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Molecular systematics and Pleistocene biogeography of Mesoamerican flying squirrels

Nicholas J. Kerhoulas* and Brian S. Arbogast

Department of Biological Sciences, Humboldt State University, 1 Harpst Street, Arcata, CA 95521, USA (NJK, BSA)
Department of Biology and Wildlife, University of Alaska Fairbanks, Fairbanks, AK 99775, USA (NJK)
Present address of NJK: Department of Mammalogy, University of Alaska Museum, 907 Yukon Drive, Fairbanks, AK 99775, USA
Present address of BSA: Department of Biology and Marine Biology, University of North Carolina, Wilmington, NC 28403, USA

* *Correspondent: nicholaskerhoulas@gmail.com*

Populations of flying squirrels from the Mesoamerican highlands represent the least understood members of the genus *Glaucomys*. Traditionally, these populations have been considered to be southern disjuncts of the southern flying squirrel (*G. volans*), a species that is widespread across the deciduous and mixed-deciduous forests of eastern North America. The limited number of museum specimens of Mesoamerican flying squirrels has made discerning the systematic and biogeographic relationships of these populations a challenge. We used ancient DNA techniques to extract, amplify, and sequence a 571-base pair segment of the mitochondrial DNA cytochrome-*b* gene from 22 of 34 available museum specimens. Mesoamerican flying squirrel data were combined with homologous sequences from representative populations of *Glaucomys* from the United States and Canada. This combined data set was analyzed using maximum-likelihood and Bayesian methods. Results indicate that *G. volans* is monophyletic and contains 2 monophyletic subclades, 1 from Mesoamerica and the other from eastern North America. Our results have important implications regarding the nature of the historical biogeographic connection between the temperate biotas of Mesoamerica and eastern North America. The divergence of populations of *G. volans* in eastern North America from those in Mesoamerica appears to have occurred in the middle Pleistocene (approximately 0.75–0.5 $\times$ 10^6 years ago), considerably earlier than a late-Pleistocene connection previously hypothesized. Our analyses also show that populations of *G. volans* from eastern North America exhibit a clear signature of recent, rapid population expansion and that Mesoamerican populations of *G. volans* exhibit higher levels of genetic variability than those found across eastern North America. The documentation of substantial genetic diversity and population structure in Mesoamerican populations of *G. volans* is especially noteworthy because these populations face ongoing habitat loss due to human activities. Anthropogenic habitat degradation of the high-elevation forests these mammals inhabit likely will be exacerbated by global climate change. Therefore, we suggest that the conservation status of Mesoamerican flying squirrels be considered data deficient at a minimum with a high potential for future studies to reveal that many populations are near threatened or vulnerable. DOI: 10.1644/09-MAMM-A-260.1.

Key words: biogeography, cytochrome *b*, flying squirrels, *Glaucomys volans*, Mesoamerica, mitochondrial DNA, phylogeography, Pleistocene

Over the last decade molecular phylogenetic studies have greatly clarified the systematics and biogeography of the New World flying squirrels (genus *Glaucomys*—Arbogast 1999; Bidlack and Cook 2001, 2002; Demboski et al. 1998; Petersen and Stewart 2006). These studies indicate that at least 3 distinct evolutionary lineages of *Glaucomys* exist in North America (2 within the northern flying squirrel [*G. sabrinus*] and 1 corresponding to populations of the southern flying squirrel [*G. volans*] from the eastern United States and southeastern Canada); and that the evolutionary history of *Glaucomys* appears to have been shaped importantly by Quaternary changes in the geographic distributions of the

Fig. 1.—Geographic distribution of *Glaucomys volans* in North America and Mesoamerica. Eastern North American subspecies are 1) *G. v. volans*, 2) *G. v. texensis*, 3) *G. v. saturatus*, and 4) *G. v. querceti*. Geographic distributions of Mesoamerican subspecies are shown in Fig. 2. Adapted from Dolan and Carter (1977) and Wilson and Ruff (1999).

coniferous and deciduous forest biomes of North America. However, a critical gap remains in our knowledge of the evolution and biogeography of New World flying squirrels because no previous molecular phylogenetic study has included populations from the highlands of Mesoamerica (Mexico to Honduras). Only 6 populations and approximately 65 specimens of Mesoamerican flying squirrels are known, all from high-elevation oak and pine–oak forests of Mexico, Guatemala, and Honduras (Dolan and Carter 1977; Fig. 1). Analysis of morphological traits suggests that these populations represent 4–6 disjunct subspecies of *G. volans* (Braun 1988; Diersing 1980; Goodwin 1961). However, the evolutionary relationships among populations of Mesoamerican flying squirrels, and their relationships to populations of *Glaucomys* from North America, remain enigmatic due to the small number of specimens, sparse distributional information, and lack of genetic data. Furthermore, no published data exist on genetic diversity, population dynamics, or conservation status of Mesoamerican flying squirrels. This lack of information is especially troubling given that much of the high-elevation pine–oak forests these flying squirrels inhabit have suffered extensive damage over the last century. The major impact has been from human land use, in which large areas of these highlands have been deforested for commercial logging, cultivation, and grazing. Consequently, the conservation status of pine–oak forests found in the Mesoamerican highlands is considered critical or endangered (Olson and Dinerstein 2002).

