Animal Conservation (2005) **8**, 123–133 © 2005 The Zoological Society of London. Printed in the United Kingdom doi:10.1017/S1367943004001830

Conservation genetics of endangered flying squirrels (*Glaucomys*) from the Appalachian mountains of eastern North America

Brian S. Arbogast[1]*, Robert A. Browne[2], Peter D. Weigl[2] and G. J. Kenagy[3]

[1] Department of Biological Sciences, Humboldt State University, Arcata, CA 95521, USA
[2] Department of Biology, Wake Forest University, Winston-Salem, NC 27109, USA
[3] Burke Museum and Department of Biology, University of Washington, Seattle, WA 98195, USA

(Received 28 January 2004; accepted 25 August 2004)

Abstract

We assessed the genetic status of two endangered subspecies of the northern flying squirrel (*Glaucomys sabrinus*) that are restricted to isolated stands of high elevation spruce-fir and adjacent spruce-fir-hardwood ecotonal habitat in the Appalachian Mountains of eastern North America. We used mitochondrial DNA (mtDNA) and allozyme data to estimate levels of genetic variability in the two subspecies of interest and then evaluated this information in the context of large-scale phylogeographical structure and overall genetic variability for the entire species and for the closely related and partially sympatric southern flying squirrel (*Glaucomys volans*). This broader analysis involves much of North America's northern coniferous forest biome, together with the deciduous forest biome of eastern North America. Our results support the evolutionary distinctness of the endangered Appalachian populations of *G. sabrinus*. These populations possess several private alleles and have levels of genetic variability that are substantially lower than those observed in conspecific populations found elsewhere. However, the endangered Appalachian populations of *G. sabrinus* have higher levels of genetic variability than those observed in populations of *G. volans* from across eastern North America. These results highlight the utility of evaluating the conservation genetics of small and isolated populations within a broad-scale comparative evolutionary and biogeographical framework.

The high altitude spruce-fir forest of the southern Appalachian Mountains is one of the rarest and most threatened habitats in North America (White *et al*., 1993; Wear & Greiss, 2002). This forest type is dominated by red spruce (*Picea rubens*) and Fraser fir (*Abies fraseri*) and occurs as a series of high-elevation (>1300 m) habitat islands in the Appalachians. These islands are characterised by high levels of endemism and support many boreal-adapted species found nowhere else in the southeastern part of the continent. A number of factors have contributed to the extreme decline of the Appalachian spruce-fir forest over the past century. These include timber harvests, fires and grazing of the resulting grasslands, as well as the invasion of the balsam woolly adelgid (Homomptera: *Adelgis piceae*), which has eliminated 95% of the Fraser fir from the southern Appalachians (Wear & Greiss, 2002). In many areas, ongoing acid rain and heavy metal deposition may be further inhibiting forest regeneration and contaminating the understory (Bruck, Robarge & McDaniel, 1989). As a result, the spruce-fir islands of the Appalachians are considered to represent the most extreme case of ecological decline in southeastern North America (Wear & Greiss, 2002).

*All correspondence to: B. S. Arbogast. Tel: 707 826 4180; Fax: 707 826 3201; E-mail: bsa2@humboldt.edu

The naturally small and fragmented nature of the Appalachian spruce-fir forest, combined with a high degree of habitat destruction, has generated concern over the long-term viability of populations of many plants and animals associated with this forest-type (e.g. Harp, 1992; Wear & Greiss, 2002). This is especially true for taxa that have important ecological roles, are characterised by relatively low dispersal rates and already have small population sizes. The northern flying squirrel, *Glaucomys sabrinus*, fits all of the above criteria (Weigl, Knowles & Boynton,1999). This small, nocturnal gliding mammal is an important dispersal agent for mycorrhizal fungi as well as an important prey item, especially for owls (*Strix* spp.: Wells-Gosling & Heaney, 1984). Although this species is broadly distributed throughout the coniferous forests of northern North America, two subspecies, *G. s. fuscus* and *G. s. coloratus*, are geographically isolated and endemic to the high-elevation spruce-fir and adjacent spruce-fir-hardwood ecotonal areas of the southern Appalachians (Fig. 1). Both subspecies are currently listed as endangered; populations of *G. s. coloratus* from the extreme southeastern edge of the species range in North Carolina and Tennessee (which are the smallest and most fragmented) are considered to be particularly vulnerable (Weigl *et al*., 1999). The small population sizes and insular distributions of these two subspecies make reduced levels

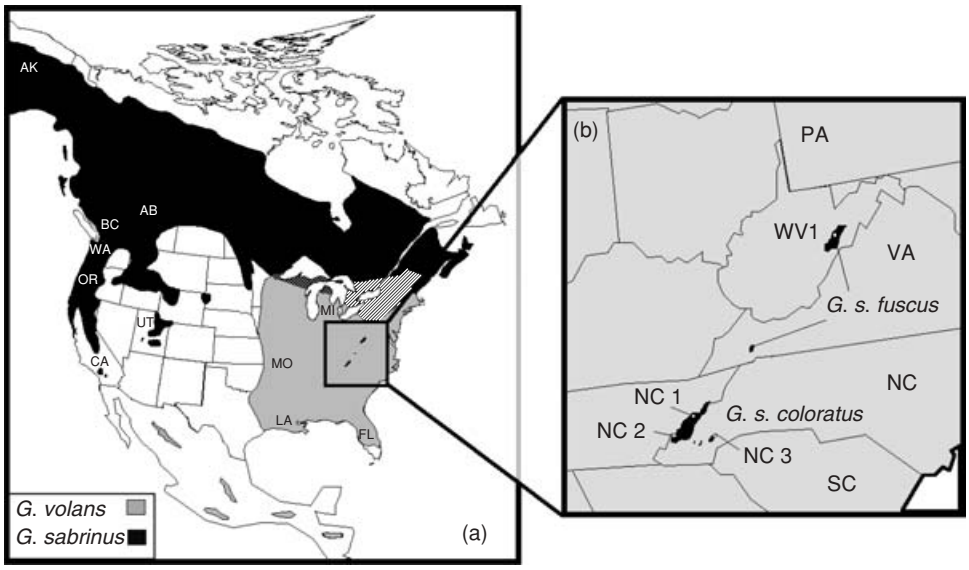

Fig. 1. Range maps of the two North American flying squirrels of the genus *Glaucomys*. (a) North American geographical distributions of the northern flying squirrel, *G. sabrinus* (black) and the southern flying squirrel, *G. volans* (grey); cross-hatching represents an area of potential sympatry. Sampling localities are given in Table 1 and the Appendix. (b) Expanded map of the southern Appalachian Mountains in eastern North America showing co-distribution of high-elevation spruce-fir habitat (black) and endangered subspecies of *G. sabrinus* (note that these subspecies are isolated from conspecific populations and although they occur within the range of *G. volans*, the two species are typically separated elevationally in the southern Appalachian region). Endangered forms of *G. sabrinus* are indicated as *G. s. fuscus* (West Virginia and Virginia) and *G. s. coloratus* (three populations in North Carolina). Geographical distributions have been modified from Wells-Gosling & Heaney (1983), Dolan & Carter (1977) and Browne *et al*. (1999). Two-letter abbreviations indicate selected states and provinces (see the Appendix).

of genetic variability and the potential for inbreeding depression also of high concern (Browne *et al*., 1999).

