygosity values below 0.7, and mean number of alleles at 6.25 (Table 1; Supplementary Data SD2). Potential null alleles were detected at 2 loci (FO11 and FO58), only in *S. n. shermani*. All remaining 30 tests were nonsignificant for genotyping errors or null alleles. Controlling for differences in sample size, allelic richness remained similar across *S. n. niger*, the intergrade zone, and *S. n. shermani* (A_{20} range from 8.50 to 8.93), but was lower for *S. n. avicennia* (5.94; Table 1). Deviation from Hardy–Weinberg proportions was detected at locus

Table 1.—Summary statistics by subspecies (including intergrades) for microsatellite loci and mitochondrial sequence data from fox squirrels (*Sciurus niger*) sampled across Florida. For microsatellite results: sample size (n), number of private alleles (A_P), average number of alleles per locus (A), and allelic richness rarefied to $n = 20$ (A_{20}), observed heterozygosity (H_O), and expected heterozygosity (H_E). Mitochondrial sequence data: sample size (n), number of haplotypes (n_H), number of private haplotypes (n_{PH}), polymorphic sites (P), haplotype diversity ($h \pm SD$), and nucleotide diversity ($\pi \pm SD$).

	Microsatellite DNA						Mitochondrial DNA					
Population	n	A_P	A (± *SD*)	A_{20}	H_O (± *SD*)	H_E (± *SD*)	n	N_H	N_{PH}	P	h (± *SD*)	π (± *SD*)
niger	20	5	8.50 (0.74)	8.50	0.744 (0.105)	0.776 (0.098)	10	7	4	17	0.911 (0.077)	0.0071 (0.0041)
niger-shermani	26	1	9.37 (4.00)	8.89	0.788 (0.111)	0.807 (0.096)	13	12	8	25	0.987 (0.035)	0.0075 (0.0042)
shermani	155	32	13.63 (4.84)	8.93	0.756 (0.089)	0.778 (0.110)	87	52	42	73	0.977 (0.006)	0.0079 (0.0041)
avicennia	25	2	6.25 (2.82)	5.94	0.658 (0.115)	0.674 (0.152)	25	8	7	12	0.812 (0.054)	0.0020 (0.0013)

FO67 ($P = 0.0002$) in *S. n. shermani* following B-H-adjusted P-values ($P_{adj} = 0.0016$).

Taxa were significantly differentiated (F_{ST}) at microsatellite loci (Table 2). Variation among taxa (AMOVA) was low but highly significant, with the majority of genetic variation distributed within individuals. For the microsatellite data, only a small (2.6%) though significant ($P < 0.0001$) percentage of the variation could be explained by variance among subspecies.

Structure results identified a modest increase in likelihood [ln$P(K)$] from $K = 1$ (−7,270.27 ± 0.04) to $K = 2$ (−7,163.21 ± 8.72), with low variance for the latter, followed by declines in likelihood for subsequent K values (e.g., $K = 3$, −7,195.36 ± 25.73; $K = 4$, −7,229.76 ± 69.96). $K = 2$ also had the highest ΔK value (15.95) reflecting the relatively large change in variance from $K = 1$ to $K = 2$, relative to subsequent steps (all other $\Delta K < 1.9$). Thus, $K = 2$ appears to represent the best model to describe structuring at the highest hierarchical level. At $K = 2$, there was clear separation between a *S. n. avicennia* cluster and all other fox squirrels (Fig. 2). However, even when examining $K = 3$ (shown) and higher K values (not shown) *S. n. avicennia* was consistently represented as being a distinct cluster from fox squirrels north of the Caloosahatchee River, and the remaining genetic groups were represented within individuals from all other subspecies (Fig. 2). In addition, a subtle change in K-value representation between *S. n. niger* × intergrade and *S. n. shermani* was revealed at greater K values, particularly at $K = 3$. As we did not include a sample "prior" in our structure analyses, this may represent weak differentiation of samples distributed on either side of the Suwannee River, though across K-values examined there was no clear evidence of a lack of admixture, as was seen between *S. n. avicennia* and all other fox squirrels (Fig. 2).