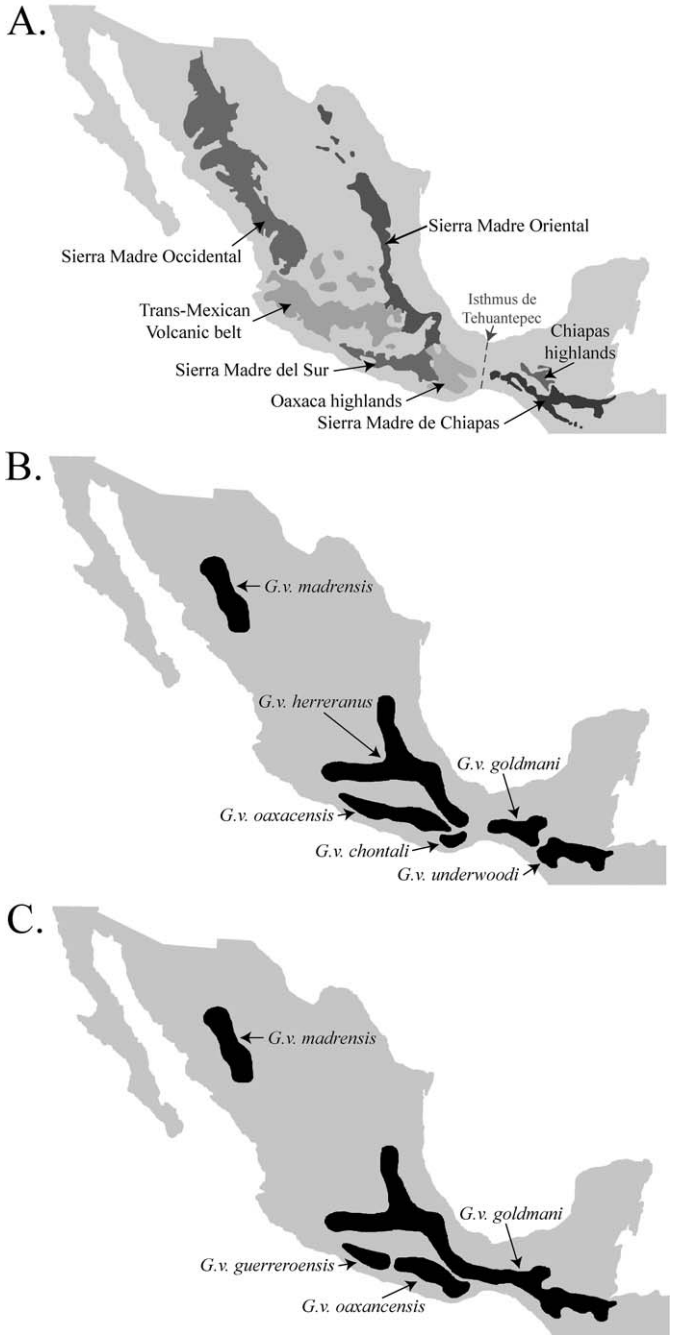

Fig. 2.—A) Generalized distribution of Mesoamerican oak and pine–oak forests shown in shades of gray. Labels refer to the 7 mountain and highland regions referred to in this study, and the Isthmus de Tehuantepec is indicated by a dashed line. B) Aerial view of Mesoamerica, with shaded areas representing the 6 Mesoamerican subspecies of *Glaucomys volans* recognized by Goodwin (1961). C) Aerial view of Mesoamerica, with shaded areas representing the 4 Mesoamerican subspecies of *G. volans* recognized by Diersing (1980).

The oak and pine–oak forests of Mesoamerica occur as a series of disjunct, high-elevation ''islands'' ranging from Mexico to Honduras (Fig. 2A). In Mexico this includes the Sierra Madre Occidental, Sierra Madre Oriental, Trans-Mexican Volcanic Belt, Sierra Madre del Sur, Oaxaca highlands, Chiapas highlands, and the Sierra Madre de

Table 1.—Collection localities, source museums, and GenBank accession numbers for *Glaucomys* samples used in this study. Boldface accession numbers denote samples for which the entire 571-bp segment was sequenced or available via GenBank. Museum abbreviations are as follows: Field Museum of Natural History (FMNH), Kansas University Natural History Museum (KU), Louisiana State University Museum of Natural Science (LSU), University of Alaska Museum (UAM), National Museum of Natural History (NMNH), and University of Washington Burke Museum of Natural History and Culture (UWBM). SDP = S. D. Peterson voucher numbers listed in GenBank. Abbreviations for Mesoamerican populations are given in Fig. 3.

Species	Country	State or province	Locality	GenBank accession no.(s)	Museum accession no.(s)
G. volans	Guatemala	Chimaltenango	Tecpan (SMdC)	GU473172, GU473184, GU473173	FMNH-41762, **FMNH-41763**, FMNH-41764
G. volans	Mexico	Chiapas	Comitian (ChiHi)	GU473174, GU473175, GU473185	KU-61315, KU-61316, **KU-61317**
G. volans	Mexico	Chiapas	Ococingo (ChiHi)	GU473187, GU473188, GU473176, GU473189, GU473190	**FMNH-64179**, **FMNH-64181**, FMNH-64182, **FMNH-64183**, **FMNH-64185**
G. volans	Mexico	Chiapas	San Christobal de las Casas (ChiHi)	GU473186, GU473191, GU473177, GU473192, GU473193, GU473194	**KU-61318**, **KU-66571**, KU-66573, **KU-66574**, **KU-66575**, **KU-66576**
G. volans	Mexico	Chiapas	Teopisca (ChiHi)	GU473195, GU473196	**KU-68833**, **KU-68834**
G. volans	Mexico	Guerrero	Agua del Obisbo (SMdS)	GU473197	**KU-99758**
G. volans	Mexico	Guerrero	Omilteme (SMdS)	GU473198, GU473199, GU473200, GU473201, GU473202, GU473203	**NMNH-329701**, **NMNH-329702**, **NMNH-329703**, **NMNH-329704**, **NMNH-329705**, **NMNH-329706**
G. volans	Mexico	San Luis Potosi	Santa Barbarita (SMOr)	GU473182, GU473183	**LSU-4189**, **LSU-4190**
G. volans	Canada	Nova Scotia	Kejimikujik National Park	AY942859	SDP SP00-025
G. volans	Canada	Nova Scotia	Middleton	AY942860, AY942862	SDP SP01-171, SDP SP01-177
G. volans	Canada	Nova Scotia	Wallbrook	AY942861	SDP SP01-176
G. volans	Canada	Ontario	Ontario	AY942863, AY42864, AY942865	SDP EB02-002, EB02-004, EB02-009
G. volans	United States	Florida	Highland County	AY703890, GU473204, GU473205, AY703884, AY703889, GU473206, AY703887, GU473207, AY703885, GU473208	FL-3,[a] **FL-6**,[a] **FL-8**,[a] FL-10,[a] FL-11,[a] **FL-100**,[a] FL-101,[a] **FL-102**,[a] FL-103,[a] **FL-104**[a]
G. volans	United States	Louisiana	East Baton Rouge Parish	GU473178, GU473179	LSU M-5768, LSU M-1957
G. volans	United States	Missouri	Girardeau County	GU473209, GU473210, GU473211, GU473212	**AJS-059**,[a] **AJS-060**,[a] **AJS-061**,[a] **AJS-062**[a]
G. volans	United States	Pennsylvania	Westmoreland County	GU473213, GU473214, GU473215, GU473216, GU473180, AY703893, GU473181, AY703894	**PA-2**,[a] **PA-5**,[a] **PA-7**,[a] **PA-9**,[a] PA-12,[a] PA-13,[a] PA-14,[a] PA-15[a]
G. volans	United States	Tennessee	Carter County	AF63063, AF63065	LSU M-5765, LSU M-5766, LSU M-5767
G. volans	United States	Texas	Harrison County	GU473217, GU473218	**NMNH-569025**, **NMNH-569026**
G. sabrinus	Canada	Alberta	Near Edmonton	GU473219, GU473220	**LSU-M3442**, **LSU-M3444**
G. sabrinus	United States	Alaska	Anita Bay	AF359213	**UAM-53845**
G. sabrinus	United States	Alaska	Dall Island	AF359238	**UAM-51181**
G. sabrinus	United States	Alaska	Heceta Island	AF271672	**UAM-38309**
G. sabrinus	United States	Alaska	Helm Bay	AF359217, AF359218, AF271669	**UAM-49445**, **UAM-49453**, **UAM-54550**
G. sabrinus	United States	Alaska	Juneau	AF271668, AF359220	**UAM-54640**, **UAM-50360**
G. sabrinus	United States	Alaska	Klondike Gold Rush National Park	AF359211	**UAM-35160**
G. sabrinus	United States	Alaska	Kosciusko Island	AF359232	**UAM-51180**
G. sabrinus	United States	Alaska	Prince of Wales Island	AF271670	**UAM-51093**
G. sabrinus	United States	Alaska	Revillagigedo Island	AF271673, AF359216	**UAM-50629**, **UAM-49443**
G. sabrinus	United States	Alaska	Suemez Island	AF359233, AF359234, AF359235, AF359236, AF359237	**UAM-48328**, **UAM-48332**, **UAM-48425**, **UAM-48430**, **UAM-48443**
G. sabrinus	United States	Alaska	Wrangell Island	AF359212	**UAM-68888**
G. sabrinus	United States	Idaho	Latah County	GU473232	**JRD-025**[b]
G. sabrinus	United States	Michigan	Alger County	GU473221	**LSU-M5760**
G. sabrinus	United States	North Carolina	Mitchell County	GU473223	**LSU-M5748**