In this study we examine the conservation genetics of the endangered Appalachian populations of *G. sabrinus* using data from two molecular markers: the mitochondrial DNA (mtDNA) cytochrome-*b* gene and allozymes. To provide the broadest possible context for evaluating these small regional populations, we also examined the large-scale population structure of *G. sabrinus* and that of the closely related southern flying squirrel (*G. volans*). *Glaucomys volans* occupies deciduous hardwood forests of eastern North America and occurs at lower elevations in the Appalachians than *G. sabrinus* (Fig. 1). Taking this two-marker approach provides two different perspectives on genetic variation and population structure; whereas mtDNA is a maternally inherited organelle, allozymes primarily survey bi-parentally inherited nuclear loci (Wilson *et al*., 1985). One major advantage of this approach (as opposed to examining either marker alone) is that it permits much stronger inferences regarding the role of historical bottlenecks in shaping contemporary genetic variation (Gaines *et al*., 1997). Extending our analysis to include populations of *G. volans* from throughout eastern North America also provides a much broader biogeographical and evolutionary framework within which to interpret our data on the endangered Appalachian populations of *G. sabrinus*. Our ultimate goals were to use this multi-marker, multi-species comparative approach: (1) to assess the distinctness of the endangered Appalachian populations of *G. sabrinus*, (2) to determine whether these populations have reduced levels of genetic variability relative to other populations of flying squirrels in North America, as might be expected given their small contemporary population sizes and insular distribution and (3) to provide important genetic information that can be used to address the current concerns for the viability and maintenance of these endangered populations.

METHODS AND MATERIALS

Molecular techniques

We analysed mtDNA variation in a 315 base-pair (bp) fragment of the cytochrome-*b* gene of the mtDNA from 63 individuals of *Glaucomys* (43 individuals of *G. sabrinus* and 20 of *G. volans*). These included 25 new sequences and 38 reported previously (Arbogast, 1999). Locality information and GenBank accession numbers for all 63 individuals are given in the Appendix. Laboratory protocols for extraction and amplification followed those described by Arbogast (1999). Sequencing was performed using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA) at the Burke Museum, University of Washington. All sequences were aligned using Sequencher (Version 3.11; Gene Codes Corp., Ann Arbor, MI) and visually inspected for errors.

Allozyme data were collected for 127 flying squirrels from North America (Table 1). Geographical sampling was similar to that for the mtDNA analysis, although not identical. Seventy-seven individuals of *G. sabrinus* were sampled from 12 geographical areas. These included 41 individuals of the endangered Appalachian subspecies

G. s. coloratus (19 from Roan Mt., Mitchell Co., North Carolina (NC1), 19 from Haw Knob, Graham Co., North Carolina (NC2) and four from Richland Balsam, Jackson Co., North Carolina (NC3)) and four individuals of the endangered subspecies *G. s. fuscus* from Spruce Knob, West Virginia (WV1). Eight populations of *G. sabrinus* from outside the Appalachians were sampled: Snohomish and Pierce Counties, Washington (WA), Douglas Co., Oregon (OR), San Bernardino Co., California (CA); E. Bloodfoot Provincial Park, Edmonton, Alberta, Canada (AB), Otsego and Mackinac Counties, Michigan (MI); Fairbanks area, Alaska (AK); Summit Co., Utah (UT) and the Vancouver area of British Columbia, Canada (BC). For comparison, we also examined 50 individuals of the southern flying squirrel, *G. volans*, from six areas: lower elevations of Mitchell Co., North Carolina (NC1b); Aiken Co., South Carolina (SC); Highland Co., Florida (FL), East Baton Rouge Par., Louisiana (LA), Kanawha Co., West Virginia (WV2) and Westmoreland Co., Pennsylvania (PA). Blood or tissue samples were run on starch gels on both continuous (Tris-Borate-EDTA) and discontinuous (Poulik's) buffers, and stained following the recipes of Selander *et al*. (1971) and Hillis, Mortiz & Mable (1996). Twenty-one loci were scored: aconitate hydratase (ACOH-1), EC 4.2.1.3; alcohol dehydrogenase (ADH-1) EC 1.1.1.1; aspartate aminotransferase (AAT-1), EC 2.6.1.1; cytosol aminopeptidase (CAP-1), EC 3.4.11.1; esterase (EST-1, EST-2, EST-3, EST-4), EC 3.1.1.- (Colorimetric); glycerol-3-phosphate dehydrogenase (G3PDH-1), EC 1.1.1.8; glucose-6-phosphate isomerase (GPI-1) EC 5.3.1.9; haemoglobin (HGB-1), no EC number; lactate dehydrogenase (LDH-1) EC 1.1.1.27; malate dehydrogenase (MDH-1, MDH-2), EC 1.1.1.37; peptidase using leucylglyclyglycine as the substrate (PEP-1), EC 3.4.-.-.; phosphoglucomutase (PGM-1, PGM-2), EC 5.4.2.2; phosphogluconate dehydrogenase (PGDH-1), EC 1.1.144; sorbitol dehydrogenase (SDH-1), EC 1.1.1.14; superoxide dismutase (SOD-1), EC 1.15.1.1; xanthine dehydrogenase (XDH-1), EC 1.1.1.204.

Data analysis

We used likelihood and distance methods to infer a mtDNA gene phylogeny of *Glaucomys* and to examine the large-scale phylogeographical structure of both species. Unless otherwise noted, all phylogenetic analyses were performed using PAUP* (Swofford, 1998). Major mtDNA clades (those reciprocally monophyletic and relatively divergent from other such clades) were treated as separate units when estimating nucleotide diversity and effective population size (see below). We conducted an initial analysis to examine the evolutionary relationships of the following Old World flying squirrels to *Glaucomys*: *Hylopetes phayrei*, *Petaurista petaurista* (two individuals), *Petaurista philippensis*, *Petaurista leucogenys*, *Petaurista alborufus* and *Pteromys volans* (GenBank numbers AB030259, AF063067, AB023909, AB023907, AB023906, AB023902 and AB023910, respectively). Parsimony, maximum-likelihood and distance methods all consistently supported *Hylopetes phayrei* as the most closely related of these Old World flying squirrels to *Glaucomys*. This result is consistent with the findings of Oshida *et al*. (2000). However, because all of the potential outgroups we examined, including *H. phayrei*, are relatively divergent from *Glaucomys* in terms of nucleotide sequence divergence, their inclusion has the potential to add high levels of homoplasy and long-branch attraction into the analyses (Halanych *et al*., 1999; Arbogast, Browne & Weigl, 2001). Therefore, we used only *H. phayrei* as an outgroup in subsequent phylogenetic analyses. We used the computer program MODELTEST (Version 3.0: Posada & Crandall, 1998), which employs a series of hierarchical likelihood ratio tests (LRTs) to evaluate the fit of nested models of nucleotide substitution, to determine which model provided the best fit to the cytochrome-*b* data. The neighbour-joining method was then used to infer a cytochrome-*b* gene tree under the best-fit model and nodal support was estimated by performing 1000 bootstrap replicates. To evaluate whether different haplotype trees might be supported under different optimality criteria, parsimony analysis also was performed (with character state changes weighted equally and with transitions down-weighted to reflect the empirical transition: transversion ratio).

