

2005

Genetic Variability, Pathogen Susceptibility, Subspecies Identity and Conservation of the Endangered Northern Flying Squirrel (*Glaucomys sabrinus*) in Virginia

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**Genetic variability, pathogen susceptibility, subspecies identity, and conservation
of the endangered northern flying squirrel (*Glaucomys sabrinus*) in Virginia.**

**A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science at Virginia Commonwealth University.**

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September 2005**

Acknowledgments

I am indebted to Dr. John Pagels and Dr. Bonnie Brown who supplied both the raw material and the intellectual refinery for this project. Dr. Rodney Dyer and Dr. D'arcy Mays were also instrumental to the completion of this thesis. Dr. Ralph Eckerlin of NVCC and Dr. Eugene Rowe DVM contributed to project design and analysis. All VCU faculty were very helpful, particularly Dr. Charles Blem, Dr. Donald Young, Dr. Michelle Peace, Ms. Anne Wright, and Dr. Karen Kester. VCU Life Science staff, Stephanie Millican and Bonnie VanDuesen assisted greatly by coordinating logistics and funding. Graduate colleague Mundy Hackett introduced me to the MRNRA study site and the flying squirrel trapping technique. I am indebted to VCU student volunteers Maureen Ostlund, René L. Cabaniss, Diana Ocampo, Rebecca Doane, Julia Rowe, and especially Lynne Hassel who created a flying squirrel mascot and illustrations for public outreach and scientific publication. I received assistance with molecular DNA techniques from Colleen Higgins, Antoine Nicolas, and Li Li. USFS MRNRA, VaDCR Grayson-Highlands State Park, VaDGIF, Virginia Academy of Sciences, and the Explorers Club Washington Group provided financial support. USFS chief ranger Beth Merz and Cecil Thomas organized the field research. Tom Blevins and George Norris cracked wise and kept things moving on the mountain. VaDCR personnel Harvey Thompson, Kevin Kelley, and Deana Blevins coordinated volunteers to perform the "Sparky's Great Adventure" outreach play. VDGIF biologists John Baker, Allen Boynton, Mike Fies, Rick Reynolds, and Ray Fernauld contributed logistical support. Craig Stihler and Jack Wallace of West Virginia DNR contributed specimens from their state. DNA samples from outgroup subspecies were provided by the Smithsonian Institution through Mr. Robert Fisher. Denise Naim helped me with the final syntax edit.

This effort is dedicated to my grandparents John and Anna Sparks, and Carl and Grace Scheidt, to my parents Lincoln and Grace Sparks, and to my two sisters Carolyn and Elinor.

To all my friends and all my relations...

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Abstract

Genetic variability, pathogen susceptibility, subspecies identity and conservation of the endangered northern flying squirrel (*Glaucomys sabrinus*) in Virginia.

James Lincoln Sparks, Jr.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2005.

Thesis Directors: Dr. John F. Pagels, Professor
Dr. Bonnie L. Brown, Professor
Department of Biology

I examined the population genetic structure of three known subspecies of *Glaucomys sabrinus* from Appalachia, Washington State, and two previously unexamined populations from Mount Rogers National Recreation Area (MRNRA), in Southwestern Virginia. Mean F_{ST} (0.107) and an AMOVA ($P < 0.001$), indicated that *G. sabrinus* subspecies populations in the southern Appalachians are genetically differentiated. *Glaucomys sabrinus* at MRNRA were less inbred than expected. Gene flow, a consensus tree based on Nei's genetic distance, elevated heterozygosity and morphometric data suggest that the MRNRA *G. sabrinus* population is an intergrade of the two recognized Appalachian subspecies, *G. s. fuscus* and *G. s. coloratus*. I compared inbreeding and the level of parasite infestation in the two MRNRA populations of *G. sabrinus* and found that Whitetop Mountain (150 ha habitat) was more inbred than the population on Mount Rogers (400 ha habitat, $P < 0.001$). The egg counts of the parasitic helminth *Strongyloides robustus* were greater in the more fragmented Whitetop Mountain population, although the difference was not statistically significant ($P = 0.278$). A Mantel comparison of genetic diversity and parasite infestation among individuals did show a highly significant negative correlation ($P < 0.0001$). The MRNRA *G. sabrinus* form a unique insular population with high genetic diversity that is nonetheless susceptible to increased inbreeding, and elevated parasitism caused by fragmentation. MRNRA *G. sabrinus* should retain endangered species status.

INTRODUCTION

The southern Appalachian northern flying squirrel, *Glaucomys sabrinus*, exists in isolated mountaintop Pleistocene relict habitat populations (Fies and Pagels 1991; Weigl et al. 1992; Browne et al. 1999; Reynolds et al. 1999). Their high degree of association with insular, relict habitats encourages vicariance, subspeciation, and genetic isolation (Slatkin 1987; Browne et al. 1999). Reduced genetic variation has been documented in maritime insular populations of an Alaskan northern flying squirrel subspecies, *G. s. griseifrons*, in the Alexander Archipelago (Bidlack and Cook 2001). Furthermore, because inbreeding has been linked to increased risk of pathogenic infectious disease (Scott 1988; Ralls et al. 1988; Mills and Smouse 1994), it is possible that reductions in genetic diversity due to inbreeding may be associated with the level of parasitism by *Strongyloides robustus*. Meagher (1999) correlated reduced genetic variability of *Peromyscus maniculatus* with elevated levels of the nematode parasite *Capillaria hepatica* in Michigan. Loss of genetic variation due to fragmentation and insularity is believed to increase the risk of extirpation (Slatkin 1987; Vrijenhoek 1989; Meagher 1999; Hale et al. 2001).

The Mount Rogers National Recreation Area population of *G. sabrinus* is the most geographically isolated one in the eastern United States. Mount Rogers is 37 km (23 mi) northwest of the nearest population of *G. s. coloratus* in Long Hope Valley, North Carolina (Weigl et al. 1992) and 273 km (170 mi) southeast of the nearest *G. s. fuscus* population in Highland County, Virginia (USFS 1990). The late systematist Charles O. Handley Jr., who originally described *G. s.*

coloratus in Tennessee and North Carolina, viewed the *G. sabrinus* from southwestern Virginia as an intergrade between the two subspecies (Fies and Pagels 1991). Weigl et al. (1992) recommended revisiting the taxonomic standing of *G. sabrinus* in southwestern Virginia because of the population's proximity to *G. s. coloratus* in North Carolina.

The Endangered Species Act (ESA) has protected the two northern flying squirrel subspecies found in middle and southern Appalachia since 1986 (USFWS 1990). A potential ESA ruling may down-list *G. s. fuscus* to threatened status (Pagels pers. com.). The subspecies identity of the MRNRA population is, therefore, relevant to local recovery plans. We address the subspecific standing of the MRNRA population using analysis of gene flow, genetic distances and biometric comparison.

Evolution of the North American Flying squirrel

Flying squirrels, family Sciuridae, sub-family Petauristinae, are nocturnal tree squirrels that are adapted for gliding as a means of locomotion. This action, known as volplaning, is made by extension of a parachute of elastic skin, the patagium. The patagium is suspended from a retractable cartilage styliform process at the wrist and attached to each corresponding ankle (Gupta 1966).

The origin of the flying squirrel genus *Glaucomys* remains obscure (Pratt and Morgan 1989; Skwara 1985; Thorington et al. 1996). Paleontological evidence suggests the concurrent arrival of two Petauristinae genera, *Petauristodon* and *Eomys*, from Asia via the Bering isthmus during the second

Hemingfordian faunal exchange, 18 mya (Webb and Opdyke 1995). Kurtén and Anderson (1980) report a single genus and species, *Cryptopterus webbi*, by the late Pliocene, 3 mya. The earliest fossil *Glaucomys* is *G. volans*, known from a single faunal deposit in Florida 80,000 ybp (Martin 1974). *Glaucomys sabrinus* debuts in the fossil records of northern California and southern Appalachia concurrently during the Wisconsinan circa 18,000 ybp (Furlong 1906; Guilday et al. 1978).

The two species of flying squirrel occurring in North America today, the northern flying squirrel, *G. sabrinus*, and the southern flying squirrel, *G. volans*, can be distinguished from each other in the field on the basis of body size and coloration. *Glaucomys sabrinus*, is the larger of the two species (Wells-Gosling and Heaney 1984) although large adult *G. volans*, may infrequently overlap the lower size range of sub-adult *G. sabrinus* (Dolan and Carter 1977). *Glaucomys sabrinus* is further distinguished by ventral coloration. The ventral guard hairs of *G. sabrinus* are tipped white distally and dark gray proximally, with charcoal gray under hair (Wells-Gosling and Heaney 1984), while the ventral guard hairs of *G. volans* are uniformly cream colored from base to tip, with plumbeous gray under hair occurring only near the legs (Dolan and Carter 1977). *Glaucomys volans* also possesses a flattened, feather shaped tail (Dolan and Carter 1977), while *G. sabrinus* tail is more like a bottle-brush (Wells-Gosling and Heaney 1984).

Hight et al. (1974) described *G. sabrinus* arising from and belonging to a genus distinct from *G. volans* based on studies using serum immunodiffusion. The findings of Thorington et al. (1996) refuted Hight et al. (1974) by employing

multiple trait phylogenetic systematics to re-assert that a single parent genus did diverge into the two extant *Glaucomys* species. Conversely, Arbogast (1999) used cladistic analysis based on a single conservative trait, cytochrome *b* mitochondrial DNA (cyt-*b* mtDNA), to suggest that *G. sabrinus* was the progenitor, in contrast to the fossil evidence (Furlong 1906; Martin 1974; Guilday et al. 1978). Since single trait phylogenies have frequently led to homoplastic error, the evolutionary history of the flying squirrel in North America is still open to debate.

Ecology and current distribution of northern flying squirrels in Virginia

Glaucomys sabrinus exhibits a strong affinity for mature mixed boreal conifer/hardwood forests throughout its range (Wells-Gosling and Heaney 1984; USFWS 1990; Weigl et al. 1992; Carey 1995). This highly specific habitat association has contributed to the natural genetic isolation and vicariant subspeciation of *G. sabrinus*, especially in elevation limited extra-zonal boreal forest islands (Slatkin 1987; Browne et al. 1999; Steele and Powell 1999). As a result of the tendency for subpopulation isolation, there are 25 recognized subspecies of *G. sabrinus* (Hall 1981; Wells-Gosling and Heaney 1984; USFWS 1990).

The habitat of *G. sabrinus* in the middle to southern Appalachian Mountains has been described as the narrow ecotone band between high elevation relict boreal conifer and northern hardwood forests (Payne et al. 1989; Fies and Pagels 1991; USFWS, 1990; Odom, 1995; Reynolds et al. 1999; Menzel 2004, Hackett and Pagels 2003, Ford et al. 2004). During the most

recent glacial maximum, 18,000 ybp, *G. sabrinus* populations were part of a large swath of boreal forest that extended well into the Ozark Plateau preceding the leading edge of the Laurentide ice shield and the great eastern tundra (Davis 1969; Guilday 1970; Kurtén and Anderson 1980; Delcourt and Delcourt 1984; Ehlers and Gibbard 2004). When the climate shifted to post-glacial conditions, deciduous broadleaf forests gained dominance over most of the formerly boreal terrain (Delcourt and Delcourt 1984). Mid-Atlantic boreal forests retreated to higher elevations, above 915 m (3,000 ft), where climate conditions have remained cool and mesic (Pielke 1981; Delcourt and Delcourt 1984).

These high elevation extra-zonal boreal habitat anomalies have become insular refugia for Pleistocene relict communities in the southern Appalachians, including *G. sabrinus* (Steele and Powell 1999; Menzel 2004; Ford et al. 2004). The palynological record provides strong evidence that boreal forests have retreated to even greater degrees of montane insularity during the post-Wisconsinan hypsithermal event 4,000-7,000 ybp (Whitehead 1972). *Glaucomys sabrinus* were restricted to higher elevation, more isolated refugia in the southern Appalachian Mountains at that time than they are at present (Guilday 1970). Periodic isolation, on a geological timescale, appears to be part of the natural history of *G. sabrinus*. Timbering in the early 1900's fragmented naturally occurring remnants of insular boreal habitats (Hassinger 1967; Pielke 1981; White 1984).