TABLE 1.—Continued.

Species	Country	State or province	Locality	GenBank accession no.(s)	Museum accession no.(s)
G. sabrinus	United States	Utah	Summit County	GU473233, GU473234	**LSU-M3013**, **LSU-M3014**
G. sabrinus	United States	West Virginia	Webster County	GU473222	**LSU-M5722**
G. sabrinus	United States	California	Plumas County	GU473225, GU473226	**HSU VM-2547**,[b] **HSU VM-2562**[b]
G. sabrinus	United States	California	San Bernardino County	GU473224	**LSU-M5742**
G. sabrinus	United States	Oregon	Douglas County	GU473227, GU473228	**LSU-M5732**, **LSU-M5736**
G. sabrinus	United States	Washington	Jefferson County	GU473229	**UWBM-75766**
G. sabrinus	United States	Washington	Kittitas County	AF030389	**UAM-35106**
G. sabrinus	United States	Washington	Pierce County	GU473229, GU473231	**LSU-M5727**, **LSU-M5709**
E. fimbriatus	Kashmir			AB126248	
H. phayrei	Thailand			AB126252	
P. setosus	Indochina peninsula			AB030260	

[a] Blood samples, no voucher specimen.
[b] Humboldt State University specimens not yet accessioned.

Chiapas, which extends from Chiapas, Mexico, to Honduras. These disjunct regions of high-elevation oak and pine–oak habitats are considered to be remnants of more contiguous forests that formerly extended throughout lower elevation areas during cooler periods of the Pleistocene (Dolan and Carter 1977; Martin and Harrell 1957). Recent phylogeographic studies of mammals inhabiting this region (e.g., *Reithrodontomys*, *Peromyscus*, *Habromys*, *Cratogeomys*, *Pappogeomys*, and *Neotoma*) have found high levels of genetic diversity within and between the high-elevation mountain ranges of Mesoamerica and in many cases have revealed the presence of cryptic species (Carleton et al. 2002; Demastes et al. 2002; Edwards and Bradley 2002; Harris et al. 2000; Sullivan et al. 2000). Thus, it seems likely that additional cryptic species and subspecies of mammals inhabit the Mesoamerican highlands, awaiting scientific documentation.

The Isthmus de Tehuantepec (Fig. 2A), a lowland region separating the Sierra Madre de Chiapas and Chiapas highlands from the remaining mountain ranges in northern and western Mexico, also appears to have played an important role in the evolutionary diversification of several groups of small mammals in Mesoamerica (Carleton et al. 2002; Sullivan et al. 2000). For example, Sullivan et al. (2000) found that populations of *Reithrodontomys sumichrasti*, *Peromyscus aztecus*, and *P. hylocetes* divided by the Isthmus de Tehuantepec formed distinct mitochondrial DNA (mtDNA) clades. Populations of *Reithrodontomys microdon* also were found to form distinct clades on each side of the Isthmus (Arellano et al. 2005). As such, it might be expected that Mesoamerican flying squirrels also would exhibit a phylogeographic break at the Isthmus de Tehuantepec.

Because of the discontinuous nature of the high-elevation oak and pine–oak habitats in Mesoamerica, and the presence of flying squirrels both north and south of the Isthmus de Tehuantepec, the number and geographical boundaries of distinct evolutionary lineages of Mesoamerican flying squirrels are unclear. Using morphological differences, Goodwin (1961) proposed that Mesoamerican populations of *G. volans* could be divided into 6 subspecies: *G. v. chontali* from the southeastern portion of the Sierra Madre del Sur, *G. v. goldmani* from the northern Sierra Madre de Chiapas, *G. v. underwoodi* from the southern Sierra Madre de Chiapas, *G. v. herreranus* from the southern Sierra Madre Oriental, *G. v. madrensis* from the middle Sierra Madre Occidental, and *G. v. oaxacensis* from the eastern and northwestern Sierra Madre del Sur (Fig. 2B). Subsequent investigation led Diersing (1980) to propose that *G. v. herreranus* and *G. v. underwoodi* be synonymized with *G. v. goldmani*; eastern populations of *G. v. oaxacensis* be combined with *G. v. chontali* under the *G. v. oaxacensis* subspecies epithet; and westernmost populations of *G. v. oaxacensis* be recognized as a new subspecies, *G. v. guerreroensis* (Fig. 2C).