For the mtDNA data we used Arlequin (Version 2.000: Schneider, Roessli & Excoffier, 2000) to estimate π (mtDNA nucleotide diversity, a measure of genetic variability) and θ (the population parameter $2N_e\mu$, where N_e is effective population size and μ is mutation rate; for haploid mtDNA data $\theta = 2N_f\mu$, where N_f is female effective populations size). Assuming equal values of μ, estimated values of θ can be used to compare N_e (or N_f) between populations. For the allozyme data we calculated allele frequencies for each locus, the percentage of polymorphic loci (%*P*; at the 0.95 level) and average individual heterozygosities ($\bar{H}$) for each population and species (Table 1). Goodness-of-fit tests were performed to test for conformance to Hardy–Weinberg equilibrium. Due to the small sample sizes for *G. s. fuscus* (4), we did not treat this group separately from the other Appalachian subspecies *(G. s. coloratus)* when calculating diversity indices and estimating relative values of θ and N_e.

RESULTS

Phylogeny and phylogeography

We found 26 unique mtDNA cytochrome-*b* haplotypes among 63 individuals of *Glaucomys* (Appendix; Fig. 2). Three of these haplotypes were found in the endangered populations of *G. sabrinus*; two out of the three only in the Appalachian populations and one out of the three was also found in one population from Michigan. All methods of phylogenetic inference converged on essentially the same haplotype tree (Fig. 2). Because the analyses appear to be robust to differences in the assumptions of the phylogenetic methods and rooting strategies, we present

Table 1. Allelic frequencies at 16 variable loci and estimates of average individual heterozygosity (H) and percentage of loci that are polymorphic (%P) for populations of *Glaucomys sabrinus* and *G. volans*

		AAT-1		ADH-1		CAP-1		EST-1		EST-3		EST-4			G-3PDH-1		GPI-1			HGB-1	
	n	1	2	1	2	1	2	1	2	1	2	1	2	3	1	2	1	2	3	1	2
G. sabrinus																					
NC1	19	1.00		1.00			1.00		1.00		1.00		1.00		1.00			1.00		1.00	
NC2	19	1.00		1.00			1.00		1.00	0.11	0.89		1.00		0.95	0.05		0.97	0.03	1.00	
NC3	3	1.00		1.00			1.00		1.00		1.00			1.00	1.00			1.00		1.00	
WV1	4	1.00		1.00		0.75	0.25		1.00		1.00		1.00		1.00			1.00		1.00	
MI	4		1.00		1.00	0.25	0.75	0.25	0.75		1.00		1.00		0.75	0.25		0.38	0.62	1.00	
AK	3	0.50	0.50		1.00		1.00	0.50	0.50		1.00		1.00		0.17	0.83		0.33	0.67	1.00	
WA	7	0.43	0.57		1.00		1.00	0.14	0.86		1.00		1.00		1.00			1.00		1.00	
OR	5	0.20	0.80		1.00		1.00		1.00		1.00		1.00		1.00			1.00		1.00	
CA	2		1.00		1.00		1.00	0.75	0.25		1.00		1.00		0.50	0.50			1.00	1.00	
AB	3	0.33	0.67		1.00		1.00	0.50	0.50		1.00		1.00		1.00			0.50	0.50	1.00	
UT	2	0.25	0.75		1.00		1.00	0.25	0.75		1.00		1.00		1.00			0.75	0.25	1.00	
BC	6		1.00		1.00		1.00	1.00			1.00		1.00		1.00			0.67	0.33	1.00	
Total	77	0.68	0.32	0.58	0.42	0.05	0.95	0.17	0.83	0.03	0.97		0.96	0.04	0.96	0.04		0.88	0.12	1.00	
G. volans																					
FL	10	1.00		1.00		1.00		1.00		1.00		1.00			1.00		1.00				1.00
SC	12	1.00		1.00		1.00		1.00		1.00		1.00			0.96	0.04	1.00				1.00
NC1b	16	1.00		1.00		1.00		1.00		1.00		1.00			1.00		1.00				1.00
LA	2	1.00		1.00		1.00		1.00		1.00		1.00			1.00		1.00				1.00
WV2	1	1.00		1.00		1.00		1.00		1.00		1.00			1.00		0.50	0.50			1.00
PA	9	1.00		1.00		1.00		1.00		1.00		1.00			1.00		1.00				1.00
Total	50	1.00		1.00		1.00		1.00		1.00		1.00			0.99	0.01	0.99	0.01			1.00

All individuals of *G. sabrinus* sampled from West Virginia correspond to *G. s. fuscus* and all those sampled from North Carolina correspond to *G. s. coloratus*. Locality information is shown on the maps in Fig. 1 and is listed in the Appendix.

only the neighbour-joining tree based on the model that provided the best fit to the cytochrome-*b* data set, consisting of the 26 unique *Glaucomys* haplotypes plus *Hylopetes phayrei* (the TrN model (Tamura & Nei, 1993) with a gamma correction ($\alpha = 0.0214$)). The analyses support three reciprocally monophyletic lineages within *Glaucomys*, two within *G. sabrinus* and one within *G. volans*. The two mtDNA lineages within *G. sabrinus*, a 'Pacific Coastal' lineage comprised of populations from western Washington, western Oregon and California and a 'Continental' lineage comprised of populations from the remainder of the species' range, including the eastern slope of the Washington Cascades. All phylogenetic analyses support a paraphyletic association of the Continental and Pacific Coastal mtDNA clades of *G. sabrinus*. The *G. volans* lineage and the Continental lineage of *G. sabrinus* were consistently found to be sister to one another, with the Pacific Coastal lineage of *G. sabrinus* assuming a basal position in the tree.

Within the Continental clade of *G. sabrinus*, haplotypes found in the endangered Appalachian populations (North Carolina and West Virginia) and those from the southern Great Lakes region (Michigan) occupy a basal position in the tree (Fig. 2). Within the clade corresponding to *G. volans*, haplotypes found in Louisiana and Florida occupy a basal position in the tree relative to haplotypes found further north. Within the Pacific Coastal clade of *G. sabrinus*, haplotypes from coastal Oregon are basal to those from western Washington and California. Mean levels of sequence divergence between haplotypes within the Continental and Pacific Coastal clades of *G. sabrinus* are 1.07% and 1.21%, respectively, based on the two-parameter model of Kimura (1980). Within the former clade, levels of sequence divergence between haplotypes of *G. sabrinus* found in the Appalachians (haplotypes 1–3) and those found only outside the Appalachians (haplotypes 4–10 and 18) range from 1.03–2.58%. Within the lineage corresponding to *G. volans*, the mean level of sequence divergence between haplotypes is 0.702%. Minimum and maximum levels of sequence divergence between the three major lineages are: 4.66–7.22% between the two lineages of *G. sabrinus*; 3.61–6.5% between the Continental lineage of *G. sabrinus* and *G. volans*; and 4.31–8.311% between the Pacific Coastal lineage of *G. sabrinus* and *G. volans*.