The diet of *G. sabrinus* is composed primarily of truffle fungi, lichens, and invertebrates (Weigl 1968; Maser et al. 1985; Rosentreter et al. 1997). Truffle

density has been shown to exhibit a greater influence on *G. sabrinus* distribution than does the availability of cavity nesting sites (Waters and Zabel 1995).

Ransome and Sullivan (1997) found that sub-optimal habitat in truffle-depleted second growth stands required artificial food supplementation to increase northern flying squirrel populations in the spruce-fir forests of British Columbia.

Zabel and Waters (1997) found that *G. sabrinus* in northeastern California preferred truffles of the genera *Gautieria* and *Alpova* to other naturally occurring foods, including other truffle species. *Glaucomys sabrinus* has highly selective dietary requirements (Maser et al. 1985). Truffle abundance could be an important limiting factor at Mount Rogers National Recreation Area, MRNRA (Loeb et al. 2000, Hackett and Pagels, 2003).

Truffles are mycorrhizal fungi that pair in mutualistic relationships with specific tree symbionts (Arora 1986). It may be that the summer flush of *Elaphomyces granulatus*, a truffle commonly eaten by *G. sabrinus* (Weigl et al. 1992; Mitchell 2001), and associated with *Picea rubens* in the southern Appalachians (Loeb et al. 2000), acts as a dietary anchor for insular populations of *G. sabrinus* further restricting gene flow during the mild season when emigration is most likely. Although the food caching habits of *G. sabrinus* are poorly documented (Wells-Gosling and Heaney 1984), we surmise that they very likely do cache food, as this behavior is common to all tree squirrels (Smith and Reichman 1984). Animals that cache food stores are more likely to establish and defend territories and less likely to abandon those territories during optimal foraging seasons (Smith and Reichman 1984).

Therefore, *G. sabrinus* should exhibit high degrees of genetic structuring even in contiguous habitat, but especially in naturally isolated landscapes that have been fragmented. We describe the genetic structure of five isolated populations of the two ESA-listed endangered subspecies of *G. sabrinus* in southern and middle Appalachia plus two outgroup populations from a contiguous forest in the state of Washington. We assessed gene flow and genetic distances in *G. sabrinus* populations across contiguous, isolated, and fragmented habitats. We also assess and compare the relationship between inbreeding and parasite resistance in the two MRNRA populations. We hypothesize that the smaller Whitetop Mountain population will be more inbred and therefore carry a greater parasite burden than the population inhabiting in the larger more contiguous habitat on Mount Rogers. We also speculate on an alternative origin of pathogenicity in the parasitic relationship between *Strongyloides robustus* and *G. sabrinus*. We determine the subspecies standing of the MRNRA population in this study. Our results contribute to the growing body of knowledge regarding inbreeding, habitat fragmentation, and population viability assessment of endangered species.

MATERIALS AND METHODS

Study Area

The primary study area for *G. sabrinus* was located on Mount Rogers (36° 39' 35" N, 81° 32' 41" W) and Whitetop Mountain (36°38'19"N, 81°36'20"W)

in southwest Virginia in the Mount Rogers National Recreation Area (MRNRA). These two peaks are of volcanic origin, formed by rhyolite intrusions during the late Proterozoic Period, 760 mya (Rankin 1993). Mount Rogers is the tallest summit in Virginia at 1,746 m, (5,729 ft). Whitetop Mountain lies 6.4 km (4 mi) to the southeast of Mount Rogers and ranks second at 1,658 m (5,540 ft).

Glaucomys sabrinus habitats at Mount Rogers and Whitetop Mountain were contiguous before heavy timbering separated the two peaks, creating Elk Garden pasture, in the early 1900's (Hassinger 1967). A secondary oak-hickory sere and grazing meadow, 3.2 km (2 mi) wide at the narrowest point, now separates the two peaks (Pyle and Shafale 1985). The remnants of the boreal spruce forest in southwest Virginia have been protected from industrial logging by steep scarps and difficult terrain (Pyle and Shafale 1985). Today there are 400 ha (1.5 mi²) of spruce forest remaining on Mount Rogers (MR) and 150 ha (0.6 mi²) of spruce-fir forest on Whitetop Mountain (WT) (Rheinhardt 1984). The MRNRA population of *G. sabrinus* inhabits portions of the ecotone between mature northern hardwood and mature boreal conifer forest (Reynolds et al. 1999; Hackett and Pagels 2003). The MRNRA population is 37 km (23 mi) from the nearest conspecifics in Long Hope, North Carolina (Weigl et al. 1992).

Picea rubens was found to be the dominant tree in these boreal conifer stands. *Abies fraseri* was found only on Mount Rogers (Rheinhardt 1984). Mature northern hardwood stands at MRNRA are characterized by an overstory of *Betula lutea*, *Acer rubrum*, and *Fagus grandifolia* (Payne et al. 1989). Woody understory composition in the ecotone consists of arctic alpine elements, *Rubus*

canadensis, *Viburnum alnifolium*, *Acer pennsylvanicum*, *Rhododendron maximum*, and *Vaccinium erythrocarpum*. (Rheinhardt and Ware 1984; Stephenson and Adams 1984). The herbaceous layer contains prolific stands of *Clintonia borealis*, *Dryopteris campyloptera*, and *Lycopodium lucidulum* (Rheinhardt 1984; Stephenson and Adams 1984). *Erythronium americanum*, *Anemone quinquefolia*, *Oxalis acetosella*, and other boreal relict wild flowers were also observed during this survey.

Outgroup samples of *G. sabrinus* were collected from 3 subspecies across 7 sample sites in 3 states. Five sample sites, including MRNRA, were from the focus region of southern Appalachia that contains two federally listed endangered subspecies of *G. sabrinus*. Specimens of *G. s. coloratus* were originally collected from 1937 to 1963 on Roan Mountain, Tennessee (RM) (36°19'42"N, 82°06'84"W; n = 5), located 109 km along a fragmented habitat archipelago, from the nearest congeners sampled at Whitetop Mountain, Virginia. Specimens of *G. s. fuscus* originated in West Virginia from Cheat Mountain (CM: 38°44'08"N, 80°00'24"W; n = 11) and Blackwater Falls (BW: 39°00'50"N, 79°41'90"W; n = 5). These two populations are separated by 147 km of severely fragmented habitat. The most geographically distant specimens were derived from museum specimens collected from two populations 125 km apart in nearly pristine contiguous habitat in the state of Washington, Easton (Ea: 47°15'14"N, 121°11'19"W; n = 11) and Keechelus Lake (KL: 47°31'56", 121°10'20"; n = 7).

Sampling procedure

We used live traps and nest boxes to capture 43 *G. sabrinus* in MRNRA from October 2002 to August 2004. An additional 21 hair samples, 10 from Whitetop and 11 from Mount Rogers were provided by the Virginia Department of Game and Inland Fisheries (VDGIF) from an earlier *G. sabrinus* survey at MRNRA (Reynolds et al. 1999). The West Virginia Department of Natural Resources (WVDNR) provided 20 hair samples and 7 fecal samples from *G. s. fuscus*. The National Museum of Natural History (NMNH) provided preserved tissue specimens of *G. s. coloratus* from Tennessee and *G. s. fuliginosus* from Washington State.

Microsatellite DNA analysis

We extracted DNA samples from three sources, tail tissue and hair follicles obtained from live specimens, and tissue from museum specimens. The highest quality DNA came from the small amount of tissue sometimes collected with hair pulled from the tip of a squirrel's tail. Hair follicles and tissue from museum study skins required additional treatment using GenomiPhi™ linear DNA amplification product (Amersham Bioscience Piscataway, New Jersey). DNA was extracted from all three sources using the Puregene™ method (Gentra Biosystems Minneapolis, Minnesota). We diluted the DNA extracted from tail-tip tissue to a 1:10 concentration with 0.25x TAE. GenomiPhi™ processed hair follicles and tissues from museum skins were likewise diluted to 1:1000 and 1:2000 concentrations, respectively. We used four microsatellite loci presumed

to be selectively neutral. Three of the loci were initially developed for the southern flying squirrel, *Glaucomys volans* (Fokidis et al. 2003); SFS 3, SFS 7, and SFS 14. These primers amplified loci at 235-255, 202-272, and 167-179 base pairs (bp), respectively. We developed one additional locus; a tetra-nucleotide repeat designated GSA 9 (upper 5' TTTCCTGTAAGCATGGTGTGACCT-3'; lower 3'-CAGTTTCGAGGACCAACCGC-5'), which generated amplicons in the range of 155-171 bp.

To amplify microsatellites we used 6µl reactions consisting of 1.0 µl DNA template, 1.2 µl H₂O, 0.2 µl of 4 mM spermidine, 0.6 µl of 5 µM primer mix and 3.0 µl JumpStart RediMix™ *Taq* DNA polymerase (Sigma-Aldrich St. Louis, Missouri). We used MJ Research PTC 100 thermal cyclers (MJ Research Waltham, Massachusetts) with 200 µl tubes to perform 'touchdown' Polymerase chain reaction (PCR) with an initial annealing temperature of 60° C, as described by Fokidis et al. (2003).

Amplified products were revealed by electrophoresis on 25-lane 7.5% native polyacrylimide gels. All gels were stained with SYBRGreen™ nucleic acid stain (Molecular Probes Eugene, Oregon) and digitally documented using ultraviolet transillumination. Fluorescent amplified alleles were scored against a 20 bp molecular ruler (BioRad Hercules, California) according to corresponding published lengths and the number of base pair repeats.

Parasite assay

We used a variation of the fecal egg count (FEC) technique to quantify the sciurid-specific intestinal parasite *Strongyloides robustus* in order to estimate immune resistance in *Glaucomys sabrinus* (n = 41), *G. volans* (n = 13), and *Tamiasciurus hudsonicus* (n = 13) from MRNRA with an additional sampling of *G. s. fuscus* (n = 7) from West Virginia. Because parasite fecundity and abundance is inversely related to the strength of the immune response of the host animal (Stear et al. 1997), FEC is assumed to serve as a non-invasive indicator of the overall condition of the major histocompatibility complex, MHC (Coltman et al. 1999; Casinello et al. 2001). The MHC has been identified as one of the primary genomic casualties of inbreeding (Yukhi and O'Brien 1990; Hedrick and Parker 1998; Hedrick et al. 2000). Fecal pellets were collected directly from live squirrels or bedding during live trapping and nest box checks. Pellets were placed in a dry vial and inspected on the day of collection since freshly expelled *S. robustus* eggs can hatch in as little as 3 h at 25°C (77°F) (Eckerlin 1974; Wetzel and Weigl 1994). Fecal pellets from West Virginia were shipped overnight on ice based on Wetzel and Weigl (1994) who found that a temperature of 10°C (50°F) effectively suppressed the hatching of *S. robustus* eggs for 6-7 days. To estimate the level of *S. robustus* infestation we used a variation of the McMaster FEC method derived from Stoll (1930). Fecal pellets were macerated in 30 ml of Feca Med™ (Vedco Inc. St. Joseph, Missouri) sodium nitrate solution in a 100 ml beaker. This mixture was poured through a kitchen grade strainer to remove macroscopic flotsam and then through a small

funnel into a 40 ml test tube. Sodium nitrate solution was added to raise the meniscus above the top of the test tube and an 18x18 mm glass cover slip was placed over the meniscus and left untouched for 30-45 minutes to allow the parasite eggs to adhere to the cover slip. The cover slip was then placed on a standard microscope slide and all *S. robustus* eggs were counted using a standard sweeping S pattern movement of the slide across the 400x lens of a compound light microscope set with a calibrated reticule. All parasite eggs were identified to genus using the reference manual "Veterinary Clinical Parasitology" (Sloss et al. 1994). A voucher drawing was made of each parasite species encountered. Mean parasite egg counts were compared using the independent samples t-test in SPSS™ version 10 (SPSS Chicago, Illinois). Length and diameter of the fecal pellets were measured and cylindrical volume was derived using the formula; length x circular area (πr^2). The level of infestation was calculated by dividing egg count by total fecal volume.