Previous biogeographic studies suggest that populations of both *Glaucomys* species were forced into southern refugia in response to glacial advances during the Pleistocene (Arbogast 1999; Arbogast et al. 2005; Dolan and Carter 1977). Using

genetic data to examine the systematics of flying squirrels north of Mexico, Arbogast (1999) found 3 distinct evolutionary lineages: a widespread continental lineage of *G. sabrinus*, a more restricted lineage of *G. sabrinus* confined to the Pacific coast, and a lineage comprising *G. volans* populations from eastern North America. The continental *G. sabrinus* lineage appears to have persisted in 1 or more coniferous forest refugia in the southeastern United States during glacial maxima. Consistent with this hypothesis, the most basal members of the continental lineage of *G. sabrinus* are those presently restricted to high-elevation mountaintops in the southern Appalachian Mountains, at the extreme southeastern edge of the species' range (Arbogast 1999). *G. volans* appears to have followed a similar trend, migrating to 1 or more southern refugia in the southern United States or Mesoamerica, or both. Arbogast (1999) noted surprisingly low levels of within-species mtDNA variation in populations of *G. volans* from across the eastern United States, a pattern that is consistent with a recent population expansion following a severe bottleneck. However, the lack of genetic data on flying squirrels from Mesoamerica has made it impossible to evaluate an equally plausible hypothesis: that the lack of genetic variation in populations of *G. volans* in eastern North America is due to rapid population expansion following a recent founder event from Mesoamerica. Thus, determining the evolutionary position of Mesoamerican flying squirrels within *Glaucomy*s has important implications for understanding the biogeographic history of this genus and the forest systems with which they are associated.

The use of molecular markers provides an opportunity to examine geographic patterns of genetic variation in Mesoamerican flying squirrels and help clarify the systematics and biogeography of the group. The cytochrome-*b* (*Cytb*) gene of mtDNA is widely used in molecular phylogenetic studies of mammals. Mitochondrial markers are well suited for examining intrageneric and intraspecific phylogenetic relationships because they undergo rapid geographic sorting. This is due to several factors, principally the relatively high mutation rate and small effective population size of the mtDNA genome relative to the nuclear genome (Avise 2000). In particular, the widespread use of the *Cytb* gene has led to a wealth of data on levels of genetic diversity in mammals and estimated rates of molecular evolution for this region of mtDNA (Bradley and Baker 2001; Irwin et al. 1991). When used in conjunction with corrected estimates of genetic divergence between 2 evolutionary lineages, these rates of mutation can be used to estimate the approximate time of divergence between 2 lineages (Arbogast et al. 2002; Avise 2000). Extensive use of the *Cytb* gene region of mtDNA in previous phylogenetic analyses of flying squirrels (Arbogast 1999; Arbogast et al. 2005; Bidlack and Cook 2001; Demboski et al. 1998; Petersen and Stewart 2006; Yu et al. 2006) also makes it an attractive marker for this study because it allows new data to be combined with existing data to provide a broad evolutionary and geographic framework for analyzing the systematics and biogeography of Mesoamerican flying squirrels.

We used mtDNA *Cytb* sequences generated from museum specimens to address 4 major goals: to determine the phylogenetic and biogeographic relationship of Mesoamerican flying squirrels to other populations of *Glaucomys* in North America; to determine the number of genetically distinct flying squirrel lineages in Mesoamerica; to evaluate whether genetic data support subspecies boundaries based on morphology; and to evaluate whether Mesoamerican flying squirrels exhibit the same phylogeographic break at the Isthmus de Tehuantepec as observed in other small mammals.

Materials and Methods

Samples.—All samples of Mesoamerican flying squirrels examined in this study ($n = 34$) came from museum specimens (Table 1). Remaining samples came from previously extracted template (43 samples of *G. volans* and *G. sabrinus* from north of Mexico—see Arbogast [1999] and Arbogast et al. [2005] for methods) and frozen tissues (2 individuals of *G. volans* from Texas). Finally, 28 sequences of *G. sabrinus* were obtained from GenBank (Bidlack and Cook 2001; Cook et al. 2001; Demboski et al. 1998). For phylogenetic analyses we used all individuals of *Glaucomys* for which we had the complete 571-base pair (bp) segment of the *Cytb* gene ($n = 73$). When calculating genetic diversity, analysis of molecular variance (AMOVA), Fu's *F*-statistic, and mismatch distributions, we used a 315-bp subset of the 571-bp segment; this truncation allowed us to include 28 Mesoamerican samples plus previously published sequences from an additional 21 individuals of *G. volans* for which only a 315-bp segment had been sequenced (Arbogast 1999; Arbogast et al. 2005; Petersen and Stewart 2006). As a result, we were able to use a total of 64 individuals of *G. volans* in the AMOVA, Fu's *F*-statistic, and mismatch distributions calculations for this species.

The risk of contamination is elevated when working with low-yield DNA from museum specimens (Hofreiter et al. 2001). To minimize this risk all extractions and polymerase chain reactions were conducted in a facility dedicated to low template procedures. Polymerase chain reaction thermal-cycling, gel visualization, and polymerase chain reaction product storage were conducted in a separate facility. Negative controls were included throughout all aspects of the extraction and polymerase chain reaction process.

DNA extractions.—Extraction of DNA from museum skin samples of Mesoamerican flying squirrels was performed in a Labconco Purifier/filtered hood with ultraviolet (Labconco Corp., Kansas City, Missouri) irradiation located in a designated low-copy laboratory at Humboldt State University. The hood and all tools were wiped down with RNase AWAY (Sigma-Aldrech Corp., St. Louis, Missouri) and irradiated for 30 min with ultraviolet light both before and after each use. A small portion (1–2 mm^3) of each museum specimen skin sample was subsampled and used for DNA extraction. Sterilized scissors and forceps were used to clip the subsample and place it into a 1.5-ml microcentrifuge tube. Between each

Table 2.—Primers used to amplify 4 overlapping segments of the cytochrome-*b* gene for members of the genus *Glaucomys*. The primer L14724 is from Irwin et al. (1991); all other primers were designed for this study.

Name	Sequence
L14724	5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′
Gvo-L2	5′-TCGTTCAAATCGTCACAGGA-3′
Gvo-L3	5′-AGGCCGAGGACTCTATTATG-3′
Gvo-L4	5′-GAGGACAAATATCATTCTGAG-3′
Gvo-R1	5′-GAGGAGAAGGCGGTTATTGTG-3′
Gvo-R2	5′-ACTCCAATGTTTCAGGTTTC-3′
Gvo-R3	5′-CCTCAGATTCATTCTACAAG-3′
Gvo-R4	5′-GATGGGTTATTGGATCCTGTTT-3′

sample, scissors and forceps were flamed, the laboratory bench was wiped with RNase AWAY, and gloves were changed. Before lysis, samples were exposed to a 24-h ethanol wash regime consisting of 1 ml of 100% ethanol being changed every 3 h for 24 h. These ethanol washes were used to leach out contaminants held over from specimen preparation and preservation and to remove surface contaminants. To facilitate the release and subsequent removal of any surface contaminants, samples were vortexed vigorously for 15 s before and after each ethanol exchange. Upon completion of the final wash cycle, the ethanol was removed and samples were air-dried in the hood. Once dry, DNA was extracted following the standard DNeasy Kit tissue extraction protocol (Qiagen Inc., Valencia, California) with the following exceptions: buffer AE was diluted 1:10 and preheated to 70°C, the 1st elution was 50 μl, and the 2nd elution 100 μl with a 10-min period between adding the elution buffer and centrifuging the sample. Washes and extractions were performed on 8 or fewer samples at any given time, with 1 of those samples being a negative control.