Genetic variability

Estimates of mtDNA nucleotide diversity are 0.951% and 0.905% for the Continental and Pacific Coastal lineages of *G. sabrinus*, respectively, and 2.92% for the two clades combined (Fig. 3). Nucleotide diversity within the southern Appalachian populations of *G. sabrinus* is 0.500%, which is about 60% that of the Continental lineage and about one-sixth that of the species as a whole. Nucleotide diversity within *G. volans* is 0.576%, which is substantially less than the summed

Table 1. (contd.)

MDH-1			MDH-2		PEP-1			PGDH-1				PGM-1		PGM-2		SOD-1			
1	2	3	1	2	1	2	3	1	2	3	4	1	2	1	2	1	2	P (%)	$\bar{H}$ (%)
	1.00			1.00			1.00			1.00		0.90	0.10		1.00	1.00		4.76	1.00
	1.00			1.00			1.00			0.95	0.05	0.97	0.03		1.00	1.00		14.29	2.51
	1.00			1.00			1.00			1.00		1.00			1.00	1.00		0	0
	1.00			1.00			1.00			1.00		0.75	0.25		1.00	1.00		9.52	0
1.00				1.00	0.25	0.38	0.38			1.00		0.88	0.12		1.00	1.00		28.57	10.7
1.00				1.00	0.33	0.33	0.33			1.00		1.00			1.00	1.00		23.81	4.77
0.50	0.50			1.00		0.07	0.93				1.00	0.93	0.07		1.00	1.00		23.81	4.77
0.90	0.10			1.00			1.00			0.40	0.60	0.90	0.10		1.00	1.00		19.05	3.81
1.00				1.00			1.00				1.00	0.75	0.25		1.00	1.00		14.29	9.52
0.83	0.17			1.00			1.00			0.17	0.83	0.83	0.17		1.00	1.00		28.57	11.11
0.75		0.25		1.00			1.00				1.00	1.00			1.00	1.00		19.05	9.52
0.83	0.17			1.00			1.00				1.00	1.00			1.00	1.00		9.52	4.77
0.34	0.65	0.01		1.00	0.03	0.04	0.93			0.69	0.31	0.92	0.08		1.00	1.00		42.86	3.58
1.00			1.00				1.00		1.00			1.00		1.00		1.00		0	0
1.00			1.00				1.00		1.00				1.00	1.00		0.96	0.04	0	0.79
1.00			1.00				1.00		1.00			1.00		1.00		1.00		0	0
1.00			1.00				1.00		1.00			0.75	0.25	1.00		1.00		4.76	2.38
1.00			1.00				1.00		1.00			0.50	0.50	1.00		1.00		9.52	9.52
1.00			1.00				1.00	1.00				1.00		1.00		1.00		0	0
1.00			1.00				1.00	0.18	0.82			0.74	0.26	1.00		0.99	0.01	9.52	0.48

diversity for all populations of *G. sabrinus* (0.576% versus 2.92%) and only slightly more than that within the endangered southern Appalachian populations of *G. sabrinus*. Estimates of θ are 3.68 for *G. volans* and 12.5 for *G. sabrinus* (7.01 and 4.04 for the Continental and Pacific Coastal lineages, respectively). When just the Appalachian populations of *G. sabrinus* are considered, $\theta = 1.52$; for all populations of *G. sabrinus* except those from the Appalachians, $\theta = 10.5$.

Out of the 21 allozyme loci examined, five were non-variable for all populations of both species (ACOH-1, EST-2, LDH-1, SDH-1, and XDH-1: Table 1). No significant deviations from Hardy–Weinberg equilibrium occurred at any locus for any population. Private alleles occurred in two populations of *G. s. coloratus*, NC2 (EST-3) and NC3 (EST-4) and in the one examined population of *G. s. fuscus* (WV1). One locus (ADH-1) exhibited allelic fixation between the endangered populations of *G. sabrinus* from the southern Appalachians and all the remaining conspecific populations. The average percentage of loci polymorphic for the four endangered populations of *G. sabrinus* from the southern Appalachians is significantly lower than for the remaining eight conspecific populations (14% versus 38%; $P < 0.02$ by ANOVA using arcsine transformed data). Although sample size can affect estimates of polymorphism, sample size was larger for the summed southern Appalachian populations than for the summed central and western populations ($n = 45$ and 32, respectively). Average individual heterozygosity values, which are not as dependent upon sample sizes as polymorphism estimates, also are significantly lower for southern Appalachian populations of *G. sabrinus* compared to the remaining conspecific populations (1.48% versus 6.55%, respectively; $P < 0.02$ by ANOVA using arcsine transformed data).

For the southern flying squirrel, *G. volans*, %*P* is zero for South Carolina and both %*P* and $\bar{H}$ were zero for Florida, Pennsylvania and North Carolina (NC1b) (Table 1). Polymorphism and heterozygosity are apparent in the Louisiana and West Virginia-2 populations, where sample sizes were extremely low. Overall, the average individual heterozygosity and the average level of polymorphism for *G. volans* are only 14% and 22%, respectively, of that observed in *G. sabrinus* (Fig. 3). Both these indices of nuclear diversity are also lower in *G. volans* than in populations of the two endangered subspecies of *G. sabrinus* from the southern Appalachians.

DISCUSSION

Population structure and historical biogeographic context

The geographically extensive set of genetic data presented here provide a comparative evolutionary and biogeographical context in which to evaluate the conservation genetics of endangered populations of the northern flying squirrel that inhabit the high elevation spruce-fir islands of the southern Appalachian mountains. The data presented here support the hypothesis that the contemporary large-scale population genetic structure of *G. sabrinus* has been strongly influenced by historical changes in the distribution of coniferous forest throughout the late Pleistocene (Arbogast, 1999). During the Wisconsinan glacial maximum, the northern coniferous forest of