Statistical methods

We estimated gene frequency, Hardy-Weinberg equilibrium, genic differentiation, allele linkage, probability of fragmentation, and rate of effective migration with GENEPOP version 3.1 software (Raymond and Rousset 1995). Allele frequency, based on expected numbers of homozygotes and heterozygotes, was calculated using Levene's correction to adjust for small sample size (Levene 1949). Adherence to the Hardy-Weinberg equilibrium was tested with exact *P*-values based on a Markov chain method analysis of

heterozygote deficiency for single loci in each population. Genic differentiation across population pairs was tested using a Markov chain analysis. Potential linkage of alleles was tested with a genotypic disequilibrium probability test based on the Markov chain method and P -values ($\alpha = 0.05$) were determined using Fisher's exact test. We confirmed negative results on Hardy-Weinberg estimates, allele heterogeneity, linkage disequilibrium, and genic differentiation with sequential Bonferroni corrections for Type I errors (Rice 1989). All Markov chain parameters were set to 1,000 dememorizations, 100 batches and 1,000 iterations per batch, all bootstrap and jackknife values were set to 1,000.

We calculated Wright's inbreeding coefficient F according to Ayres and Balding (1998). F – statistics, based on homozygous allele fixation, were described with both GENEPOP and FSTAT (Goudet 1994). Mean population fragmentation was extrapolated from pairwise comparisons of the subpopulation fixation index F_{ST} generated by GENEPOP (Raymond and Rousset 1995). Pairwise rates of effective migration ($N_e m$) were calculated using the standard relationship ($N_e m = (1/F_{ST}) - 1) / 4$) to estimate the number of immigrants per generation. We also calculated gene flow with the rare allele method (Barton and Slatkin 1986).

AMOVA analysis, based on Φ_{ST} , was performed according to Excoffier et al. (1992).

We used the MICROSAT program (Minch 1997) to define Nei's genetic distance (D_S) which was in turn used to construct a majority rule strict consensus tree using PHYLIP (Felsenstein 1995). Mantel tests (Mantel 1967) of the

correlation between genetic dissimilarity and geographic distance and between genetic dissimilarity and parasite load were conducted according to the matrix correspondence algorithm of Smouse et al. (1986).

RESULTS

Within and among genetic analyses

We successfully genotyped 100 out of 107 individual *G. sabrinus*, plus 17 *G. volans*, with up to 4 loci (mean: 3.5 typed loci per sample). All loci were polymorphic as follows; SFS-3 had 4 alleles; SFS-7 had 12 alleles, SF-14 had 11 alleles, and GSA-9 had 6 alleles distributed among populations (Å) as shown in Table 1. The mean number of alleles across all loci per population (mean Å) ranged from 2.25 at RM to 5 at WT (Table 1). The overall average number of alleles manifested per individual animal sampled was 4. Hardy-Weinberg Equilibrium was consistently violated across each locus, even after the application of the Bonferroni adjustment ($P < 0.013$, Table 1). Hardy-Weinberg expectations were also violated across each population, except for BW and RM (Bonferroni $P < 0.05$, Table 1).

A chromosome linkage disequilibrium seemed evident between SFS 3 and GSA 9 ($\chi^2 = 23.79$, $P = 0.048$). This linkage disequilibrium appeared most pronounced within the WT population ($P = 0.002$). The linkage disequilibrium was no longer significant after sequential Bonferroni correction, both across locus pairs ($P < 0.008$) and within populations ($P < 0.001$). Since the linkage disequilibrium was barely significant across all populations and manifested only

in a single population, it is likely that it was merely a sampling artifact. Therefore there was no violation of the required assumptions underlying genetic drift and there was no impact on the statistical basis of our study.

Allele frequencies were heterogeneous across all samples, except for WT vs. CM ($\chi^2 = 19.74$, calculated $P = 0.011$, Bonferroni $P < 0.006$). The mean estimate for overall allele fixation ($F_{ST} = 0.090$) was significantly less than zero ($P < 0.001$) indicating a moderate intraspecific genetic differentiation among the *G. sabrinus* populations we sampled. AMOVA analysis of Φ_{ST} indicated a high degree of genetic differentiation among populations ($P > 0.001$), most likely reflecting the three distinct subspecies in our survey. We considered single locus $F_{ST} > 0.150$ to represent significant differentiation among populations (Frankham et al. 2002). Mean F_{ST} among southern Appalachian demes was 0.107. The populations with the greatest mean rate of allelic fixation were MR and RM ($F_{ST} = 0.188$, Table 2). The populations with the least mean rate of allelic fixation were MR vs. WT and EA vs. KL ($F_{ST} = 0.023$ and 0.027, respectively). Pairwise estimates of allele fixation between populations are listed in Table 2.

Gene flow across all populations was $N_e m = 2.048$ (rare alleles) or $N_e m = 3.255$ (F_{ST}), moderate to high. Pairwise comparisons of gene flow $N_e m$ (Table 2) based on F_{ST} were greatest between MR and WT ($N_e m = 10.388$) and least between MR and RM ($N_e m = 1.075$). Although MR is closer to RM than CM (115 km vs. 402 km), it has a greater gene flow with more distant CM ($N_e m = 6.127$). CM has less gene flow with neighboring BW (148 km, $N_e m = 2.144$) than it does

with MR. Gene flow between contiguous EA and KL (125 km, $N_e m = 8.841$) was greater than gene flow between fragmented CM and BW over similar distance.

Subspecies identity

Estimated Nei's genetic distance (D_S) between population pairs ranged from $0.023 \pm (0.022)$ between MR and WT to $1.319 \pm (1.625)$ between RM and CM (Table 3). The large standard errors most likely are an artifact of the small sample size and possibly large standard deviation values from populations outside of the primary study site. Mantel correlation of genetic isolation by geographic distance was inconclusive ($r^2 < 0.01$, $P = 0.616$). Figure 2 presents a neighbor joining (UPGMA) consensus tree based on D_S . RM appears to fall outside of the southern Appalachian sub-group bootstrapped to 61.4% confidence interval, suggesting that *G. s. coloratus* may be more closely related to the Washington state subspecies, *G. s. fuliginosus* than the more proximate West Virginia subspecies, *G. s. fuscus*. The population of *G. sabrinus* residing at MRNRA also seems to be distinct from the West Virginia subspecies bootstrapped to a 51.9% confidence interval. Strong bootstrap confirmation is generally considered to exceed 70% certainty (Hillis and Bull 1993).

Handley (1953) used tail lengths to distinguish *G. s. coloratus* (135 mm) from *G. s. fuscus* (115 mm). Weigl et al. (1992) found mean tail length of *G. s. coloratus* to be 129 mm. Reynolds et al. (1999) reported MRNRA *G. sabrinus* tail lengths to be 128 mm. We found mean tail lengths of MRNRA *G. sabrinus* to be 135 mm ($n = 36$). It therefore seems likely, given the differing origins suggested

by the morphological and genetic data, that the population at MRNRA is an intergrade between the two subspecies, *G. s. fuscus* and *G. s. coloratus*.

Inbreeding and intensity of parasite infection

Mean inbreeding across all of the *G. sabrinus* populations sampled was $F = 0.103$ (Figure 3). The highest level of inbreeding was detected within the *G. s. coloratus* population at RM ($F = 0.152$), indicating that half-sib mating was commonplace. The lowest level of inbreeding was detected at MR ($F = 0.022$), suggesting a near absence of consanguineous mating. An ANOVA test revealed that the coefficient of inbreeding F for each population differed significantly from the overall mean coefficient of inbreeding F ($P < 0.001$). Least squares difference post hoc analysis revealed that the coefficient of inbreeding failed to differ between CM, an insular population in a fragmented landscape, and EA, a contiguous population within a forested landscape ($P = 0.336$).

A significant correlation was observed between genetic dissimilarity and parasite load ($r^2 = -0.22$, $Z = 4726$, $P = 0.007$). We found that the *G. sabrinus* on Whitetop Mountain had a significantly greater estimated inbreeding (Figure 4) than did the nearby Mount Rogers population (median $F = 0.085$ and 0.016 , respectively; $P < 0.001$, Figure 4). The more inbred Whitetop Mountain population carried a larger mean FEC ($0.562 \text{ eggs/mm}^2 \pm 0.205$) than did the Mount Rogers population ($0.228 \text{ eggs/mm}^2 \pm 0.071$), although the difference was not statistically significant ($P = 0.278$, Figure 5).

Strongyloides robustus eggs were absent in all four fecal samples obtained from BW, West Virginia ($F = 0.057$), a population that also was significantly less inbred than WT ($P < 0.001$). Of the two fecal samples that were collected at CM ($F = 0.144$), one had no FEC and the other 0.037 eggs/mm². Sample sizes from individual West Virginia populations were not sufficient to determine mean FEC. There was no difference in the level of *S. robustus* infestation among three sympatric arboreal sciurid species that we encountered in the boreal conifer/northern hardwood ecotone, Figure 6 ($P = 0.579$). A Mantel test of matrix correspondence revealed that there was a correlation between pairwise genetic distance and FEC ($P = 0.006$, $\rho = 0.270$).

DISCUSSION

Allelic diversity

Reduction of allelic diversity in isolated wild populations has been well established (Soulé 1972; Lacy 1987; Stangel et al. 1992; Coltman et al. 1999). Maritime insular populations of *G. sabrinus* in the Alexander Archipelago exhibited reduced genetic variation when compared to mainland Alaska populations (Bidlack and Cook 2001). Similar effects have been described for *Peromyscus maniculatus* in the lacustrine islands of Lake Michigan (Meagher 1999) and *P. leucopus* on the barrier islands of the Delmarva Peninsula (Loxterman et al. 1998). We observed reduced heterozygosity in only two of the *G. sabrinus* populations that we studied.

Two populations of *G. sabrinus* exhibited greater mean observed heterozygosity than expected, one in the Pacific Northwest and one at our study

site. Both had very high to panmictic gene flow with one or more neighboring populations. The Northwestern population was located in an undisturbed contiguous forest in Easton, Washington where greater heterozygosity is to be expected because of its lack of insularity (Frankham 1998). The other, Mount Rogers, is part of an island that was separated from Whitetop Mountain within the last century. Genetic diversity can, initially, appear higher in fragmented populations if population fragments experience genetic drift in a uniquely different directions (Leberg 1992), seemingly increasing overall diversity across the formerly contiguous range (Frankham et al. 2002). A secondary behavioral factor may also come into play under fragmented conditions that could result in increased population levels of heterozygosity; *G. sabrinus* may increase movements as a response to proximate fragmentation. For example root voles, *Microtus oeconomus*, traveled corridor-connected habitats more frequently as fragmentation increases (Andreassen et al. 1998). Such an effect would explain the increased heterozygosity within the fragmented Mount Rogers populations due to increased levels of gene flow, while the lack of corridors and smaller habitat area contribute to increased isolation and decreased heterozygosity in the Whitetop Mountain fragment (Mills and Smouse 1994). Since a subspecies is a phenotypically, and presumably genotypically distinct vicariant, intergradation of subspecies necessarily increases allelic diversity (Frankham et al. 2002).

Higher levels of heterozygosity may also be promoted by a combination of behavioral avoidance of inbreeding (Howard 1960, May 1979, Johnson and Gaines 1990) and balancing selection (Aguilar et al. 2004). Hoogland (1982)

observed inbreeding avoidance through juvenile dispersal and parent avoidance of fertile offspring in familial coterie of the prairie dog, *Cynomys ludovicianus*. Ground squirrels, *Spermophilus columbianus*, avoid inbreeding by similar means (Weddell 1991). High gene flow results from the state of Washington populations (Table 2) suggest that continuity between *G. sabrinus* populations is improved if suitable habitat corridors exist.