To keep high-copy DNA from coming into contact with low-copy DNA, frozen tissue samples from fresh tissues (i.e., Texas samples) were extracted in a separate laboratory and building than that used for the museum skin samples. All work with previously extracted samples also was conducted in this ''high-copy'' facility. The standard DNeasy Kit tissue extraction protocol was used for the fresh tissue samples.

Polymerase chain reaction and sequencing.—Polymerase chain reaction was used to amplify a 571-bp segment of the mitochondrial *Cytb* gene. To amplify the entire 571-bp segment from the museum skin samples of Mesoamerican flying squirrels, 4 small (<250-bp), overlapping internal segments were amplified. To achieve this we used 1 universal primer (Irwin et al. 1991) and 7 *Glaucomys*-specific primers that we designed (Table 2). The conservative nature of the *Cytb* gene allowed the design of perfect or near perfect primers. Primer design was aided by the following programs: Primer3 (Rozen and Skaletsku 2000), Amplify 3X (Engels 1993), and the Sequence Manipulation Suite (Stothard 2000). The entire 571-bp segment was amplified in 1 reaction for samples from high-quality DNA extractions from previous studies (Arbogast 1999; Arbogast et al. 2005) and frozen tissue samples.

Polymerase chain reactions consisted of 12.5 μl of AmpliTaq Gold PCR master mix (Applied Biosystems, Foster City, California), 7.5–8 μl of double-distilled H_2O, 2 μl of each primer, and 0.5–1 μl of template for a total of 25 μl. Thermal-cycling parameters were: denaturation at 94°C for 5 min, followed by 50 cycles with a denaturation of 94°C for 1 min, an annealing temperature of either 40°C or 45°C for 1 min 30 s, and an extension of 72°C for 1 min. This was followed by a final 5-min extension at 72°C. Agarose (2%) gels were used to visualize polymerase chain reaction products. All polymerase chain reaction cleanup and sequencing was outsourced to the High-Throughput Genomics Unit (Seattle, Washington) with the exception of 4 samples that were purified using the QIAquick PCR purification kit (Qiagen Inc.) and sequenced at the CSUPERB Microchemical Core Facility (San Diego, California).

Data analysis.—Sequence data quality was visually inspected using the program FinchTV (Geospiza Inc., Seattle, Washington). Special care was taken to check for the presence of multiple chromatogram peaks that could indicate contamination. Consolidation of overlapping sequences and alignment was performed by hand using the program Se-Al (version 2.0—Rambaut 1995). Only unique haplotypes were used in phylogenetic analyses; duplicate haplotypes were detected using Collapse (version 1.2; http://darwin.uvigo.es). Molecular phylogenies were inferred using Bayesian and maximum-likelihood (ML) methods implemented in MrBayes (version 3.2-cvs—Huelsenbeck and Ronquist 2001) and PAUP* (version 4.0b—Swofford 2003), respectively. Best-fit models of nucleotide substitution for the Bayesian and ML analyses were selected using the Akaike information criterion (AIC) and the programs MrModeltest (version 2.2—Nylander 2004) and Modeltest (version 3.7—Posada and Crandall 1998).

Bayesian and ML phylogenetic trees were rooted initially using homologous *Cytb* sequences from 3 species of Eurasian flying squirrels (*Eoglaucomys fimbriatus*, *Hylopetes phayrei*, and *Petinomys setosus*) obtained from GenBank (Table 1; Fig. 3). Bayesian analysis consisted of 2 concurrent, independent runs with different starting trees. Four chains, 3 heated and 1 cold, were used to improve Markov chain Monte Carlo sampling of the target distribution. Sampling consisted of 2×10^6 Markov chain Monte Carlo generations sampled every 100 generations. This sampling regime resulted in an average standard deviation of split frequencies below 0.01, indicating that the 2 runs had converged. The first 5,000 trees constructed were omitted as burn-in. Nodal support of Bayesian phylogenetic trees was assessed using posterior probabilities.

The same 3 species of Eurasian flying squirrels used as outgroups in the Bayesian analysis also were used in the ML analysis. Starting trees were constructed using random addition of taxa. A heuristic search with tree-bisection-reconstruction branch swapping was used to find the best phylogenetic tree under the ML criterion. To evaluate nodal support 1,000 full-heuristic ML bootstrap replicates were performed (Felsenstein 1985). We tested whether the data