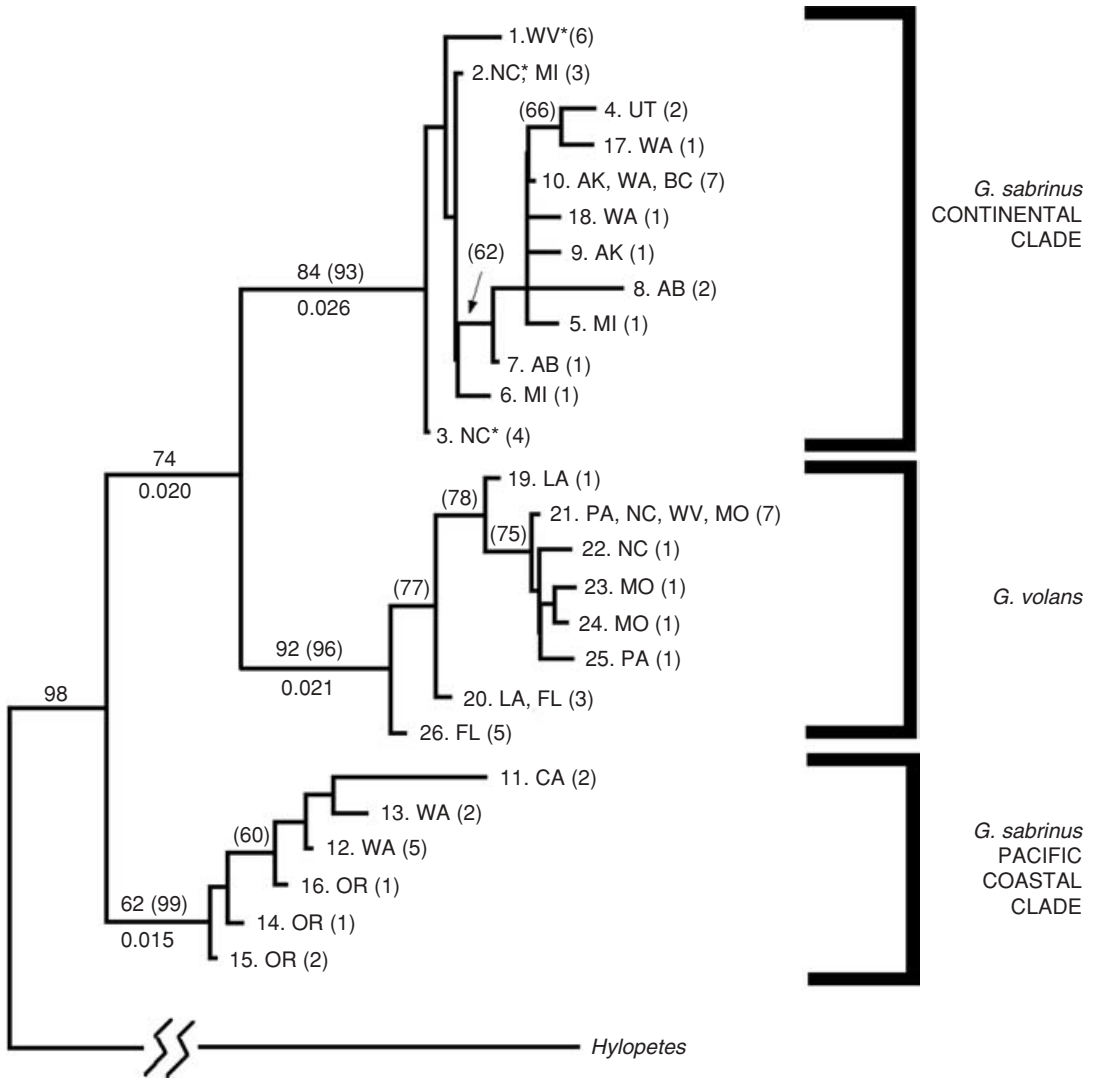

Fig. 2. Neighbour-joining tree showing evolutionary relationships of populations of the two species of *Glaucomys* based on an analysis of the mtDNA cytochrome-*b* gene. This tree is presented as a phylogram with branch lengths (substitutions per site) shown below the major branches (branch lengths are proportional except for that between *Hylopetes* and the ingroup taxa). Bootstrap values > 50% (based on 1000 replicates) for the outgroup analysis are shown above the line at each node, followed in parentheses by those estimated with the outgroup taxon removed. Branch tips indicate haplotype numbers (1–26), localities (state or province abbreviations; see the Appendix) and number of individuals showing the haplotype. Haplotypes found in the endangered southern Appalachian subspecies of *G. sabrinus* are indicated by asterisks.

North America was pushed far south of its present range (Adams & Faure, 1997). Fossil evidence indicates that the range of *G. sabrinus* was similarly displaced southward at this time (Kurtén & Anderson, 1980). The mtDNA data suggest that contemporary populations of *G. sabrinus* are derived from ancestral populations isolated in two separate coniferous forest refugia during the Wisconsinan (Arbogast, 1999). Most of northern North America is occupied by the Continental clade of *G. sabrinus*. Appalachian haplotypes are basal in this clade (Fig. 2). The general southeast-to-northwest topology of the mtDNA gene tree for this clade is consistent with a northward and westward post-Wisconsinan range expansion from a southeastern refugium. By contrast, populations of *G. sabrinus* currently found west of the Cascades and Sierra Nevada in Washington, Oregon and California appear to be derived from an ancestral population that persisted in a coniferous forest refugium that existed along the Pacific coast of the USA.

In addition to the mtDNA gene tree, the allozyme data also support a northward recolonisation of much of North America from a southeastern refugium. The predicted genetic consequences of an ice-age cycle of range contraction and expansion include: (1) the lowest levels of variation should be found at the leading (i.e. northern) edge due to repeated bottlenecks caused by founder events during range expansion and (2) private alleles are most likely to be found at the trailing edge (Hewitt, 1996). The allozyme data for *G. sabrinus* support these predictions. For example, of the populations we sampled, those

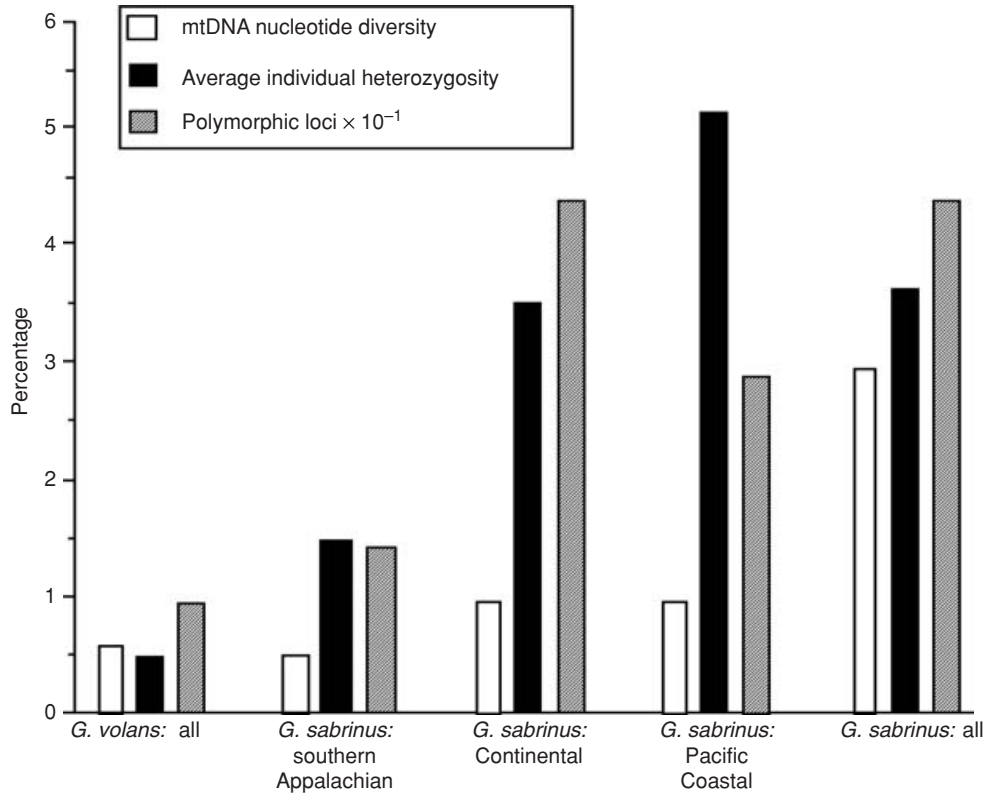

Fig. 3. Estimated levels of nucleotide diversity (based on mtDNA data) and average individual heterozygosity and percentage polymorphic loci (based on the allozyme data) for *Glaucomys volans* and various populations of *G. sabrinus*.