Isolation by distance based on microsatellite genotypes was not significant at $\alpha = 0.05$ ($r^2 = 0.003$, $P = 0.054$) across Florida. Omitting *S. n. avicennia* resulted in even less support for isolation by distance among fox squirrels from north of the Caloosahatchee River to the western panhandle ($r^2 = 0.001$, $P = 0.208$). However, the stratified Mantel's test was nonsignificant regardless of whether *S. n. avicennia* was included (with, $r^2 = 0.001$, $P = 0.650$; without, $r^2 = 0.001$, $P = 0.911$), revealing the impact of hierarchical genetic structure on affecting the strength of isolation by distance across the entire sampling distribution, or more specifically, the relative impact of *S. n. avicennia* on contributing to genetic structuring across Florida.

Table 2.—Pairwise F_{ST} based on microsatellite (lower diagonal) and cytochrome *b* (*Cytb*) (upper diagonal). Significant ($P = 0.05$) F_{ST} values based on 1,000 bootstraps are indicated with italics.

	niger	*shermani-niger*	*shermani*	*avicennia*
niger	—	0.060	0.055	*0.289*
intergrade	*0.010*	—	−0.002	*0.127*
shermani	*0.016*	*0.010*	—	*0.162*
avicennia	*0.071*	*0.055*	*0.041*	—

Importantly, the lack of an isolation by distance pattern suggested that natal dispersal is high in fox squirrels.

mtDNA analyses.—Our final mtDNA data set consisted of 1,133 bp of *Cytb* corresponding to nucleotides 8–1,140 of the fox squirrel *Cytb* gene. Representative samples of each of the unique haplotypes generated for this project were deposited in GenBank (accession numbers MH766401–MH766474). Diversity as measured by h and π was lowest within *S. n. avicennia* but similar among the remaining subspecific groups (Table 1). Private haplotypes were common within each subspecific group (Table 1), resulting in AMOVA identifying 10.6% ($P < 0.000$) of genetic variation distributed among subspecies at *Cytb*. Only pairwise comparisons (F_{ST}) with *S. n. avicennia* were significantly different from zero (Table 2).

The Bayesian phylogenetic tree was shallow with limited node support within the ingroup, and there was no subspecific monophyly (Fig. 3). The TCS minimum-parsimony network highlighted the reticulate nature of the mtDNA haplotypes. There were 2 main clusters of *S. niger* Florida haplotypes representing a mix of subspecies separated by 8–9 mutational steps (Fig. 4), and these groups were not geographically structured. The 2 northern haplotypes (1 and 2) and haplotype 63 (from Putnam County, Florida) were also highly divergent. Lineage A consisted of *S. n. niger* and *S. n. shermani* haplotypes sampled from the panhandle to as far south as Marion County. Lineage B consisted of a similar distribution but included haplotypes from south Florida including all 8 haplotypes from *S. n. avicennia*. One *S. n. avicennia* haplotype was shared with *S. n. niger* and *S. n. shermani*. Many of the remaining 7 *S. n. avicennia* haplotypes were differentiated from one another by more than 1 mutation (Fig. 4).

The included haplotypes from Iowa and Wyoming rooted lineage A in the Bayesian phylogenetic tree but were more closely allied with lineage B based on the 95% limits of