Comparative reductions in heterozygosity that we detected in isolated *G. sabrinus* populations may be associated with genetic bottlenecks. Jaarola and Teglestrom (1996) identified genetic bottlenecks in post-glacial remnant populations of the field vole, *Microtus agrestis*. A similar natural history can be ascribed to the majority of southern Appalachian *G. sabrinus* populations, where boreal forests were greatly fragmented by numerous glacial advances and retreats (Guilday 1970; Whitehead 1972; Webb and Oppdyke 1995). Federov (1999) described differing bottleneck rates corresponding to varying postglacial climate shifts and the ecological plasticity of two genera of Arctic lemmings, *Dicrostonyx* and *Lemmus*. *Glaucomys sabrinus* is similarly susceptible to the reduction of allelic heterozygosity through bottlenecks because of the species high degree of niche specialization (Zabel and Waters 1997; Loeb et al. 2000; Hackett and Pagels 2003).

Allelic diversity within populations can be restored by genetic mutation and drift in subsequent generations (Wright 1931). According to Lacy (1987), heterozygosity can be restored to a bottlenecked population in 150 generations with a minimum of 100 individuals. In *G. sabrinus*, with a generation time of 2-3

years (Wells-Gosling and Heaney 1984, Villa et al. 1999), allelic restoration could occur in as little as 300 years, providing that sufficient habitat is available. Our analysis supports the recommendation of re-establishing habitat contiguity (Lande 1988, Frankham 1995, Hess 1996) of the extra-zonal boreal forests where they have been fragmented in southern Appalachia. Establishment of habitat corridors would increase long-term genetic health of associated boreal fauna including *G. sabrinus*.

Genetic differentiation

Population fixation (F_{ST}) is the probability of the fixation of homozygous alleles at given loci due to inbreeding within subpopulations (Wright 1931; Frankham et al. 2002). Isolated populations that are below idealized population levels, fragmented, or of singular origin will have elevated F_{ST} due to inbreeding (Wright 1931; Weir and Cockerham 1984; Frankham et al. 2002). Beaumont and Nichols (1996) suggested a minimum sample size of $n = 50$ individuals and $n = 20$ loci for ideal assessment of F_{ST} . We were limited by the rare nature of our subject species (Tear et al. 1995).

With our limited samples we found mean genetic differentiation among the southern Appalachian populations of *G. sabrinus* to be around 11% ($F_{ST} = 0.107$). This is comparable to the level of genetic differentiation recorded among high elevation boreal relict populations of southern Appalachian red-backed voles ($F_{ST} = 0.100$, Reese et al. 2001) and among disjunct prairie dog populations in New Mexico ($F_{ST} = 0.103$, Chesser 1983). By way of comparative illustration, a

similar level of moderate genetic structuring can be found among the three aboriginal human populations in Pacific Meganesia ($F_{ST} = 0.105$, Nasidze et al. 2001).

Significant genetic differentiation was evident among the *G. sabrinus* populations we sampled. Genetic differentiation between proximate populations of rodents has been well documented (Patton and Yang 1977; Chesser 1983; Faulkes et al. 1997). Spruce timber harvesting, the major anthropogenic impact affecting *G. sabrinus* distribution, is only a century old (Pielke 1981; White 1984) or approximately 50 generations in the life history of the northern flying squirrel (Wells-Gosling and Heaney 1984, Villa et al. 1999). We expect that genetic differentiation will increase over time given the prevalence of deforestation (Whitlock 1992; Frankham 1998). Indeed, elevation restricted extra-zonal boreal habitats in southern Appalachia will become further isolated with increased global warming (Delcourt and Delcourt 1984) and may, in fact, be extirpated (Delcourt and Delcourt 1998). The pending climate change also lends urgency to corrective action.

Overall gene flow was moderate to high for the southern Appalachian *G. sabrinus* populations. Gene flow between the two Washington state populations was very high (Table 2). This was expected since both population samples were collected in contiguous boreal forests of the northern Cascade Mountains from 1897 – 1901 (Nelson 1969). There was high gene flow between the populations at Blackwater, West Virginia and Cheat Mountain, West Virginia, but it was considerably less than the gene flow between the two Washington state

populations that were nearly the same distance apart (Table 3). We attributed this difference to the fragmented nature of the West Virginia habitats (Menzel 2004). Gene flow was unimpeded, panmictic ($N_e m > 10$), between the two primary study sites, Mount Rogers and Whitetop Mountain, Virginia (Table 2). These two sites are essentially a single potentially isolated *G. sabrinus* population that has been decimated and fragmented within the past century (Hassinger 1967; Pielke 1981; Rheinhardt 1984). *Glaucomys sabrinus* on Whitetop Mountain are gradually differentiating from those on Mount Rogers, as evidenced by the significantly increased inbreeding on Whitetop Mountain (Figure 4).

Population structure and historical biogeography

Gene flow and genetic distance must be interpreted in the context of biogeography and the geologic time scale (Slatkin 1987). High historical gene flow was indicated between MRNRA *G. sabrinus* populations and distant *G. s. fuscus* populations at Cheat Mountain, West Virginia, 402 km away. Gene flow across fragmented habitat is not unusual (MacArthur and Wilson 1963; Slatkin 1987; Lande 1988). However, gene flow between the MRNRA populations of *G. s. coloratus* and those at Roan Mountain, Tennessee, only 116 km away was considered only moderate to low (Table 2). The MRNRA populations are more closely related to Tennessee *G. s. coloratus* and Washington state *G. s. fuliginosus* than they are to Cheat Mountain, West Virginia, *G. s. fuscus* based on Nei's genetic distance matrix (Table 3), although, standard error is considerable

for most pairwise Nei's genetic distance values. The cladogram based on bootstrap re-sampling of Nei's D_S reiterates the gene flow analysis, placing *G. s. coloratus* and *G. s. fuliginosus* together and lumping MRNRA populations with *G. s. fuscus* (Figure 2). These results support the identification of *G. sabrinus* from MRNRA as an intergrade between the two southern Appalachian subspecies.

There are few historical records regarding the location of boreal conifer forests before timbering at the turn of the last century (Pielke 1981; Rheinhardt 1984; White 1984). Information regarding recent boreal habitat corridors can only be inferred. Pielke (1981) estimated the current potential range of *Picea rubens* to be limited to elevations above 915 m (3,000 ft). *Glaucomys s. coloratus* inhabited boreal forests that were once part of a contiguous stand that stretched from the northern Unaka - Blue Ridge Mountains across the 732,952 ha (2,830 mi²) French Broad River Basin (mean elevation 716 m (2350')), to the southern Unaka - Great Smoky Mountains during the Wisconsinan period (Whitehead 1972; Delcourt and Delcourt 1984). Mount Rogers, in the northern Unaka range has been separated from Roan Mountain, in the southern Unaka range since the post glacial retreat gave way to mixed mesophytic forests in the lower elevation of French Broad River Basin prior to the hypsithermal event, 7,000 ybp (Whitehead 1972). This provides a likely explanation of how *G. s. coloratus* became stranded on Mount Rogers and Whitetop Mountain in the northernmost part of the volcanic Unaka range. Sipe and Browne (2004) used the French Broad River Basin as an elevation boundary to define

phylogeography of boreal conifer associated shrews in the Great Smoky Mountains.

The *G. s. coloratus* specimens we sampled were more closely related to the *G. s. fuliginosus* from the Cascades than they were to *G. s. fuscus* from Cheat Mountain, West Virginia in the Alleghenies, based on Nei's genetic distance and gene flow (Table 3). Although boot strapped values applied to the consensus tree and gene flow analysis suggest otherwise, paleogeography offers support. West Virginia *G. s. fuscus* inhabit boreal forests that did not exist during the most recent glacial maximum 18,000 ybp (Guilday 1970; Whitehead 1972; Delcourt and Delcourt 1984). During this time *G. s. coloratus* was likely safely ensconced in a boreal refugia that extended south to the Georgia piedmont, but gave way to tundra north of the Unaka Range (Whitehead 1972; Delcourt and Delcourt 1984). Similarly the boreal forests of the Cascade Range also remained intact (Ehlers and Gibbard 2004). *Glaucomys s. coloratus* are true relicts originating from the super boreal forest that existed prior to the Wisconsinan glacial advance (Whitehead 1972; Delcourt and Delcourt 1984; Ehlers and Gibbard 2004). *Glaucomys s. fuscus* most likely belongs to the boreal community that reforested the Allegheny tundra during the Wisconsinan retreat. This would explain the genetic similarity of distant Unaka and Cascade Range populations, and also points to the origin of dissimilarity between more proximate subspecies. Thus, our analysis provides additional detail that complements the mitochondrial DNA phylogeography for *G. sabrinus* as proposed by Arbogast (1999).

Despite the great difference in Nei's genetic distance, populations of Cheat Mountain and MRNRA *G. sabrinus* had high levels of gene flow and were closely related according to the bootstrapped consensus analysis (C.I. < 70%). This high gene flow likely contributed to the large standard error in Nei's genetic distance and may also be indicative of a population reunion caused by the Laurentide glacial retreat.

The northern Unaka range is separated from the southern Allegheny Mountains by the 977,961 ha (3,776 mi²) Holston - New River Basin, mean elevation 610 m (2,000'). Given the proximity to southern Appalachia and the direction of the Laurentide ice shield retreat (Dyke and Prest 1987; Ehlers and Gibbard 2004) it is likely that post-glacial *G. s. fuscus* populations from Cheat Mountain, West Virginia enjoyed habitat connectivity with Mount Rogers and Whitetop Mountain *G. s. coloratus* for some time after the other *G. s. coloratus* populations in the southern Unaka range had been disjoined from Mount Rogers and Whitetop mountain by the French Broad River basin.

Historical biogeography often reveals the underlying causes of population relationships that are described with indirect molecular genetics techniques (Slatkin 1987). For example, although the MRNRA population is currently described as *G. s. fuscus* for legislative purposes, our analyses indicate that the *G. sabrinus* population at MRNRA is an intergrade based on paleobiogeography and the molecular genetic data we have compiled. A number of other investigators have suggested on the bases of various other data types that the population is an intergrade. C. O. Handley Jr. was the first to propose that the

MRNRA population was a subspecific intergrade between *G. s. coloratus* and *G. s. fuscus* based on subtle differences in coloration and caudal metrics (Fies and Pagels 1991). Weigl et al. (1992) urged that the MRNRA population be re-evaluated and grouped with *G. s. coloratus*. Our results support Handley's contention of intergradation, however we suggest that *G. sabrinus* populations in MRNRA should be managed as *G. s. coloratus* given their closer geographic proximity and greater genetic proximity based on Nei's genetic distance.

Inbreeding

The mean level of inbreeding for *G. sabrinus* ($F = 0.103$) was seven times greater than mean inbreeding described among prairie dogs, *Cynomys ludovicianus*, at Wind Cave National Park, South Dakota ($F = 0.014$, Dobson et al. 1997) and four times less than inbreeding within naked mole rat, *Heterocephalus glaber*, colonies in Southeast Kenya ($F = 0.450$, Reeve et al. 1990). Ralls et al. (1988) found that members of the Order Rodentia exhibited high levels of inbreeding tolerance compared to members of other mammalian orders she surveyed that are commonly maintained in captivity and pedigreed. Given the paleohistorical expansions and contractions of the boreal forest, especially in the southern Appalachians (Guilday 1970; Whitehead 1972; Delcourt and Delcourt 1984; Ehlers and Gibbard 2004), *G. sabrinus* may be naturally adapted to tolerate levels of inbreeding similar to other rodent species (Ralls et al. 1988, Reeve et al. 1990, Dobson et al. 1997).

Cheat Mountain, West Virginia and Easton, Washington populations of *G. sabrinus* exhibited similar elevated levels of inbreeding, although each is located in a vastly different habitat context. The specimens from Easton were collected circa 1900, when the northern Cascade boreal forests were largely intact (Nelson 1969). Conversely, the Cheat Mountain population has existed for a century in an anthropogenically-fragmented habitat (Pielke 1981, White 1984). Suitable contiguous habitat corridors should facilitate juvenile dispersal, inbreeding avoidance and genetic heterogeneity (Gilpin and Soulé 1986; Mills and Smouse 1994), we would expect the more contiguous habitat to be less inbred.