from Alaska (leading edge) exhibit much lower levels of polymorphism and average individual heterozygosity than those from Alberta and Michigan further to the south and east (Table 1). In addition, private alleles were found only within the southern Appalachian populations (trailing edge of range). Overall, the combined mtDNA and allozyme data support the view that the endangered southern Appalachian populations of *G. sabrinus* are late Pleistocene relicts that became isolated from more northern populations as the distribution of coniferous forests shifted northward following glacial retreat (Arbogast, 1999; Weigl *et al*., 1999).

Relationships of haplotypes within the mtDNA clade corresponding to *G. volans* (Fig. 2) suggest a south-to-north pattern of post-glacial recolonisation in this species as well. Haplotypes from Florida and Louisiana are basal in this clade, while more northern haplotypes are all closely related to one another. These results are consistent with the paleovegetation record (Davis, 1983) in supporting a northward post-glacial recolonisation of eastern North America by *G. volans* out of a southeastern deciduous forest refugium that existed in the Gulf Coast region of North America during the most recent glacial maximum. It has recently been suggested (Adams & Faure, 1997) that such a refugium may have been smaller and more fragmented than previously considered (Davis, 1983). Because the genetics of populations of *G. volans* from west of the Mississippi River, Mexico and Central America have not been examined, it remains to be seen how they will contribute to the overall historical biogeographical picture for *Glaucomys*.

The three mtDNA clades recovered among the *Glaucomys* populations we sampled (Fig. 2) indicate that the Continental and Pacific Coastal mtDNA clades of *G. sabrinus* are paraphyletic, supporting the findings of Arbogast (1999). However, all three mtDNA clades are separated by similar amounts of sequence divergence from one another. This suggests that the three clades may have experienced a relatively contemporaneous divergence. The paraphyly could also reflect incomplete lineage sorting, especially given that these clades probably originated in the early–mid Pleistocene (Arbogast, 1999).

Genetic variability and historical bottlenecks

Overall, levels of genetic variability in *G. sabrinus* are relatively high (Fig. 3). This is especially true when compared to those observed in *G. volans*. This suggests that, as a species, *G. sabrinus* has not experienced strong historical bottlenecks. Our estimates of θ also suggest a value of N_e for *G. sabrinus* that is about three times larger than that for *G. volans* ($\theta = 12.5$ versus 3.68, respectively). Our most extensive allozyme data, from populations of *G. s. coloratus* at the extreme southeastern edge of the species range, suggest that some loss of genetic variability has occurred in these endangered Appalachian populations of *G. sabrinus*. However, levels

of genetic variability in the Appalachian populations of *G. sabrinus* are actually similar to (based on mtDNA), or higher than (based on allozymes) those observed in populations of the widespread *G. volans* (Fig. 3). Thus, our comparative approach suggests that the endangered southern Appalachian populations of *G. sabrinus* have levels of polymorphism, heterozygosity and mtDNA nucleotide diversity that are actually intermediate, rather than extremely low. This is somewhat surprising given the small effective population sizes for these populations. Our estimates of θ (based on the mtDNA data) indicate that N_e for the summed Appalachian populations of *G. sabrinus* is about one-eighth that of the species as a whole and about one-half that of *G. volans*. However, the present-day functional N_e for the Appalachian populations of *G. sabrinus* may be much lower given their highly fragmented distribution. For example, field studies suggest that many local populations of *G. s. coloratus* have an N_e of < 100 individuals (Weigl *et al*., 1999). Recent historical connections during cooler climatic periods, such as in the late Pleistocene, may have contributed to the maintenance of intermediate levels of genetic diversity in these now-isolated populations.

The apparent lack of major genetic variation in the southern flying squirrel over a broad area of eastern North America is one of the most surprising results of our study. Both the mtDNA and allozyme data for *G. volans* reveal surprisingly low levels of variability within and between populations of this species (Table 1, Fig. 3). This combination of low variability in nuclear and mtDNA is predicted for species that have undergone a severe or prolonged historical bottleneck (Wilson *et al*., 1985; Gaines *et al*., 1997). Adams & Faure (1997) have suggested that the extent of deciduous forest habitat in the southeastern United States was either extremely small, highly fragmented, or both during the Wisconsinan glacial maximum. Thus, the low levels of genetic variability observed in *G. volans* may be related to a historical bottleneck at this time. No such severe bottleneck appears to have affected *G. sabrinus*, suggesting that coniferous forest refugia may have been relatively large during the Wisconsinan glacial maximum.

The phylogeographical architectures of *G. sabrinus* and *G. volans*, while differing markedly from one another, share much in common with those of other vertebrate taxa associated with the northern coniferous and eastern deciduous forest habitats of North America, respectively. For example, several taxa of mammals occupying the coniferous forest of North America share a phylogeographical discontinuity in the Pacific Northwest similar to that observed in *G. sabrinus* (Arbogast & Kenagy, 2001). Similarly, many mammals associated with the deciduous forest of eastern North America appear to be relatively depauperate in terms of genetic variability, e.g. the eastern gray squirrel, *Sciurus carolinensis*, and the fox squirrel, *S. niger* (Moncreif, 1998). Additional genetic studies of North American forest taxa, including trees, will test the generality of our conclusions. Such studies, combined with continued advances in palynology and paleobotany, promise to provide many additional insights into the complex relationship between the Quaternary dynamics of North American forests and the genetic diversity of associated species.