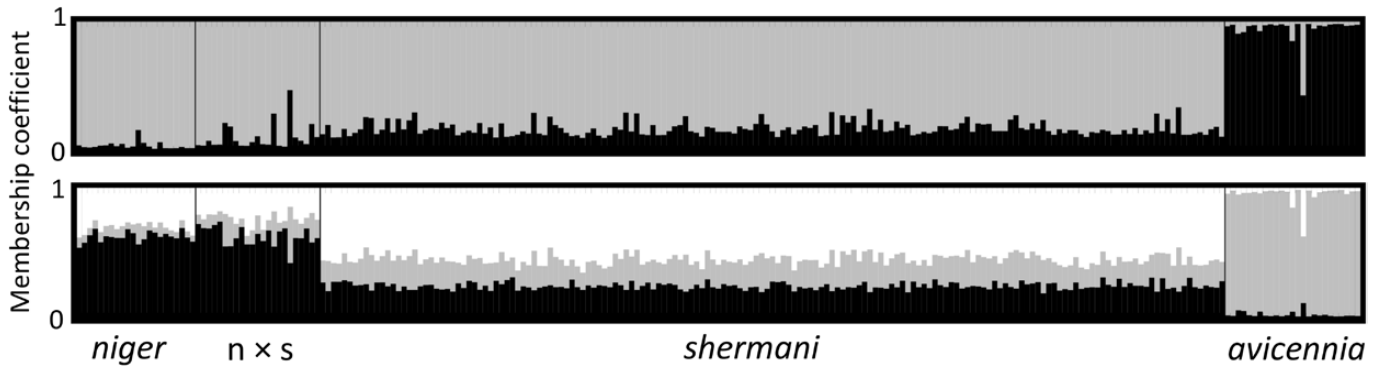

Fig. 2.—Bayesian clustering results generated using STRUCTURE, CLUMPP, and DISTRUCT. Bar plots showing individual membership coefficients (ranging from 0 to 1) for $K = 2$ (top) and $K = 3$ (bottom). Samples are ordered by subspecies designation (n × s refers to intergrade zone between *Sciurus niger niger* and *S. n. shermani*). In both models, *S. n. avicennia* was largely distinct from the remainder of the samples.