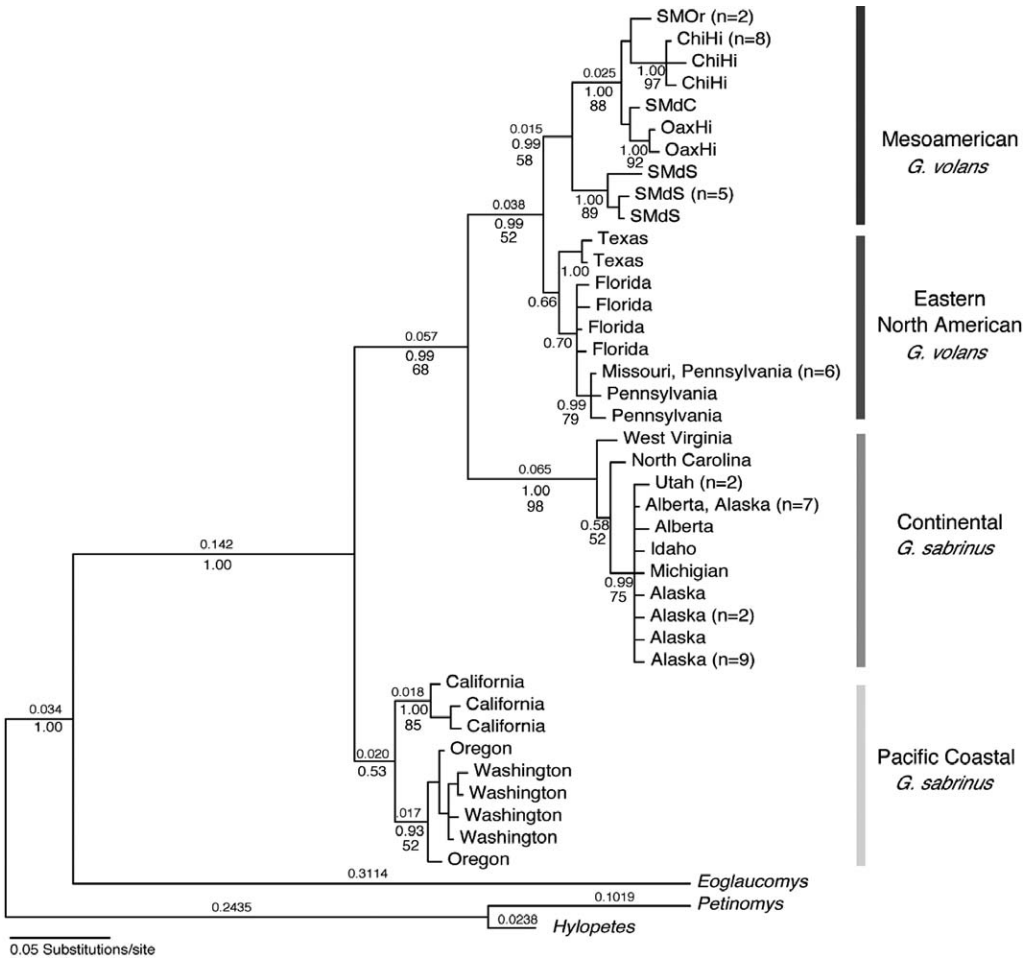

FIG. 3.—Phylogram constructed using Bayesian analysis, rooted with members of 3 genera of Asian flying squirrels. Numbers above branches represent branch lengths. The 1st number below a branch is the Bayesian posterior probability score. Maximum-likelihood (ML) scores >50 are shown below the Bayesian probability scores. Outgroup-rooted ML analysis produced an identical topology with the exception that the position of the sample from the Sierra Madre de Chiapas was not resolved with respect to samples from the Sierra Madre Oriental, Chiapas highlands, and Oaxaca highlands, and the monophyly of the Pacific coastal clade of *G. sabrinus* was not resolved in the ML analysis. Mesoamerican abbreviations: SMOr = Sierra Madre Oriental, ChiHi = Chiapas highlands, SMdC = Sierra Madre de Chiapas, OaxHi = Oaxaca highlands, and SMdS = Sierra Madre del Sur.

were consistent with a molecular clock using a likelihood ratio test (Huelsenbeck and Rannala 1997).

Because of the relatively large divergence of the Eurasian flying squirrels from *Glaucomys*, we also conducted an ML analysis wherein members of the continental clade of *G. sabrinus* were used to root a tree of all haplotypes of *G. volans* included in the study. This allowed us to examine whether the use of remote outgroups was influencing phylogenetic relationships or nodal support, or both, within *G. volans*. Except for changing the outgroup taxa, the same procedures described above were used for this ML analysis, including the performance 1,000 full-heuristic ML bootstrap replicates to evaluate nodal support.

To estimate approximate dates of divergence for important nodes in the phylogeny, we 1st estimated pairwise sequence divergence values using the Kimura 2-parameter model (Kimura 1980) implemented in PAUP* (version 4.0b—Swofford 2003). This model was used so that sequence divergence values could be compared to previous studies of *Glaucomys* and other mammals (Arbogast 1999; Bradley and Baker 2001). To estimate approximate dates of divergence we used estimated levels of sequence divergence at 3rd codon positions only and a corresponding rate of sequence divergence for 3rd codon positions of 10–15%/10^6 years (Arbogast 1999).

An AMOVA, mismatch distribution (the distribution of pairwise differences between haplotypes), Fu's *F*-statistic (Fu 1997), and nucleotide diversity were calculated using the program Arlequin (version 2.000—Excoffier et al. 2005). For these analyses we treated samples of *G. volans* from Mesoamerica and those from eastern North America as 2 separate groups. The AMOVA allowed us to assess if significant population structuring existed between these 2 groups. Fu's *F*-statistic provides information on demographic history, with significantly negative values associated with rapid population expansions. Similarly, the shape of the mismatch distribution provides insights into whether a population has had a relatively stable demographic history

or has undergone a recent population expansion (Rogers and Harpending 1992). Finally, nucleotide diversity permits a direct comparison of genetic variability between the 2 groups.

Results

The 73 individuals used in the initial Bayesian and ML analyses exhibited 39 unique haplotypes. These unique haplotypes were nearly evenly distributed among *G. volans* ($n = 19$) and *G. sabrinus* ($n = 20$). By using a combination of several primer pairs (Table 2) we obtained the entire 571-bp segment for 22 of the 34 Mesoamerican samples. Of these 22 samples, we found 10 unique haplotypes—Sierra Madre Occidental ($n = 1$), Oaxaca highlands ($n = 2$), Sierra Madre de Chiapas ($n = 4$), and Sierra Madre del Sur ($n = 3$)—representing 8 collection localities and 4 major geographic areas. Chromatograms of the sequence data had Phred quality scores >20, minimal background noise, and no double peaks. No unexpected stop codons were found, and all overlapping sequences for a given individual were identical.

The best-fit model of nucleotide substitution selected by the AIC criterion for the data set with outgroups was the GTR+G in both Modeltest and MrModeltest. Base frequencies were A = 0.25, C = 0.28, G = 0.15, and T = 0.33, with a gamma correction value of 0.2182. A likelihood ratio test found no significant difference between phylogenetic trees with and without a molecular clock enforced (−ln likelihood clock enforced = 1,639.5360, clock not enforced = 1,663.2296; $\chi_{37}^2 = 47.3872$, $P = 0.1178$).

The outgroup-rooted Bayesian and ML analyses produced similar phylogenies, albeit with different levels of resolution. Bayesian probability scores for nodes were generally much higher than ML bootstrap scores (Fig. 3). Both methods recovered a widespread continental clade of *G. sabrinus* and a sister clade containing all individuals of *G. volans*. In both analyses the monophyly of *G. volans* was well supported. Within *G. volans*, populations from eastern North America and those from Mesoamerica formed reciprocally monophyletic sister clades. In the eastern North American clade, haplotypes from Texas were basal and those from Missouri and Pennsylvania were nested within the multiple haplotypes found in Florida. Within Mesoamerica, haplotypes from the Sierra Madre del Sur represent the earliest divergence and are sister to a group of haplotypes from all other Mesoamerican populations. In both analyses populations from the Sierra Madre Oriental were sister to populations from the Chiapas highlands. In Bayesian analysis populations from the Sierra Madre de Chiapas were sister to those in the Oaxaca highland (Fig. 3). This sister relationship was not recovered in the ML analysis. Otherwise, the topologies of the Bayesian and ML trees were identical for *G. volans*. In terms of relationships within *G. sabrinus*, the Bayesian and ML trees were identical with the exception that the latter did not resolve a monophyletic Pacific coastal clade with respect to the outgroup taxa.