CONCLUSIONS

Conservation implications

Genetic data provide a means for assessing the evolutionary distinctiveness of populations of conservation concern. These data can be further used to establish management units (MUs) and/or evolutionary significant units (ESUs), two commonly used designations for threatened or endangered taxa (Moritz, 1994; Bidlack & Cook, 2001). MUs are defined by either reciprocal monophyly in mtDNA or substantial allele frequency divergence at nuclear loci; ESUs are defined by the presence of both (Moritz, 1994). The presence of several private alleles in our allozyme analysis clearly distinguishes the endangered Appalachian populations of *G. sabrinus* from conspecific populations elsewhere (Table 1). However, the cytochrome-*b* data indicate that some mtDNA haplotypes are shared between populations from the Appalachians (North Carolina) and populations outside the Appalachian region (e.g. Michigan; Fig 2). Thus, at the level of sensitivity provided by this mitochondrial gene, the Appalachian populations fall short of reciprocal monophyly relative to other conspecific populations. The combined results of the mtDNA and allozyme data thus support the continued recognition of the endangered southern Appalachian population of *G. sabrinus* as a distinct MU, although not necessarily as a distinct ESU. Two sampling issues should be addressed in the future in order to further evaluate our conclusions on the genetic distinctness and conservation status of populations of *G. sabrinus* in the Appalachians. First, more samples of *G. s. fuscus* should be examined; our sample from this subspecies is quite small, especially relative to that from *G. s. coloratus*. Second, samples of *G. sabrinus* from the more contiguous portion of the species' range just north of the fragmented Appalachian spruce-fir islands occupied by *G. s. fuscus* (i.e. from Pennsylvania, New York, etc) should be examined.

The mtDNA and allozyme data provided here give important information on whether the Appalachian populations have low genetic variability relative to other conspecific populations and relative to *G. volans*. Limited genetic variability is considered to be an important potential factor in the long-term viability of endangered taxa (Frankham, 1997). Our data suggest that levels of genetic variability in these endangered populations are reduced relative to conspecific populations, but are not low compared to populations of the widespread southern flying squirrel. Although future decreases in genetic variability are a potential problem for populations of *G. sabrinus* from the spruce-fir islands of the southern Appalachians, the most immediate threat to the persistence of these populations is likely to be habitat loss. The spruce-fir and spruce-fir-hardwood ecotonal habitats of *G. s. fuscus* and

G. s. coloratus are effectively trapped between two highly destructive processes: (1) the loss of much of the conifer zone due to introduced pests and acid precipitation and (2) disturbance of upland hardwoods by human activities, including road-building and recreational uses (Weigl *et al*., 1999). This loss of suitable habitat for populations of *G. sabrinus* in the southern Appalachians, which are already small and fragmented, would further decrease the size of local populations, thereby increasing the likelihood of local extinction. The loss of high-elevation habitat also increases the isolation of existing populations, thus restricting natural gene flow. Because global warming and climatic cycles of both short and long duration will undoubtedly affect forest distributions in the future, recognition of climate change and its implications also will be vital in constructing proactive conservation strategies designed to maintain viable populations of *G. sabrinus* in the southern Appalachians.

Acknowledgments

E. Eagleson, J. Kelly, L. Penhall, D. Ransome, D. L. Reed, J. Ridgeway, C. Stihler, A. J. Schwandt-Arbogast, J. Wallace and Elizabeth White provided field and laboratory assistance. Tissue samples were provided by M. Hafner, Louisiana State University Museum of Natural Science; J. Cook, University of Alaska Museum; C. Stihler and J. Wallace, West Virginia Department of Natural Resources; H. Pawalczyk, Monongahela National Forest; A. Carey and T. Wilson, Pacific Northwest Research Station, Olympia, Washington; J. Scheibe, Southeast Missouri State University, Cape Gerardeau; and A. Boynton, R. Hughes and D. Battle. Financial assistance was provided by the NC Wildlife Commission, U.S. Fish and Wildlife Service, NC Department of Highways, American Society of Mammalogists, a Spires Excellence grant from Wake Forest University and the University of Washington Burke Museum Mammalogy Program and Royalty Research Fund.

REFERENCES

Adams, J. M. & Faure, H. (1997). *Review and atlas of palaeovegetation: preliminary land ecosystem maps of the world since the Last Glacial Maximum*. QEN members, Oak Ridge National Laboratory, TN, USA. http://www.esd.ornl.gov/ern/qen/adams1.html

Arbogast, B. S. (1999). Mitochondrial DNA phylogeography of the New World flying squirrels (*Glaucomys*): implications for Pleistocene biogeography. *J. Mammal*. **80**: 142–155.

Arbogast, B. S. & Kenagy, G. J. (2001). Comparative phylogeography as an integrative approach to historical biogeography. *J. Biogeogr*. **28**: 819–825.

Arbogast, B. S., Browne, R. A. & Weigl, P. D. (2001). Evolutionary genetics and Pleistocene biogeography of North American tree squirrels (*Tamiasciurus*). *J. Mammal*. **82**: 302–319.

Bidlack, A. L. & Cook, J. C. (2001). Reduced genetic variation in insular northern flying squirrels (*Glaucomys sabrinus*) along the North Pacific Coast. *Anim. Conserv*. **4**: 283–290.

Browne R. A., Weigl, P., Eagleson, E., Kelly, J. & Steele, M. (1999). Mountaintops as islands: I. Genetic variation among Southern Appalachian populations of the endangered northern flying squirrel (*Glaucomys sabrinus*) (Mammalia; Sciuridae). In *Proceedings of the Appalachian biogeography symposium*: 205–214. Eckerlin, R. P. (Ed.). Martinsville, VA: Virginia Museum of Natural History Press.

Bruck, R. I., Robarge, W. P. & McDaniel, A. (1989). Forest decline in the boreal montane ecosystems of the southern Appalachian Mountains. *Water Air Soil Pollut.* **48**: 161–180.

Davis, M. B. (1983). Quaternary history of deciduous forests of Eastern North America and Europe. *Ann. Mo. Bot. Gard*. **70**: 550–563.

Dolan, P. G. & Carter, D. C. (1977). *Glaucomys volans*. *Mammalian Species* **No**. **78**: 1–6. Special Publication of the American Society of Mammalogists.

Frankham, R. (1997). Do island populations have less genetic variation than mainland populations? *Heredity* **78**: 311–327.

Gaines, M. S., Diffendorfer, J. E., Tamarin, R. H. & Whittam, T. S. (1997). The effects of habitat fragmentation on the genetic structure of small mammal populations. *J. Heredity* **88**: 294–304.

Halanych, K. M., Demboski, J. R., Jansen van Vuuren, B., Klein, D. R. & Cook, J. A. (1999). Cytochrome *b* phylogeny of North American hares and jackrabbits (*Lepus*, Lagomorpha) and the effects of saturation in outgroup taxa. *Mol. Phylog. Evol.* **11**: 213–221.

Harp, J. M. (1992). *A status survey for the Spruce-fir moss spider, **Microhexura montivaga** Crosby and Bishop (Aranaea, Dipluridae)*. North Carolina Wildlife Commission, Nongame and Endangered Wildlife Program and the U. S. Fish and Wildlife Service. Unpublished report.

Hewitt, G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc*. **58**: 247–276.

Hillis, D. M., Mortiz, C. & Mable, B. K. (1996). *Molecular systematics*. 2nd edn. Sunderland, MA: Sinauer Associates.

Kimura, M. (1980). A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–120.

Kurtén, B. & Anderson, E. (1980). *Pleistocene mammals of North America*. New York: Columbia University Press.

Moncreif, N. D. (1998). Allozymic variation in populations of fox squirrels (*Sciurus niger*) and gray squirrels (*S. carolinensis*) from the eastern United States. In *Ecology and evolutionary biology of tree squirrels*: 145–158. Steele, M. A., Merrit, J. F. & Zegers, D. A. (Eds). Special Publication of the Virginia Museum of Natural History no. **6**.