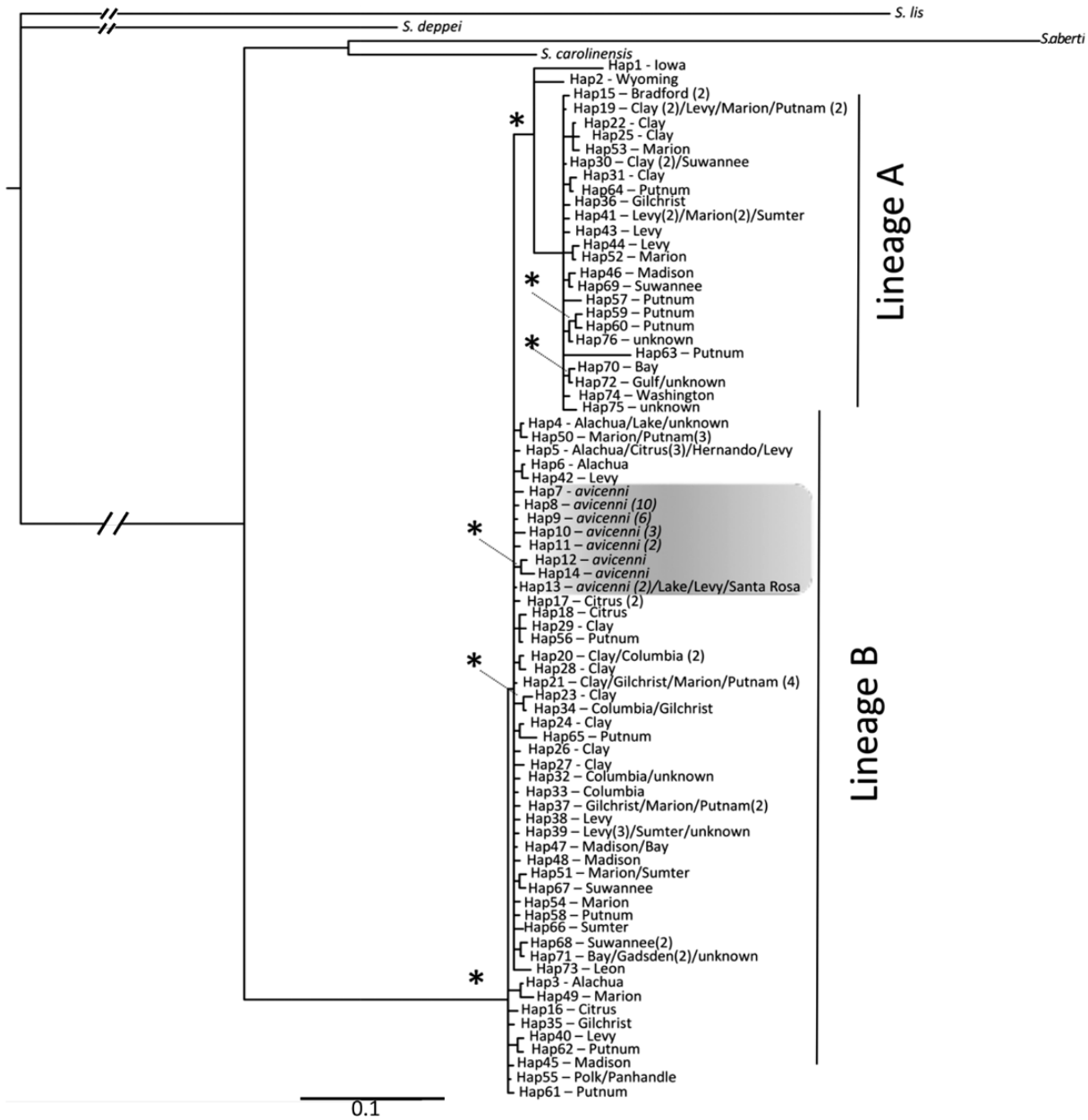

Fig. 3.—Bayesian phylogenetic tree for all unique cytochrome *b* (*Cytb*) haplotypes. * indicates posterior support > 0.95. Haplotypes identified south of the Caloosahatchee River, within the range of *Sciurus niger avicennia*, are shaded.

parsimony-based TCS network. *Cytb* diversity was generally highest in *S. n. shermani*, although haplotype diversity (*h*) was higher in fox squirrels sampled from within the putative *S. n. shermani-niger* intergrade zone (Table 1).

Gene flow and divergence timing.—Results from the IM analyses were inconsistent between nDNA and mtDNA. Significant levels of unidirectional gene flow were detected only from *S. n. avicennia* into *S. n. shermani* for microsatellite markers but not at mtDNA (Table 3). The combined data set was significantly nonzero in both directions, though notably higher from *S. n. avicennia* into *S. n. shermani* (Table 3; Fig. 5B). Coalescence analyses based on *Cytb* produced a divergence time estimate between *S. n. shermani* and *S. n. avicennia* that corresponded to approximately 15,000 years ago (Table 3). That is approximately the Holocene-Pleistocene boundary. In contrast, the microsatellite data reflected a contemporary timing of divergence (Fig. 5C). Combined, the intermediate timing of divergence would be mid-Holocene (Fig. 5C). Matrilineal effective population sizes (N_e) were considerably larger for mtDNA estimates than for microsatellites considered separately or for the combined analyses (Fig. 5A). However, estimates for ancestral N_e were flat for microsatellites and combined, reflecting the lack of historical information content inherent in microsatellite loci.

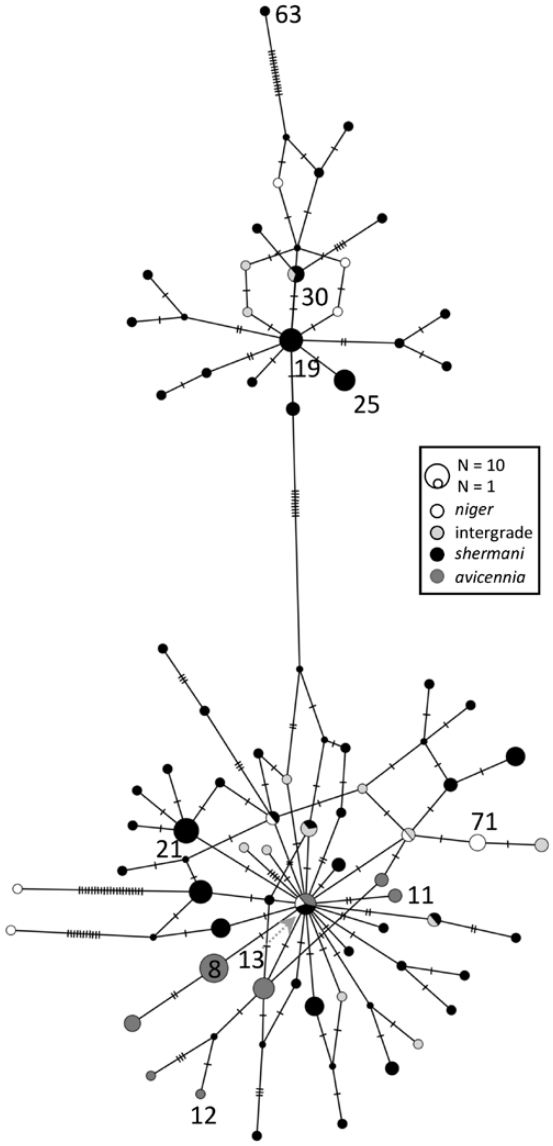

Fig. 4.—TCS parsimony network of cytochrome *b* (*Cytb*) haplotypes. Some haplotypes (corresponding to those in Fig. 3) are indicated for comparative purposes. The hash marks along branches represent the number of substitutions separating particular groups and lineages.