Given that inbreeding levels are roughly equal between the squirrels of Easton and Cheat Mountain it may be that limitations based on caching behavior (Smith and Reichman 1984) and strong habitat preference (Wells-Gosling and Heaney 1984; Maser et al. 1985; Ransome and Sullivan 1997; Zabel and Waters 1997; Reynolds et al. 1999, Mitchell 2001) can restrict *G. sabrinus* dispersal as effectively as habitat fragmentation. Washington state squirrels may tolerate inbreeding in favor of retaining proximity to rare hardwood/spruce ecotone site-specific resources. *Glaucomys sabrinus* appears to have a population structure adapted to some degree of inbreeding tolerance.

Although inbreeding may improve inclusive fitness and altruistic behavior in a population (Dawkins 1976; Wilson 1976; May 1979), it can only be advantageous if the fitness cost is low (May 1979; Ralls et al. 1988; Frankham 1995). It is generally accepted that although some species tolerate inbreeding within populations, some minimal level of outbreeding must exist or that

population will ultimately suffer reduced fitness and risk extinction (Lacy 1987; Yukhi and O'Brien 1990; Frankham 1995, Meagher et al. 2000). Even low numbers of migrants can keep inbreeding levels below the theoretical threshold, provided that the immigrants are sufficiently genetically differentiated from the host population (Lacy 1987; Frankham 1995; Frankham et al. 2002). We certainly detected evidence of low levels of outbreeding in MR ($F < 0$, Figure 4). Fossorial rodents such as naked mole rats, *Heterocephalus glaber* (Jarvis et al. 1994; Faulkes et al. 1997; Clarke and Faulkes 1999), and the pocket gopher, *Thomomys bottae* (Patton and Yang 1977) provide excellent examples of inbreeding tolerance and low gene flow in rodent species that are restricted by ecological constraints. While *G. sabrinus* demes within and among populations may function in an analogous fashion, in both the montane archipelago habitat of the southern Appalachians and the contiguous forests of the northern Cascades, inbreeding levels do not naturally approach those of the fossorial rodents.

Pathogenicity of *Strongyloides robustus*

Strongyloides robustus is a ubiquitous parasite of Sciuridae (Parker 1971; Eckerlin 1974) and has been documented in *G. sabrinus* as far north as Wisconsin (Pauli et al. 2004) and in *G. volans* as far south as Florida (Eckerlin 1974). Its known distribution suggests a much larger range, but there are few studies that have been focused on delineating the ranges of sciurid parasites. Wetzel and Weigl (1994) found that low temperature limited reproduction of *S. robustus* in vitro and proposed that this might have some mollifying effect on its

distribution within wild *G. sabrinus* populations at higher elevations in the Southern Appalachians. The discovery of *S. robustus* in *G. sabrinus* from Wisconsin (Pauli et al. 2004) would seem to counter this contention. Mean annual temperature in the Southern Superior Uplands of Wisconsin is 10°C cooler than it is in the Blue Ridge Mountains (Bailey et al. 1994).

Parker (1971) was the first to document *S. robustus* in gray squirrels, *Sciurus carolinensis*, of Southwestern, Virginia. Lichtenfels and Haley (1969) reported *S. robustus* in *Tamiasciurus hudsonicus* in the Allegheny Mountains of Maryland. We found *S. robustus* in 88% of the wild caught *G. sabrinus* from MRNRA. Pagels et al. (1990) report *S. robustus* from a *G. sabrinus* carcass found at MRNRA. Although Weigl et al. (1992) did not find *S. robustus* in wild caught *G. sabrinus* of various subspecific origins between 1968 and 1988, our findings suggest that *S. robustus* is more prevalent than previously perceived.

Recently *S. robustus* has been scrutinized as one of the primary pathogenic afflictions affecting *G. sabrinus* (Weigl 1968; Wells-Gosling and Heaney 1980; Pagels et al. 1990; Weigl et al. 1992; Wetzel and Weigl 1994; Pauli et al. 2004). Weigl (1968) documented *S. robustus* induced mortalities in captive *G. sabrinus* from several different locations within the United States. This observation gave rise to the hypothesis that *S. robustus* is an agent of competitive exclusion vectored by *G. volans* (Weigl 1968; Weigl et al. 1992; Wetzel and Weigl 1994; Pauli et al. 2004). The proposed mechanism for pathogenicity is that *S. robustus* is a novel parasite in naïve host populations of

G. sabrinus (Weigl 1968; Weigl et al. 1992; Wetzel and Weigl 1994; Pauli et al. 2004).

For parasite mediated resource competition to occur, one host species must be separated from the other long enough for its parasite to evolve qualities that are virulent to the competitor yet remain innocuous to the original host (Price et al. 1988; Klein and O'hUigin 1994). The fact that sciurid assemblage in the eastern boreal forest has been intact since the Wisconsinan, 18,000 ybp (Guilday 1970; Kurtén and Anderson 1980), and that we found similar mean levels of *S. robustus* eggs in all three sciurid species that are sympatric in the boreal ecotone at MRNRA; *G. sabrinus*, *G. volans*, and *Tamiasciurus hudsonicus* ($P = 0.579$).

The distribution, interspecies ubiquity, and evident climate tolerance of *S. robustus* in situ, belie the necessary conditions for the naïve host hypothesis. The origin of pathogenicity in the *G. sabrinus* / *S. robustus* symbiosis is perhaps more likely to be found in the breaching of inbreeding thresholds (Mills and Smouse 1994; Klein and O'hUigin 1994; Frankham 1995; Spielman et al. 2004) caused by recent deforestation and fragmentation (Pielke 1981; White 1984) of naturally isolated insular populations (Guilday 1970; Delcourt and Delcourt 1984; Weigl et al. 1992).

Inbreeding, parasites and population health

Insular populations in a managed landscape are subject to genetic drift and cumulative degradation of heterozygosity through inbreeding (Lacy 1987, Ralls et al. 1988). Mills and Smouse (1994) identified inbreeding as a critical

component to demic collapse in stochastic environments. Frankham (1995) noted that inbreeding might have little apparent effect in any population until a threshold accumulation of deleterious alleles is crossed. At that point the population may then begin to collapse with little warning. Parasite loading is considered to be a good preliminary metric for assessing immune resistance in the major histocompatibility complex (MHC) of populations that are susceptible to inbreeding (Coltman et al. 1999; Cassinello et al. 2001).

The MHC is a region on the vertebrate genome that encodes immune response to foreign antigens (Hill 1998) and is sensitive to inbreeding (Yukhi and O'Brien 1990; Klein and O'hUigin 1994). Hedrick et al. (2000) documented the degradation of the MHC with corresponding disease outbreaks in the genetically bottlenecked Arabian oryx, *Oryx leucoryx*. O'Brien et al. (1985) found that reduced MHC resistance among inbred cheetah, *Acionyx jubatus*, was revealed by their failure to reject extra-familial skin grafts. Cassinello et al. (2001) correlated high parasite levels with low individual heterozygosity in Cuvier's gazelle, *Gazella cuvieri*. Coltman et al. (1999) found greater parasite loads and higher mortality in island bound Soay sheep, *Ovis aries*. Meagher (1999) noted an increase in the prevalence of the nematode parasite, *Capillaria hepatica*, in wild populations of deer mice, *Peromyscus maniculatus*, with decreased allozyme heterozygosity.

In our study we found that Mount Rogers, the larger sized of two fragmented habitats we studied (400 ha vs. 150 ha), had significantly less inbreeding and a lower mean count of *S. robustus* eggs per volume of feces.

Although the difference in absolute parasite count was not statistically significant, our observation of a highly significant relationship between genetic constitution and parasite load corresponds to similar relationships documenting elevated parasitism in inbred populations of free-living sheep (Coltman et al. 1999), captive gazelles (Cassinello et al. 2001) and deer mice (Meagher 1999).

Coltman et al. (1999) has promulgated the idea that mortalities due to MHC reduction maybe be one way of improving heterozygosity and disease resistance in wild populations. This genetic pruning effect might contribute to the relatively high levels of allelic diversity that we found in insular *G. sabrinus* populations. Further study is warranted regarding the distribution of *S. robustus* and its pathogenicity at various inbreeding thresholds. *Strongyloides robustus* infestation will increase with greater habitat fragmentation and inbreeding severity. Because parasite levels measured by FEC are a reliable, inexpensive and non-invasive way to assess population health (Coltman et al. 1999; Meagher 1999; Cassinello et al. 2001), we believe FEC should be employed in future assessments of *G. sabrinus* throughout its range.

Management Recommendations

The significant relationship between genetic variability and parasite load in *G. sabrinus* at MRNRA, and the elevated levels of inbreeding and *S. robustus* in FEC at the smaller WT site have management implications. Contiguous habitat corridors should improve juvenile dispersal and decrease inbreeding (Gilpin and Soulé 1986; Mills and Smouse 1994) at the more impacted WT site. For this

reason, our recommendation for management is to increase *G. sabrinus* habitat connectivity at MRNRA.

Although the MRNRA *G. sabrinus* population is the most geographically isolated one in the Appalachian range, we did not find the reduced genetic diversity predicted by Browne et al. (1999). We determined that the unanticipated increased heterozygosity for the MRNRA population of *G. sabrinus* is an artifact associated with the intergradation of *G. s. coloratus* and *G. s. fuscus*. Mating between genetically differentiated populations is known to increase allelic diversity (Lacy 1987; Frankham 1995; Frankham et al. 2002). The Virginia MRNRA *G. sabrinus* population is a unique genetic resource that may function as a reservoir for future conservation of the phenotypically identical *G. s. coloratus* populations. We recommend that the endangered status be retained for the MRNRA *G. sabrinus* because it is a population of special origin and importance.

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TABLE 1. Sample sizes, number of alleles, observed and expected heterozygosities and P values for Hardy Weinberg Equilibrium in 7 populations of 3 subspecies of *G. sabrinus* and 1 population of *G. volans*.

Locus	Site Subsp.	MRNRA <i>G. vol.</i>	MR <i>G.s. int.</i>	WT <i>G.s. int.</i>	RM <i>G.s. col.</i>	CM <i>G.s. fus.</i>	BW <i>G.s. fus.</i>	KL <i>G.s. ful.</i>	EA <i>G.s. ful.</i>
<i>HWE</i>									
SFS 3	0.082								
	n	14	26	34	6	11	6	6	9
	Å	4	3	5	3	3	3	3	2
	Ho	0.57	0.61	0.67	0.66	0.54	1.00	0.33	0.33
	He	0.69	0.46	0.67	0.50	0.43	0.66	0.59	0.42
	HWE	0.234	0.169	0.004	1.000	1.000	0.198	0.144	1.000
SFS 7	<0.001								
	n	16	18	33	6	12	6	8	12
	Å	4	4	4	2	4	4	2	3
	Ho	0.31	0.16	0.09	0.00	0.16	0.33	0.00	0.16
	He	0.52	0.38	0.44	0.54	0.54	0.69	0.53	0.52
	HWE	0.093	0.003	<0.001	0.021	0.002	0.082	0.005	0.005
SFS 14	<0.001								
	n	14	26	34	6	12	6	8	12
	Å	7	5	5	2	4	6	2	2
	Ho	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00
	He	0.70	0.66	0.68	0.54	0.60	0.84	0.53	0.52
	HWE	<0.001	<0.001	<0.001	0.092	<0.001	0.110	0.026	0.002
GSA 9	<0.001								
	n	12	23	31	6	11	4	3	5
	Å	4	5	6	2	4	4	3	4
	Ho	0.41	0.78	0.58	0.33	0.27	0.50	0.66	1
	He	0.68	0.71	0.72	0.54	0.49	0.64	0.73	0.73
	HWE	0.252	<0.001	<0.001	0.480	0.035	0.422	1.000	0.739
Overall									
	Mean n	14	23.25	33	6	11.5	5.5	6.25	9.5
	Mean Å	4.75	4.25	5	2.25	3.75	4.25	2.5	2.75
	Mean Ho	0.57	0.67	0.57	0.50	0.50	0.72	0.48	0.58
	Mean He	0.64	0.56	0.63	0.53	0.52	0.72	0.57	0.52
	HWE	<0.001	<0.001	<0.001	0.083	<0.001	0.072	0.006	0.002

TABLE 2. Pairwise estimates of the rate of homozygous allele fixation, F_{ST} (upper matrix) and effective migration, $N_e m$ (lower matrix) based on allele size for each population pair across all 4 microsatellite loci of 3 *G. sabrinus* subspecies including mean level of inbreeding (F) table and ANOVA results.