Moritz, C. (1994). Defining 'evolutionary significant units' for conservation. *Trends Ecol. Evol*. **9**: 373–375.

Oshida, T., Lin, L. K., Yanagawa, H., Endo, H. & Masuda, R. (2000). Phylogenetic relationships among six flying squirrel genera inferred from mitochondrial cytochrome b gene sequences. *Zool. Sci*. **17**: 485–489.

Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Schneider, S., Roessli, D. & Excoffier, L. (2000). Arlequin: a software for population genetics data analysis. Version 2.000. Geneva: Dept of Anthropology, Genetics and Biometry Lab, Dept., University of Geneva.

Selander, R. K., Smith, M. H., Yang, S. Y., Johnson, W. E. & Gentry, J. B. (1971). Biochemical polymorphism and systematics in the genus *Peromyscus* I. Variation in the old–field mouse (*Peromyscus polionotus*). Studies in Genetics VI. *Univ. Texas Publ. Genet*. **7103**: 49–90.

Swofford, D. L. (1998). *PAUP*: phylogenetic analysis using parsimony (*and other methods)*. Computer program, version 4.0. Sunderland, MA: Sinauer Associates.

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol*. **10**: 512–526.

Wear, D. N. & Gries, J. G. (2002). *Southern forest resource assessment: summary report*. General Technical Report SRS-54. Asheville, NC: U.S. Department of Agriculture, Forest Service, Southern Research Station.

Weigl, P. D., Knowles, T. W. & Boynton, A. C. (1999). *The distribution and ecology of the northern flying squirrel, **Glaucomys sabrinus coloratus**, in the southern Appalachians*. Raleigh, NC: North Carolina Wildlife Resources Commission.

Wells-Gosling, N. & Heaney, L. R. (1984). *Glaucomys sabrinus*. *Mammalian Species* **No**. **229**: 1–8. Special Publication of the American Society of Mammalogists.

White, P. S., Buckern, E., Pittillo, J. D. & Cogbill, C. V. (1993). High-elevation forests: spruce-fir forests, northern hardwood forests, and associated communities. In *Biodiversity of the Southeastern United States; Upland Terrestrial Communities*: 305–338. Martin, W. H., Boyce, S. G. & Echternacht, A. C. (Eds). New York: John Wiley and Sons.

Wilson, A. C., Cann, R. L., Carr, S. M., George, M., Gyllensten, U. B., Helm-Bychowski, K. M., Higuchi, R. G., Palumbi, S. R., Prager, E. M., Sage, R. D. & Stoneking, M. (1985). Mitochondrial DNA and two perspectives on evolutionary genetics. *Biol. J. Linn. Soc*. **26**: 375–400.

APPENDIX. Localities, GenBank Accession numbers and mtDNA haplotype designations for all individuals of *Glaucomys sabrinus* and *G. volans* from which cytochrome-*b* data were analysed

Species and haplotype number	Locality	State/province code	GenBank accession number
G. sabrinus			
1	Pendleton Co., West Virginia	WV	AF63029
1	Pendleton Co., West Virginia	WV	AF63030
1	Webster Co., West Virginia	WV	AF63031
1	Pendleton Co., West Virginia	WV	AY703873
1	Randolph Co., West Virginia	WV	AY703874
1	Randolph Co., West Virginia	WV	AY703875
3	Mitchell Co., North Carolina	NC	AF63032
2	Mitchell Co., North Carolina	NC	AF63033
3	Mitchell Co., North Carolina	NC	AF63034
2	Mitchell Co., North Carolina	NC	AY703876
3	Mitchell Co., North Carolina	NC	AY703877
3	Mitchell Co., North Carolina	NC	AY703878
4	Summit Co., Utah	UT	AF63035
4	Summit Co., Utah	UT	AF63036
6	Mackinac Co., Michigan	MI	AF63037
2	Otsego Co., Michigan	MI	AF63038
5	Alger Co. Michigan	MI	AY703879
7	Edmonton, Alberta	AB	AF63039
8	Edmonton, Alberta	AB	AF63040
8	Edmonton, Alberta	AB	AF63041
9	Fairbanks, Alaska	AK	AF63042
10	Fairbanks, Alaska	AK	AF63043
10	Vancouver, British Columbia	BC	AF63044
10	Vancouver, British Columbia	BC	AF63045
10	Vancouver, British Columbia	BC	AF63046
10	Vancouver, British Columbia	BC	AF63047
10	Okanogan Co., Washington	WA	AF63048
17	Okanogan Co., Washington	WA	AF63049
18	Okanogan Co., Washington	WA	AF63050
10	Okanogan Co., Washington	WA	AF63051
13	Pierce Co., Washington	WA	AY703880
13	Pierce Co., Washington	WA	AF63052
12	Pierce Co., Washington	WA	AF63053
12	Snohomish Co., Washington	WA	AF63054
12	Snohomish Co., Washington	WA	AF63055
12	Mason Co., Washington	WA	AY703881
12	Mason Co., Washington	WA	AY703882
14	Douglas Co., Oregon	OR	AF63056
15	Douglas Co., Oregon	OR	AF63057
15	Douglas Co., Oregon	OR	AF63058
16	Douglas Co., Oregon	OR	AY703883
11	San Bernardino Co., California	CA	AF63059
11	San Bernardino Co., California	CA	AF63060

APPENDIX. Continued

Species and haplotype number	Locality	State/province code	GenBank accession number
G. volans			
19	East Baton Rouge Par., Louisiana	LA	AF63061
20	East Baton Rouge Par., Louisiana	LA	AF63062
21	Mitchell Co., North Carolina/Carter Co., Tennessee	NC	AF63063
21	Mitchell Co., North Carolina/Carter Co., Tennessee	NC	AF63064
22	Mitchell Co., North Carolina/Carter Co., Tennessee	NC	AF63065
21	Kanawha Co., West Virginia	WV	AF63066
20	Highland Co., Florida	FL	AY703884
20	Highland Co., Florida	FL	AY703885
26	Highland Co., Florida	FL	AY703886
26	Highland Co., Florida	FL	AY703887
26	Highland Co., Florida	FL	AY703888
26	Highland Co., Florida	FL	AY703889
26	Highland Co., Florida	FL	AY703890
21	Westmoreland Co., Pennsylvania	PA	AY703891
25	Westmoreland Co., Pennsylvania	PA	AY703892
21	Westmoreland Co., Pennsylvania	PA	AY703893
21	Westmoreland Co., Pennsylvania	PA	AY703894
23	Girardeau Co., Missouri	MO	AY703895
21	Girardeau Co., Missouri	MO	AY703896
24	Girardeau Co., Missouri	MO	AY703897

All individuals of *G. sabrinus* sampled from West Virginia correspond to *G. s. fuscus* and all those sampled from North Carolina correspond to *G. s. coloratus*. Museum voucher information for previously published sequences (AF numbers) is provided in Arbogast (1999); voucher information for new sequences (AY numbers) is provided online with each GenBank record.