To investigate phylogenetic relationships and nodal support within *G. volans* in more detail, we removed the Eurasian flying squirrel taxa and members of the Pacific coastal clade of *G. sabrinus* and conducted a 2nd ML analysis with members of the continental clade of *G. sabrinus* assigned as outgroup taxa (Fig. 4). This choice was based on the strongly supported sister relationship of the continental clade of *G. sabrinus* to *G. volans* observed in this (Fig. 3) and previous studies (Arbogast 1999; Arbogast et al. 2005). The best-fit model of nucleotide substitution selected by the AIC criterion for this reduced data set was the GTR+G. Base frequencies were A = 0.25, C = 0.26, G = 0.15, and T = 0.34, with a gamma correction value of 0.1823. Results of this ML analysis revealed strong support for the monophyly of *G. volans*, and increased support for the reciprocal monophyly of the Mesoamerican and eastern North American populations of *G. volans*.

Based on the Kimura 2-parameter model, estimated levels of pairwise sequence divergence between populations of *G. volans* from Mesoamerica and eastern North America ranged from 2.3% to 4.9% for all positions and from 7.3% to 15.7% when only 3rd positions were included (Table 3). Estimated levels of pairwise sequence divergence between individuals representing different Mesoamerican mountain and highland locations ranged from 0.7% to 4.4% for all positions and from 2.2% to 13.1% for 3rd positions only. Many of these comparisons revealed a greater amount of sequence divergence between the different Mesoamerican highland populations than was observed between recognized subspecies of *G. volans* from eastern North America (Table 4; Fig. 1).

Results of the AMOVA revealed significant population structuring between populations of *G. volans* from eastern North America and Mesoamerica (pairwise $F_{st} = 0.188$, $P < 0.00001$). The mismatch distribution for the pooled populations of *G. volans* from eastern North America was unimodal and the Fu's *F*-statistic was significant and negative ($F_s = -3.561$, $P = 0.031$). We did not calculate a mismatch distribution or estimate Fu's *F*-statistic for the Mesoamerican *G. volans* because the disjunct and highly structured nature of the populations we sampled precluded us from pooling them into a single population. Despite having a much smaller and more fragmented geographic distribution, populations of *G. volans* from Mesoamerica exhibited higher nucleotide diversity than did populations from across all of eastern North America (0.823 versus 0.735, respectively).

Discussion

Phylogenetic relationships within Glaucomys.—The phylogenetic analyses presented here agree with previous morphological studies (Braun 1988; Diersing 1980; Goodwin 1961) indicating that Mesoamerican flying squirrels are southern, disjunct populations of *G. volans* (Figs. 3 and 4). The phylogenetic analyses also suggest that Mesoamerican flying squirrels and those from eastern North America are reciprocally monophyletic sister groups. This relationship was consistently recovered in all phylogenetic analyses; however, corresponding values of nodal support were variable (Figs. 3