Site	MR	WT	RM	CM	BW	KL	EA	
Subsp.	<i>G.s. int.</i>	<i>G.s. int.</i>	<i>G.s. col.</i>	<i>G.s. fus</i>	<i>G.s. fus</i>	<i>G.s. ful.</i>	<i>G.s. ful.</i>	Mean F
MR		0.023	0.188	0.039	0.131	0.074	0.073	0.022
WT	10.388		0.118	0.056	0.079	0.076	0.048	0.088
RM	1.075	1.865		0.168	0.172	0.103	0.103	0.152
CM	6.127	4.143	1.234		0.104	0.112	0.068	0.144
BW	1.658	2.898	1.199	2.144		0.149	0.155	0.057
KL	3.132	3.026	2.174	1.98	1.418		0.027	0.117
Ea	3.169	4.958	2.170	3.404	1.358	8.841		0.141

AMOVA: $P < 0.001$

TABLE 3. Pairwise estimates of Nei's genetic distance, D_S (with standard errors) based on allele size and frequency for each population pair of *G. sabrinus* subspecies. Geographic proximity is given in kilometers (lower matrix).

Site Subsp.	MR <i>G.s. int.</i>	WT <i>G.s. int.</i>	RM <i>G.s. col.</i>	CM <i>G.s. fus.</i>	BW <i>G.s. fus.</i>	KL <i>G.s. ful.</i>	EA <i>G.s. ful.</i>
MR		0.023 (0.022)	0.384 (0.097)	0.724 (1.683)	0.240 (0.218)	0.089 (0.101)	0.102 (0.104)
WT	6.4		0.233 (0.086)	0.713 (1.682)	0.222 (0.199)	0.150 (0.100)	0.078 (0.058)
RM	115.8	109.4		1.319 (1.652)	0.546 (0.373)	0.430 (0.086)	0.460 (0.234)
CM	402.2	395.8	485.9		0.860 (1.677)	0.788 (1.696)	0.675 (1.705)
BW	511.6	505.2	603.3	148.0		0.454 (0.302)	0.368 (0.356)
KL	4308.9	4305.6	4267.0	4255.8	4173.7		0.027 (0.148)
EA	4334.6	4331.4	4294.4	4320.1	4238.1	125.5	

Figure Legends

FIG. 1. Geographic range of *G. sabrinus* with the locations of the 7 sample sites. Abbreviations are MR: Mount Rogers, VA (n = 26), WT: Whitetop Mountain, VA (n = 35), RM: Roan Mountain, TN (n = 5), CM: Cheat Mountain, WV (n = 11), BW: Blackwater Falls, WV (n = 5), Ea: Easton, WA (n= 11), KL: Keechelus Lake (n = 7).

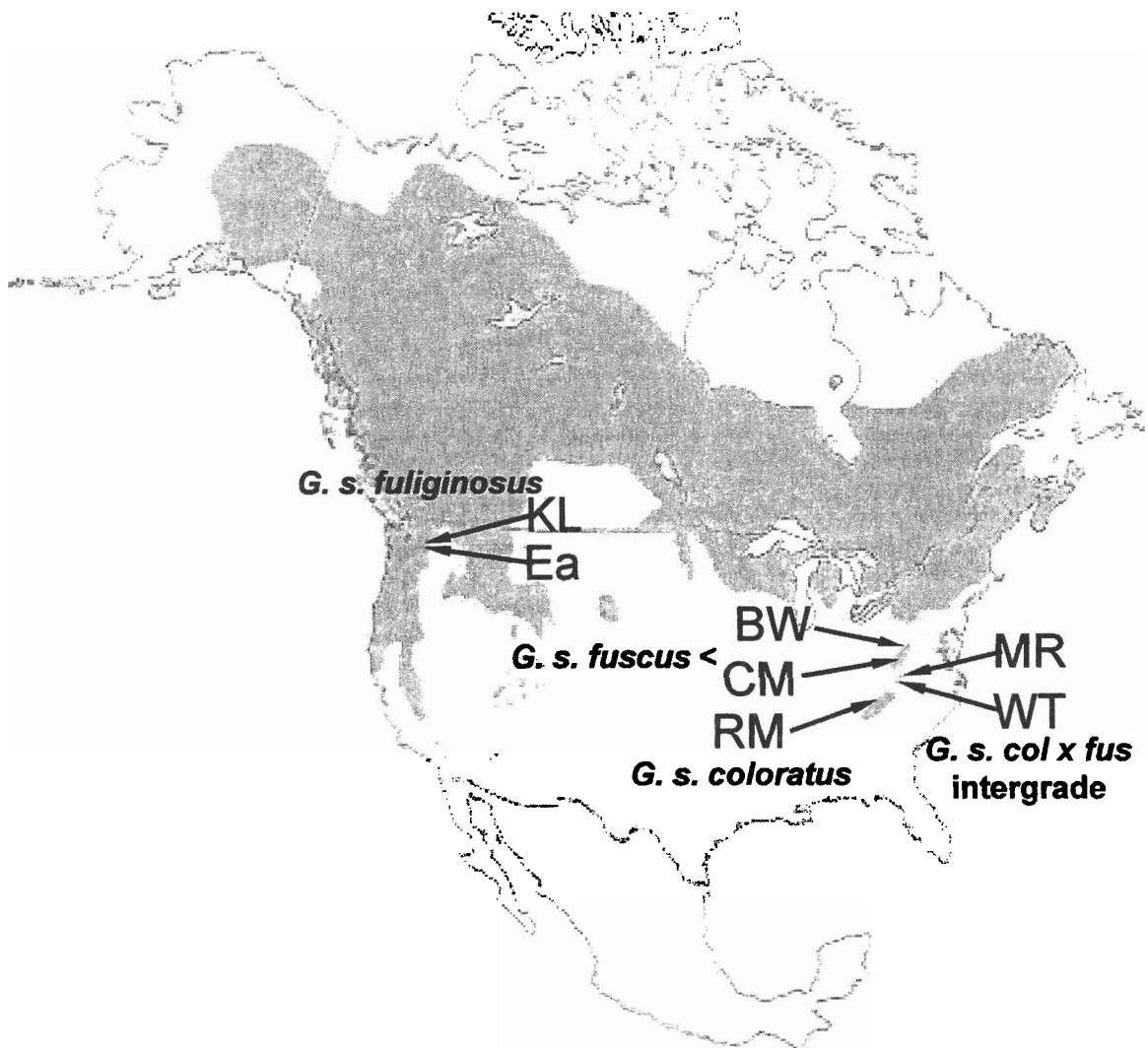
FIG. 2. Consensus tree based on Nei's genetic distances (D_S) among seven *G. sabrinus* populations. Bootstrap values at the nodes indicate the number of unambiguous branches at that point out of 1000 re-sampling events indicating the percent accuracy of the group consisting of the subspecies that are to the right of that fork. Subspecies designations are noted to the right.

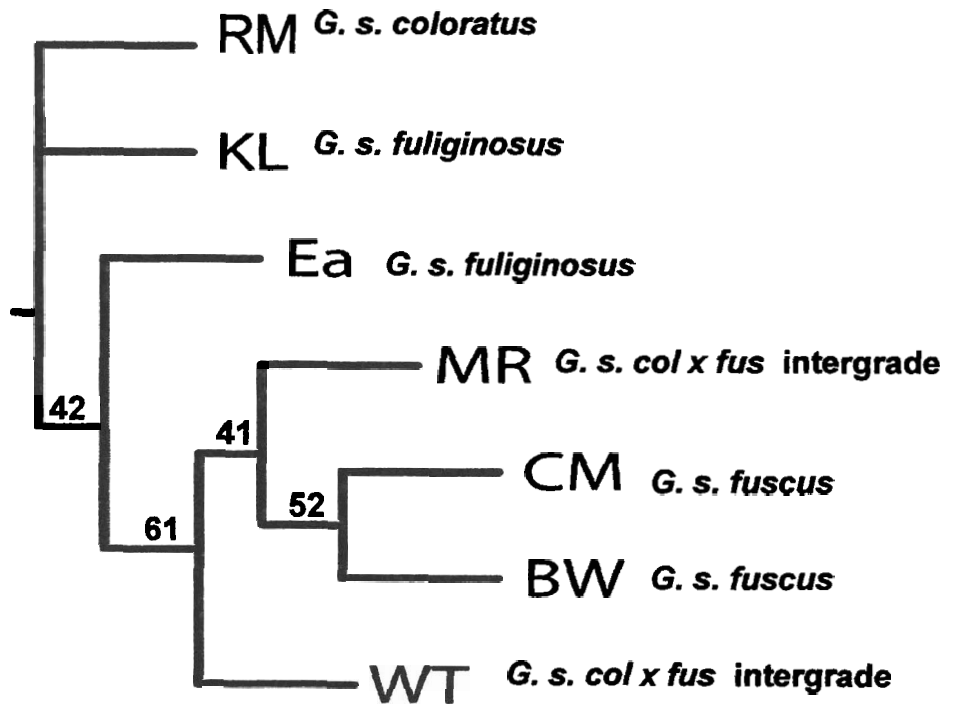
FIG. 3. ANOVA comparison of mean level of inbreeding (F) among five populations of *G. sabrinus* in the southern Appalachian region and two from Washington State ($P < 0.001$).

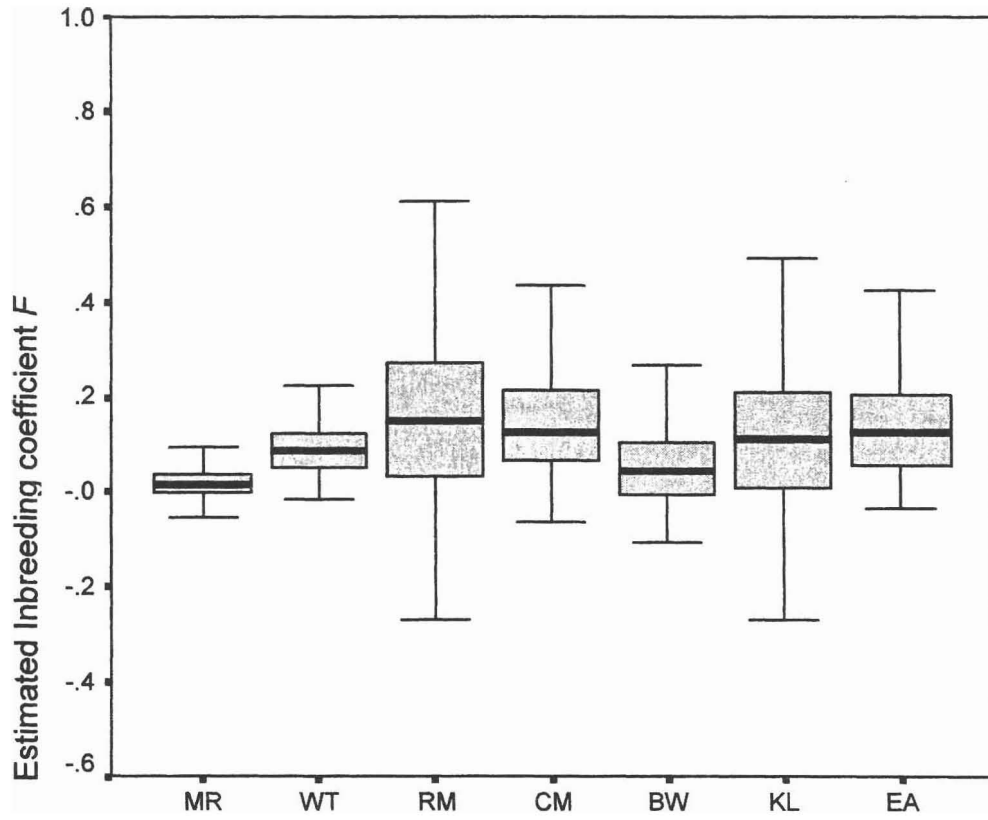
FIG. 4. Frequencies of estimated inbreeding coefficients (using 4 microsatellite loci) for *G. sabrinus* populations on Mount Rogers and Whitetop Mountain ($P < 0.001$).