Discussion

Across the range of *S. niger* in Florida, subspecies were polyphyletic at mtDNA and showed limited geographical genetic structure at putatively neutral nDNA markers. In terms of the latter, substantial genetic clustering was only observed between squirrels geographically separated in the region south of the Caloosahatchee River, which represents the recognized transition from *S. n. shermani* to *S. n. avicennia*. After controlling for the hierarchical structure represented by the split between *S. n. avicennia* and the rest of Florida, there also was a lack of isolation by distance across the entirety of Florida. The absence of nuclear or mtDNA structure further north is evidence that those taxonomic boundaries do not reflect ecological or evolutionary (i.e., restricted gene flow) distinction. More specifically, there is little evidence (the Caloosahatchee River being a possible exception) to support rivers as important isolation barriers between populations or taxonomic units. Phylogenetic results based on the mitochondrial *Cytb* gene revealed that *S. n. bachmani*, *S. n. niger*, and *S. n. shermani* are co-distributed across 2 divergent haplotype groups; however, *S. n. avicennia* is only represented in 1 of these groups. Taken together, the genetic data suggest that Florida fox squirrels represent a highly intermixed population, possibly representing historical mtDNA lineages from at least 2 refugia, and that *S. n. avicennia* has diverged in parapatry since the early Holocene. This is supported by the lack of divergent haplotypes identified from the latter and major differences in habitat between *S. n. avicennia* and fox squirrels to the north. Evidence of parapatric divergence in natural populations of mammals has been limited but is expected to be common where ecological transitions occur (Schilthuizen 2000).

The pattern and timing of divergence detected in south Florida likely is not a result of refugial dynamics, but rather ecological divergence. Matrilineal splitting times between *S. n. avicennia* and *S. n. shermani* put the onset of divergence at approximately 15,900 years ago (95% interval of 11,000–25,000 years).

Table 3.—Migration results from isolation with migration (IMa2) analyses for each genome consider separately and combined. Values are given as 2*Nm* per generation, and "na" indicates that no migration events were reported for a given genome. Asterisks indicate significant at $P < 0.01$ by the Nielsen and Wakeley (2001) test.

Migration rate	Nuclear	Mitochondrial	Combined
avicennia → *shermani*	48.68**	0[na]	28.52**
shermani → *avicennia*	1.66[na]	0[na]	1.31**