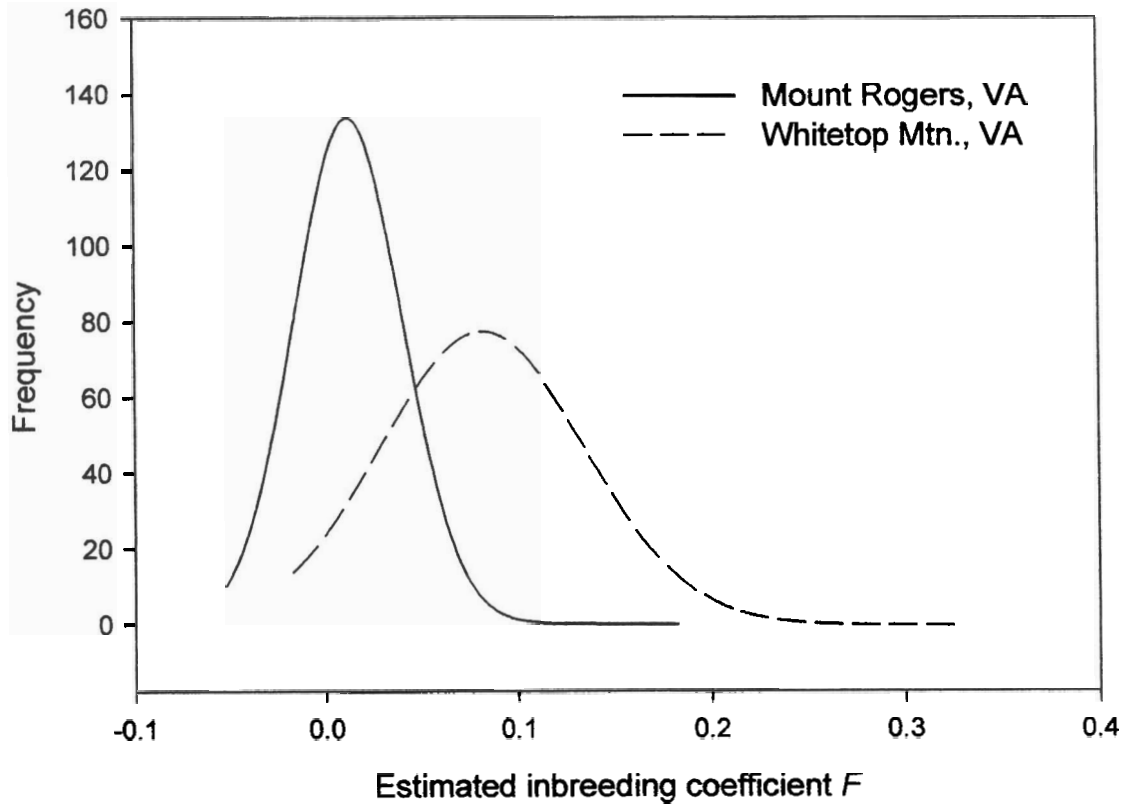
FIG. 5. Independent samples t-test comparison of mean *Strongyloides robustus* eggs per volume of feces in *G. sabrinus* populations from Mount Rogers (n=11) and Whitetop Mountain (n=23) ($P = 0.278$).

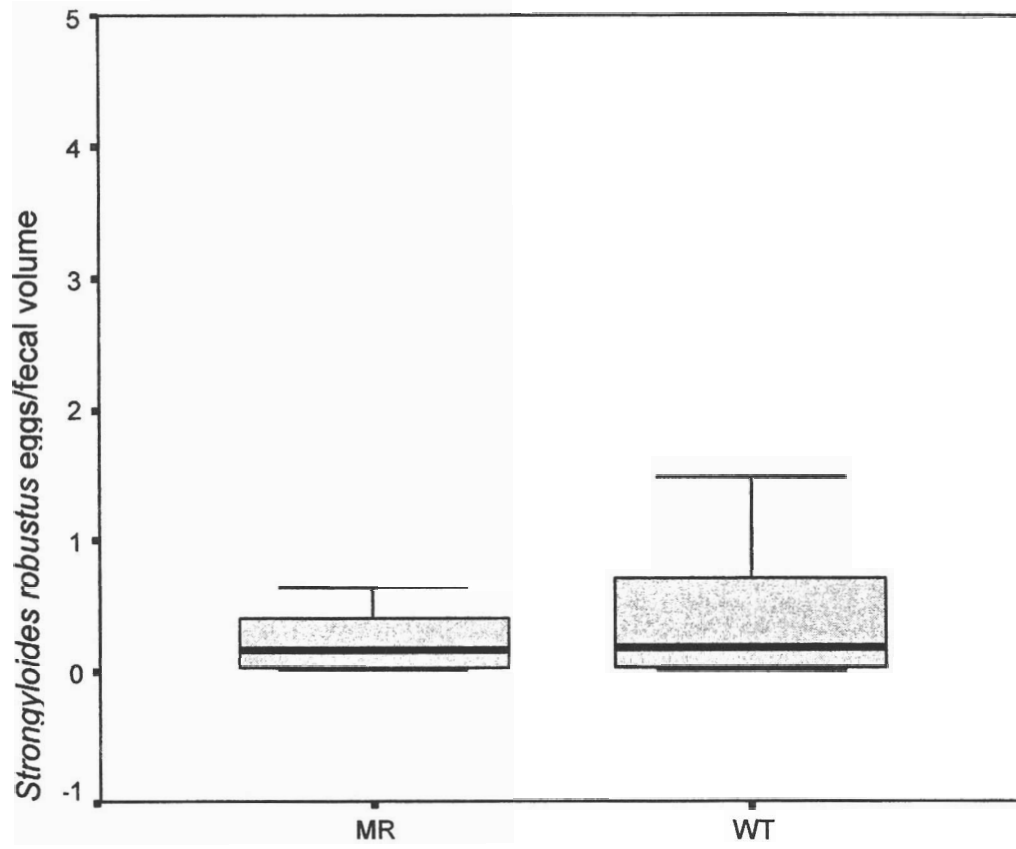
FIG. 6. Comparison of mean *S. robustus* eggs per volume of feces among three species of sympatric arboreal sciurids from MRNRA. *G. sabrinus* (n = 41), *G. volans* (n = 13) and *T. hudsonicus* (n = 13) populations ($P = 0.579$).

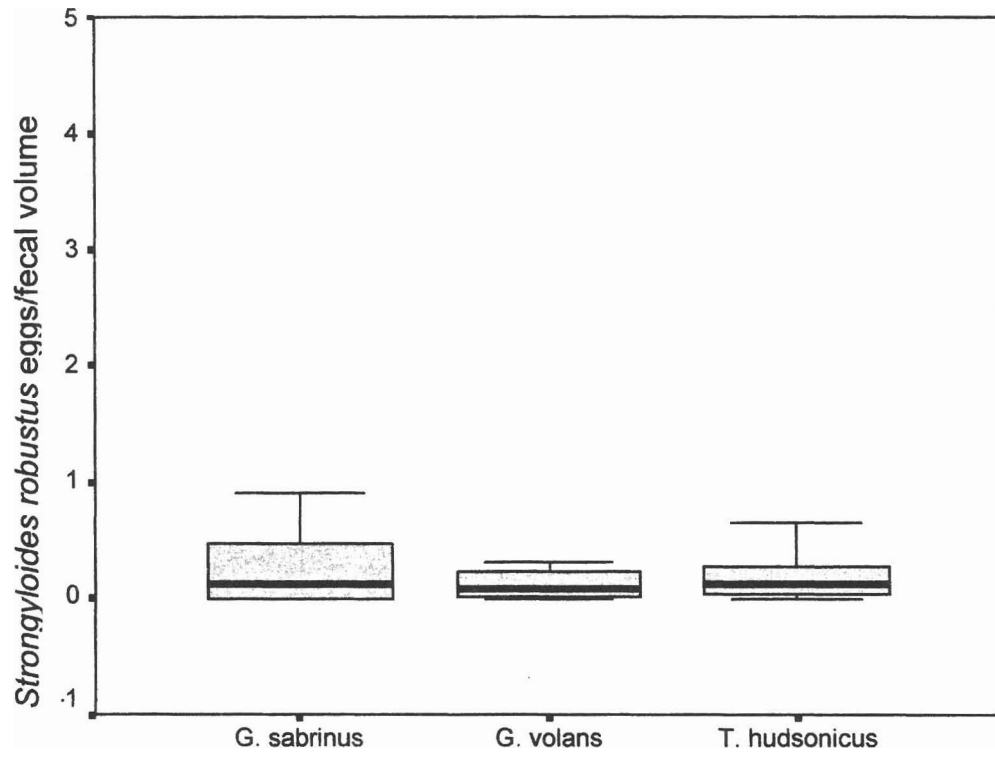












Boreal tree squirrels

Observation of an externally situated southern flying squirrel (*Glaucomys volans*) nest depredated by a rat snake (*Elaphe obsoleta*) in southwest Virginia.

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Accounts of externally situated southern flying squirrel (*Glaucomys volans*) nests are rare (Snyder 1921, Sollberger 1943). Sollberger (1943) found only three outside nests occupied by *G. volans* over six years of surveying in Ohio and Pennsylvania. Only one of these external nests was used for rearing young, whereas 35 nursery nests were found in tree cavities (Sollberger 1943). Pearson (1954) reported second hand the only other direct observation of southern flying squirrel depredation by a snake.

We witnessed a black rat snake (*Elaphe obsoleta*) dislodge a southern flying squirrel (*Glaucomys volans*) nest from an eastern hemlock tree (*Tsuga canadensis*) on 14 August 2003. The event occurred between 1700 and 1800 hr in Mount Rogers National Recreation Area, Grayson County, along Fox Creek near Fairwood Cemetery, elevation 970 m. The snake, which we later estimated to be 150 cm in total length, was wrapped around the nest and fell to the bare ground of the hemlock grove with an audible thud. It constricted the nest for nearly 15 minutes before it began eating an adult squirrel. We observed the event for 30 min and the snake regurgitated the squirrel and fled when we inadvertently disturbed it while attempting a photograph.

Uhler et al. (1939) surveyed the stomach contents of 18 species of snakes in the Virginia piedmont and found *G. volans* in *E. obsoleta*, the timber rattlesnake (*Crotalus horridus*), and the black racer (*Coluber constrictor*). More recently, Mitchell (1994) also documented *G. volans* in the diet of *E. obsoleta*. The propensity of *E. obsoleta* to climb trees for the purpose of securing prey is well documented (Uhler et al. 1939, Mitchell 1994, Neal et al. 1993, Saenz et al. 1999).

The adult squirrel killed by the snake was the mother of three hairless nestlings that we found in the nest. All three young were males, and two weighed 7.6g and the other 8.0g. All had sealed eyes, and we estimated their age to be less than 10 days based on mass and pelage state (Booth 1946, Linzey & Linzey 1979). It is likely that this was the female's second litter of that year; biannual parturition in the southern flying squirrel has long been recognized (Dolan & Carter 1977, Sollberger 1943). The largest neonate survived to adulthood and is currently being used as a live exhibit in a flying squirrel public education program sponsored by Virginia Commonwealth University, Mount Rogers National Recreation Area, and the Virginia Department of Conservation and Recreation.

The flying squirrel's nest was woven from shredded hemlock bark and appears to have been externally situated, as the surface was free of debris. Although at first irregularly shaped because of the constriction, the nest was easily reshaped to a ball-like form with a slight vase-like neck aperture. A schematized drawing of the reshaped nest is provided in Figure 2. The outer

layer of the nest was 18 cm in diameter and approximately 2 cm thick. The outer layer was constructed of coarsely shredded bark in lengths of 30 to 50 cm and was 0.5 to 1.0 cm wide. The interior chamber comprised of finely shredded bark formed a cup-shaped depression 7 cm in diameter. The inner layer was also about 2 cm thick. The finely shredded bark was 10 to 15 cm long and 0.05 to 0.2 cm wide; it was situated nearly opposite the opening and concealed the three young squirrels. The bedding was slightly damp and had the odor of urine.