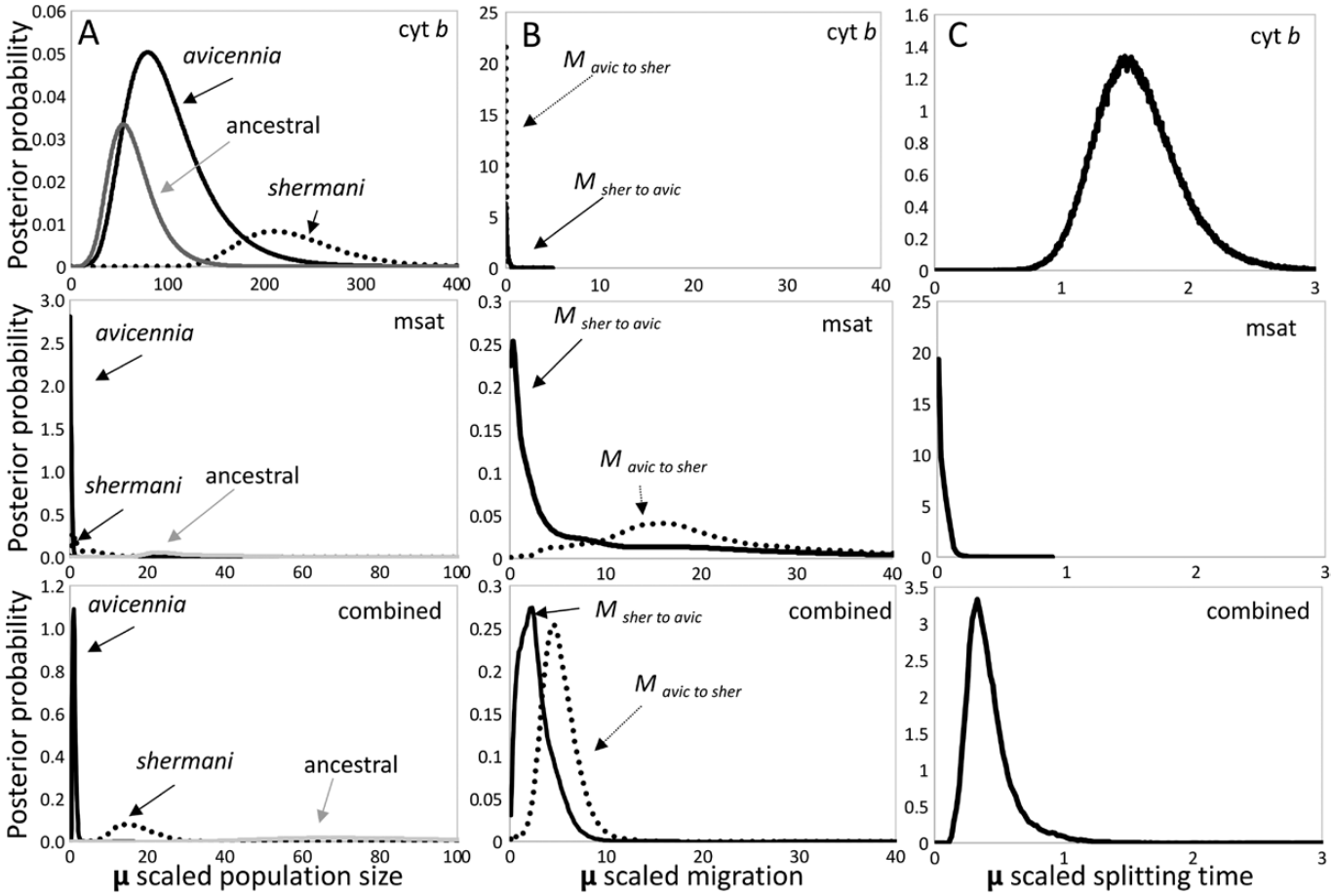

Fig. 5.—Posterior probability density histograms of the 6 parameters of the isolation with migration model: A) 3 mutation-rate scaled effective populations sizes (effective individuals in the current *Sciurus niger shermani*, *S. n. avicennia*, and the ancestral population from which both are drawn). B) Forward migration rate *M* (migrations/generation). C) Time since split (t_0). Each is provided for cytochrome *b* (*Cytb*) (top), microsatellite (middle), and combined (bottom) data.

However, the combined mtDNA and nDNA estimate of splitting was roughly in the mid-Holocene, a time when major ecological changes were occurring in south Florida (Lodge 2010). At ~10,000 years before present (ybp) rising sea levels and changing precipitation patterns resulted in the onset of the formation of Lake Okeechobee and the Everglades, as well as surrounding wetland habitats (including Big Cypress). The lack of matrilineal gene flow was interesting given that some level of permeability was anticipated, and would seem to reflect substantial ecological divergence by the early Holocene. Considered separately, gene flow was estimated at zero for maternally inherited DNA, suggesting that gene flow is heavily male-biased. Nuclear gene flow was asymmetrical, with non-zero migration occurring northward from *S. n. avicennia* into *S. n. shermani* over this period (Table 3). Male-biased dispersal is typical for mammals (Greenwood 1980), although sex bias in squirrels has been rarely documented (Larsen and Boutin 1994). The observed nuclear gene flow may reflect male-driven natal dispersal, which may be linked to multiple proximate factors (e.g., density, sex ratios, behavioral differences—Gaines and McClenaghan 1980; Clobert et al. 2009). The asymmetry measured from south to north across the Caloosahatchee River may be related to environmental heterogeneity of available resources (e.g., number of mates, available suitable habitat) and intrinsic differences between sexes, such as the asymmetry in home ranges between male and female *S. n. avicennia* (Kellam et al. 2016). In this instance, greater competition for lower-quality resources, and larger home ranges of males may lead to greater exploratory dispersal from peripheral population to the species range center, as was detected in peripheral-range red squirrels (Merrick and Koprowski 2017).

Somewhat unexpected were the contemporary estimates of isolation provided by the microsatellite data set. Divergence timing overlapped zero, with a peak corresponding to approximately 500 ybp. This may be due to the vagaries of mutation rate estimates, though even a more slowly mutating microsatellite rate (e.g., 1.0×10^{-5}) still places the timing of isolation within 100s of years rather than 1,000s. The nuclear genome is, in general, expected to have a larger effective population size (N_e) than mtDNA, resulting in slower response to genetic drift in the former. However, this may not be true in instances where male reproductive variance is substantially greater than that in females, or where strong sexual selection is occurring (Ballard and Whitlock 2004). Reproductive variance in fox squirrels is poorly understood; however, male *S. n. avicennia* do maintain larger home ranges than females (Kellam et al. 2016),

suggesting that intensive scramble competition for mating occurs (Lane et al. 2009). This could have the effect of greatly reducing N_e of nDNA relative to mtDNA, thus leading to more recent coalescent periods at microsatellite loci.

Another probable explanation for the inability of microsatellite loci to reflect deeper divergence is related to saturation effects. Microsatellite loci are highly polymorphic loci, and their stepwise-like mutation process (i.e., increases or decreases in repeat sizes) is subject to homoplasy relative to infinite allele models (Estoup and Cornuet 1999), particularly when populations are large and mutation rates are high (Estoup et al. 2002). The timing of older demographic events may be obscured by the higher polymorphism and mutational characteristics of microsatellites.

Our results build on previous phylogeographic studies on *S. niger*. Moncrief et al. (2010) argued that *S. niger* underwent recent range expansion from southern refugial area, and they implied that the mtDNA polyphyly reflected a lack of lineage sorting during the process of differentiation into recognized subspecies. They concluded that Weigl et al.'s (1998) hypothesis that *S. niger* utilized 2 distinct Pleistocene refugia (west and east) is likely incorrect due to the lack of "2 distinct stars in a haplotype network" observed in their data. Our data support the Weigl et al. (1998) scenario, given that our statewide sampling both revealed far greater haplotype diversity than detected by Moncrief et al. (2010) and identified 2 divergent mtDNA haplotype groups that are highly intermixed geographically (Fig. 3). The contrasting results likely represent a sampling artifact in that Moncrief et al. (2010) included only Florida samples from Collier County, within the range of *S. n. avicennia*, and sequenced only a 400-bp portion of the *Cytb* gene. Their haplotype D was shared across the range of *S. niger* and corresponds to our haplotype 13 (Fig. 4). They also reported 2 private haplotypes (U and V) in Collier County (see figure 2 of Moncrief et al. 2010), which correspond to our haplotypes 11 and 12, respectively (Fig. 3).

The Florida peninsula may have been an important long-term refugium where *S. niger* persisted, possibly throughout multiple glacial-interglacial cycles (Stewart and Dalén 2008; Stewart et al. 2010). The amount of haplotype variation detected within Florida (from 2 divergent groups of mtDNA) and the distribution of divergent haplotypes within recognized subspecies (except *S. n. avicennia*; Fig. 4) suggest that haplotype groups diverged in allopatry (i.e., Florida and Texas refugia—Swenson and Howard 2005), and these refugial populations subsequently expanded in range and became widespread during the Holocene. This Holocene spatial expansion is further supported by the lack of nDNA isolation by distance across Florida (north of the Caloosahatchee River), which reflects high gene flow and a lack of ecological or geographic barriers. At this same time of northern expansion from glacial refugia, ecological changes in south Florida initiated the isolation and divergence of what is currently recognized as *S. n. avicennia*. The differentiation between *S. n. shermani* and *S. n. avicennia* is emphasized by a lack of microsatellite genetic structuring (Fig. 5) north of the Caloosahatchee River.