Snyder (1921) describes a similar nest composed of red cedar bark (*Juniperus virginiana*) that contained a single juvenile *G. volans* in Ontario, Canada. Figure 2 illustrates the approximate position and scale of the nest materials from Grayson County with regard to a single neonate. The base of the nest was flattened and compressed. The base of the nest appears to have been the only contact point with the tree.

Acknowledgements:

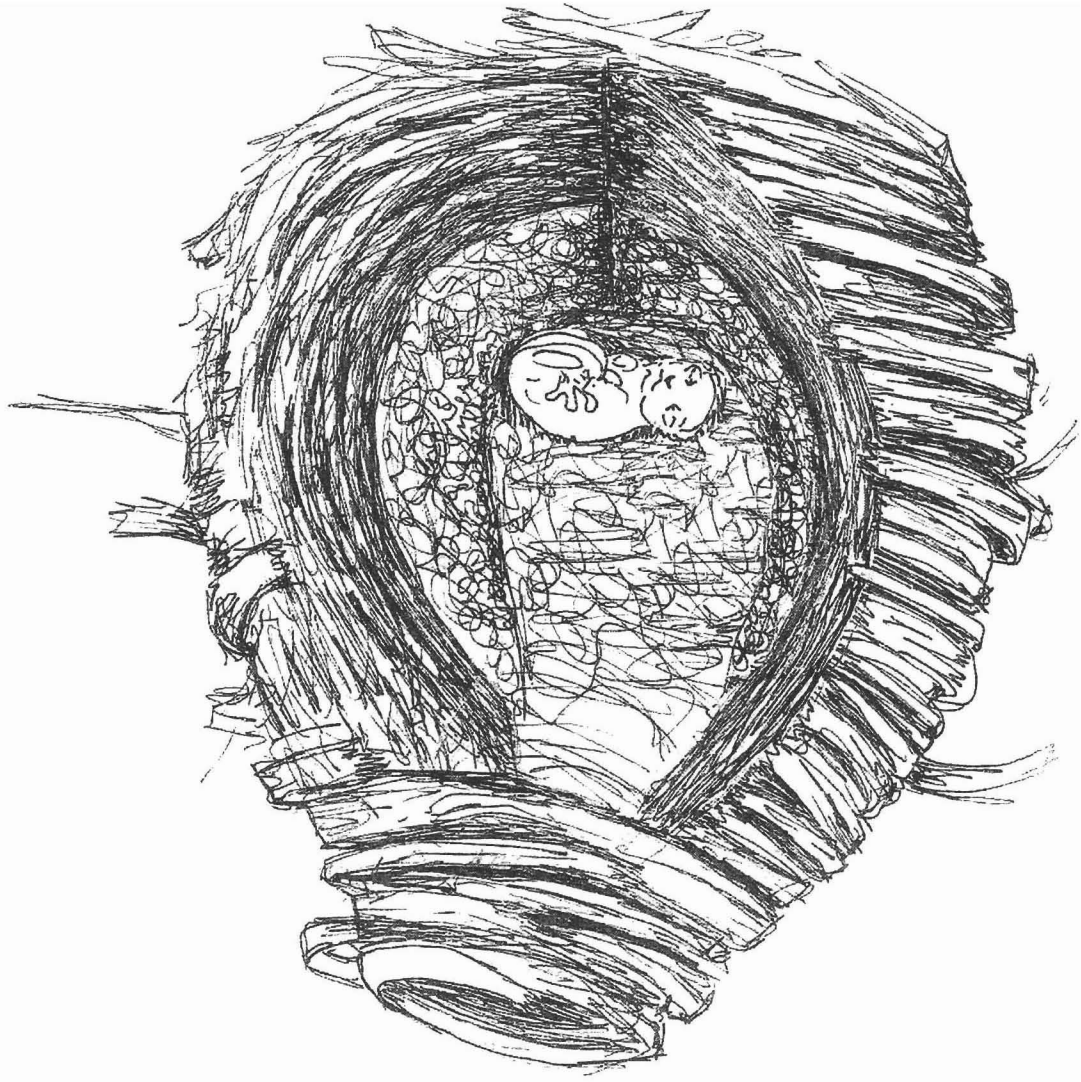
This observation made in a study on the endangered northern flying squirrel, *Glaucomys sabrinus*, was supported in part by funds to Pagels from the USDA-USFS, Mount Rogers National Recreation Area, the Virginia Department of Game and Inland Fisheries Non-Game Program, and a grant from the Small Grant Program of the Virginia Academy of Science to Sparks and Pagels. René Cabaniss raised the squirrel, and provided records regarding the initial health of all three neonates. Anne Wright developed the curriculum around Virginia's flying squirrels that serves as the basis for the public education program. Charles Blem commented on an earlier draft of the manuscript.

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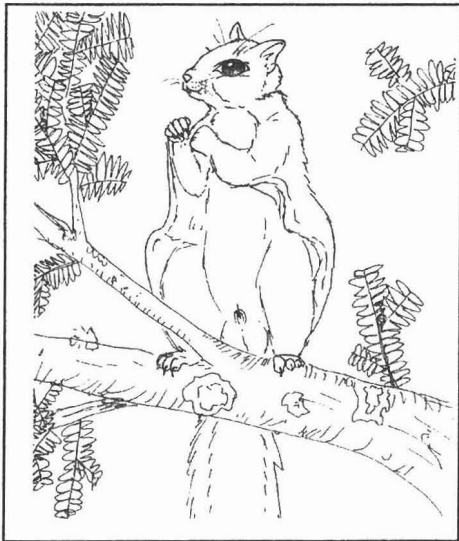
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Appendix I

Fig. 1. Schematized drawing of the nest of a southern flying squirrel (*Glaucomys volans*) depredated by a black rat snake (*Elaphe obsoleta*) in Grayson County, Virginia (drawing by Lynne Hassel).



Sparky, the Endangered Carolina northern flying squirrel from Mount Rogers National Recreation Area



in...

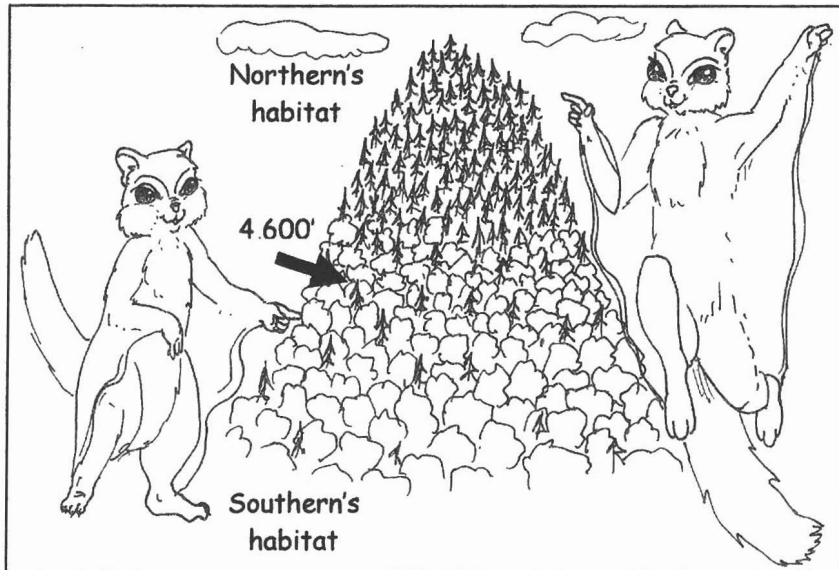
"Canadian Journey"

The northern flying squirrel in Virginia is a rare cousin of the common southern flying squirrel. Both creatures are active only at night and also share some other traits, like the ability to glide through the air using a webbed membrane called patagium. They both eat insects, mushrooms and sometimes carrion.

Northern flying squirrels are physically and ecologically distinct. They are larger than southern flying squirrels by about 1.5 ounces. Northern flying squirrels have a distinct charcoal coloration that underlies white hair on the belly, with a cinnamon coat on top. Southern flying squirrels have belly hairs that are cream colored all the way to the base.

The northern flying squirrel in Virginia is special because they are only found in rare spruce forests above

4,600ft elevation. These high elevation islands of habitat are relicts from the last glacial advances 20,000 years ago. The best examples of this habitat today are found in northern latitudes such as Canada, Alaska and Maine. There is only one place in Virginia where the Carolina northern flying squirrel can be found, and that is right here in Mount Rogers National Recreation Area!



Now, northern flying squirrels like Sparky have a special diet that ties them to these spruce islands. They are able to

find tasty mushrooms that grow under the soil associated with the roots of spruce trees. Sparky loves to eat truffles!



When Sparky gets finished eating these delectable woodland treats he spreads them all over the forest. This prepares the soil with fresh truffle spores that

help the young spruce grow strong and healthy. The relationship between a tree and a fungus is called a mycorrhizal symbiosis and it is very important because the fungus helps digest minerals and nutrients for the tree. Without Sparky and his, umm... dispersal mechanism, the spruce forests would take much longer to regenerate.

Now this all begs the question: how did Sparky and a little piece of Canada wind up in the Virginia Blue Ridge? Well the best way to understand this is to follow Sparky's family history.

Now a long, long, long time ago, nearly 2 million years, Sparky's great, great, great, great, great, great grand pappy left the tall spruce forests of Russia and made the brave journey across the Bering straits into Alaska. Spruce forests at that time stretched all the way around

the north Pacific and deep into North America.

Of course the sea level was lower on account of major ice shields being so big at the time. It was a pretty good place for northern flying squirrels at that time, but the pioneer spirit called. The climate was a lot cooler then and spruce forests reached all the way down into the Mid-Atlantic States along the Appalachian Mountains. Sparky's ancestors traveled all the way across Canada and down through the spruce into the Carolinas.

After they had arrived and settled in the cold glaciers began to retreat about 20,000 years ago. The spruce forests began to climb to the coolness of higher elevations. Before they knew it, Sparky's family had been isolated from their nearest kinfolk in West Virginia. Then, the Carolina northern

flying squirrel started to change a little.



The most noticeable thing was that their tails started getting longer than just about any other sub-species of northern flying squirrel. Sparky's family had become their own unique sub-species. For this reason, and also because of the rarity of their spruce habitat in a southern

climate zone, the Carolina northern flying squirrel was granted protection under the endangered species act in 1985.

If you have seen a flying squirrel in Virginia chances are that it was a southern. Since both squirrels are nocturnal, most people will never see either. The important ecological role of the northern flying squirrel is best witnessed by the healthy regeneration of a spruce grove.



Brought to you by: VCU Mammal Lab, USFS, VA Academy of Science, VDGIF, VA DCR Grayson Highlands State Park and the Explorer's Club Washington Group.

Illustrations by: Lyn Hassel

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Vita

James Lincoln Sparks Jr. was born on December 19, 1970, in Baltimore, Maryland. In 1989 he graduated West Morris Mendham Public High School in Mendham, New Jersey. He graduated from Antioch College in Yellow Springs, Ohio in 1993, receiving the degree of Bachelor of Arts in Environmental Science with a Biology concentration. From 1993 to 1998 he worked as an itinerant field biologist with an interest in endangered species and predator ecology. He worked as a wildlife technician with the Virginia Department of Game and Inland Fisheries Fur Bearer Project before entering VCU in 2002. While attending VCU James has won awards at the Virginia Academy of Sciences and the Virginia Chapter of the Wildlife Society 2004 annual meetings presenting research regarding inbreeding and parasite susceptibility in the Mount Rogers National Recreation Area (MRNRA) populations of the endangered northern flying squirrel *Glaucomys sabrinus*. He also executed a public relations effort to raise endangered species awareness in local communities surrounding MRNRA. He was a teaching assistant for introductory biology laboratory courses and for the technical writing intensive ecology laboratory.