Based on the microsatellite data, only weak differentiation between subspecies and regions of putative *S. n. shermani*/*niger* intergradation associated with the western Florida panhandle and the Caloosahatchee River was revealed (Fig. 1). With the exception of *S. n. avicennia*, the genetic uniformity observed herein and in other mtDNA studies (Moncrief et al. 2010) suggests that gene flow has been high. That, and the described lack of isolation by distance, suggests that the genetic distinctiveness of *S. n. avicennia* may reflect ecological divergence corresponding to reduced gene flow.

Ecological divergence is a primary mechanism of early population divergence leading to speciation (Nosil 2012). In south Florida, the transition between the longleaf pine (*Pinus palustris*) savanna ecosystem and a more mesic system with pond cypress (*Taxodium distichum* var. *imbricarium*), slash pine (*P. ellottii* var. *densa*), and wet prairies corresponds to a shift in body size in fox squirrels. *Sciurus n. avicennia* are considerably smaller than fox squirrels north of the Caloosahatchee River, potentially an adaptation to the environmental conditions south of the Caloosahatchee River. The smaller body size of *S. n. avicennia* may be ecologically important for dealing with limited and poor-quality food resources (Weigl et al. 1998). Alternatively, the larger body size of *S. n. shermani* likely lowers their vulnerability to avian predators but it also reduces their arboreal agility (Weigl et al. 1998). The greater agility in the smaller *S. n. avicennia* may be advantageous south of the Caloosahatchee River where forests are thicker and fox squirrels are seasonally restricted to dense canopies during the wet season, requiring them to be fully arboreal (Kellam et al. 2016). Furthermore, seeds from pine cones are seasonally important components for fox squirrels throughout the southeast (Loeb and Moncrief 1993), but *S. n. avicennia* do not need added size to effectively handle the large (15–25 cm) longleaf pine cones, selected by fox squirrels north of the Caloosahatchee. The smaller *S. n. avicennia* instead favors the smaller (8–16 cm) cones of slash pine that are prolific in the areas south of the Caloosahatchee (Humphrey 1992).

In their entirety, our results suggest that differentiation between *S. n. avicennia* and northern subspecies is better described by recent (Holocene) divergence in the environment than by riverine vicariance, differential refugia, or isolation by distance. Historically, numerous subspecies of North American small mammals were delineated at rivers (Hall 1981; Steele and Koprowski 2001), under the assumption that rivers are barriers to dispersal that restrict gene flow. Moncrief (1993) found evidence of both riverine effects on allozyme variation across the lower Mississippi River, and for morphological divergence between fox squirrels in the floodplain versus the surrounding upland areas. We found little support for this riverine barrier hypothesis, as there was limited differentiation and a lack of isolation by distance among *S. n. niger* and *S. n. shermani*. Our findings are further supported by the fact that there is currently little evidence that phenotypic variation in Florida's fox squirrels is geographically discrete, but rather is clinal (reviewed in Turner and Laerm 1993), as well by evidence that some small mammals can readily cross rivers and other bodies of water (Beer et al. 1954; Steele and

Koprowski 2001; Williams et al. 2001). Therefore, we recommend against assuming that rivers restrict gene flow, thus implying that rivers do not always delineate taxonomic boundaries. By extension, subspecies as "geographically defined aggregate of populations" (Mayr 1940), whose designation is supported by multiple, independent, genetically based traits (Barton and Hewitt 1989; O'Brien and Mayr 1991), is dubious for *S. n. shermani*. The limited genetic and ecological differentiation among subspecies in northern and western Florida suggests that these are not demographically independent lineages. Instead, our data support the idea that genetic population structuring of fox squirrels in south Florida is a result of unique ecological conditions and that *S. n. avicennia* is on a distinct evolutionary trajectory from fox squirrels to the north. Renewed management emphasis should be toward emphasizing ecological and genomic features that reflect evolutionary divergence (Lesica and Allendorf 1995). As such, our results support the continued recognition of *S. n. avicennia* as a distinct population segment; however, there is little support from our study for the continued recognition of *S. n. shermani* as distinct from *S. n. niger*.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—List of samples included in the genetic study of fox squirrels (*Sciurus niger* ssp.) in Florida. Data include coordinates (NA = not available), county collected from, microsatellite genotypes (Y = yes, or NA), and cytochrome *b* haplotype ID (number corresponds to Fig. 4).

Supplementary Data SD2.—Microsatellite genotypes.

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