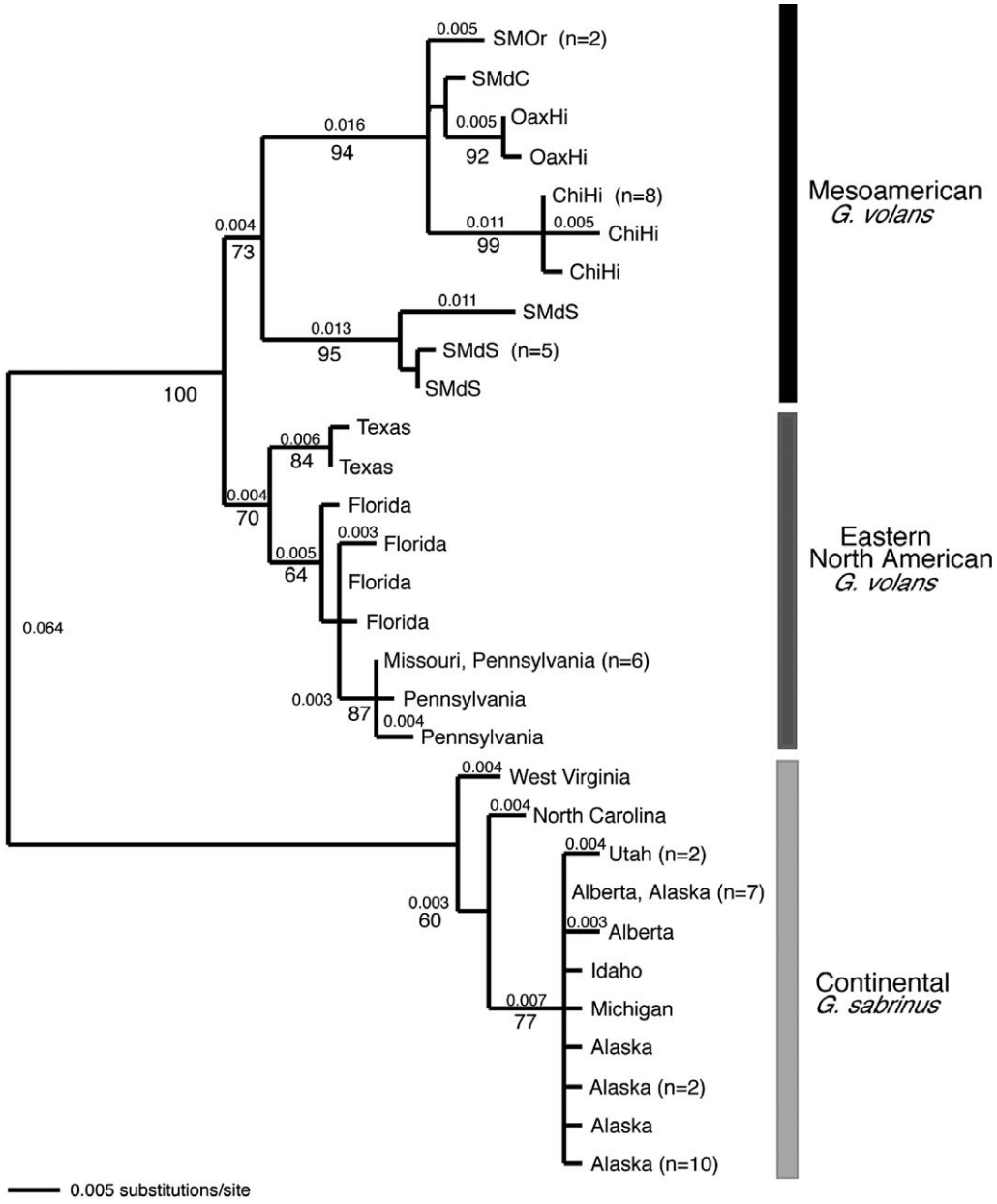

Fig. 4.—Phylogram of haplotypes of *Glaucomys volans* constructed using maximum-likelihood (ML) analysis, rooted with haplotypes from the continental lineage of *G. sabrinus* (see text for details). Numbers above branches represent branch lengths. ML bootstrap scores (based on 1,000 full heuristic replicates) are shown below each branch.

and 4). For example, although the Bayesian probability score for the monophyly of the Mesoamerican clade was 0.99 in the tree rooted with the Eurasian taxa of flying squirrels, this node received ML bootstrap support of <50 (Fig. 3). Similarly, the node defining the monophyly of the eastern North American samples of *G. volans* also received ML bootstrap support of <50. In this case the Bayesian probability score for this node also was low (0.66). To examine whether the use of distantly

Table 3.—Minimum and maximum values of percent sequence divergence based on the Kimura 2-parameter model between major evolutionary lineages of *Glaucomys volans* examined in this study.

Lineages	All positions		Third position only	
	Minimum	Maximum	Minimum	Maximum
Mesoamerica versus eastern North America	2.32	4.94	7.28	15.72
Sierra Madre del Sur versus all other Mesoamerica	2.88	4.38	7.90	11.00

Table 4.—Minimum and maximum values of percent sequence divergence based on the Kimura 2-parameter model A) between 3 North American subspecies of *Glaucomys volans* and B) between all Mesoamerican highland populations of *G. volans* examined in this study. Minimum values are given above the diagonal and maximum values are given below. Abbreviations for Mesoamerican populations are given in Fig. 3.

A. North American subspecies	*G. v. volans*	*G. v. texensis*	*G. v. querceti*		
G. v. volans		1.59	0.35		
G. v. texensis	2.14		1.24		
G. v. querceti	1.06	1.60			
B. Mesoamerican populations	SMOr	ChiHi	SMdC	OaxHi	SMdS
SMOr		1.60	0.88	1.24	3.44
ChiHi	2.15		1.42	1.60	4.00
SMdC	0.88	1.96		0.71	2.88
OaxHi	1.42	2.33	0.88		3.25
SMdS	4.00	4.38	3.44	4.19	

related outgroups might have been influencing levels of nodal support within *G. volans*, we conducted a 2nd ML analysis using members of the continental clade of *G. sabrinus* as designated outgroups (Fig. 4). This choice was based on the outgroup-rooted ML and Bayesian analyses, which recovered strong support for a monophyletic *G. volans* clade and its sister relationship to the continental clade of *G. sabrinus* (Fig. 3); this result has been found in previous studies (Arbogast 1999; Arbogast et al. 2005). Subsequently, ML bootstrap scores improved markedly, with both the Mesoamerican clade and eastern North American clade of *G. volans* receiving ML bootstrap support of >70 (Fig. 4).

Overall, the Bayesian and ML analyses produced similar relationships among the populations of *Glaucomys* sampled. However, the Pacific coastal clade of *G. sabrinus* was unresolved in the outgroup-rooted ML analysis, and populations of *G. volans* from the Sierra Madre de Chiapas were sister to those from the Oaxaca highlands in the Bayesian analyses, yet unresolved in the outgroup-rooted ML analysis (Fig. 3). These inconsistencies could be an artifact of using distantly related outgroups, because, despite being their closest extant relatives, all Asian flying squirrels are relatively divergent (i.e., 15–21% Kimura 2-parameter corrected sequence divergence) from *Glaucomys*. Likewise, the relatively large divergence between populations of *G. sabrinus* from California and those from Oregon–Washington might have promoted discordant resolution across analyses. The outgroup-rooted Bayesian analysis did recover a monophyletic Pacific coastal clade of *G. sabrinus* (Fig. 3), as did a midpoint-rooted tree (not shown) and an ML analysis with a molecular clock enforced (not shown). This suggests that issues related to rooting might have played a role in the discrepancies observed between methods with regard to the monophyly of the Pacific coastal populations of *G. sabrinus*. Alternatively, the above discrepancies simply could be stochastic given that we examined only a portion of 1 mtDNA gene. Regardless, a more detailed analysis of the phylogeographic structure of populations of *G. sabrinus* along the Pacific coast of North America clearly is warranted to resolve this issue.

With respect to the relationship between Mesoamerican flying squirrels and populations of *G. volans* from eastern North America, the results of our phylogenetic analyses (Figs. 3 and 4) are largely, although not entirely, consistent with relationships inferred in the most recent morphological study of these populations (Braun 1988). Braun also concluded that 2 major groups of *G. volans* exist; however, neither of these groups was composed exclusively of populations from Mesoamerica or eastern North America. Rather, 1 group comprises 5 eastern North American populations plus 1 Mesoamerican population, and a 2nd group comprises 4 Mesoamerican populations plus 1 eastern North American population (Braun 1988).

The clade of Mesoamerican flying squirrels sampled in this study can be divided further into 2 major lineages, 1 comprising populations found in the Sierra Madre del Sur and another containing all remaining Mesoamerican populations (Figs. 3 and 4). Using the Kimura 2-parameter model, estimated levels of sequence divergence between the 2 major Mesoamerican lineages are similar to those observed between the Mesoamerican and eastern North American lineages of *G. volans* (Table 3), and the differences could be stochastic (i.e., due to the sampling effects) or differences in the coalescent processes in the 2 groups (Arbogast et al. 2002). To obtain an approximate time frame for the divergence of the Mesoamerican and eastern North American lineages, and for the 2 major Mesoamerican lineages from one another, we used a rate of sequence divergence of 10–15%/10^6 years for 3rd codon positions and a value of sequence divergence at these positions of 7.6%; this value is the average minimum corrected sequence divergence for each comparison (e.g., 7.3% and 7.9%; Table 3). Use of the minimum value should approximate more closely the time at which gene flow ceased than would the use of the mean or maximum value (Arbogast et al. 2002). This approach resulted in a time frame of approximately 0.75–0.50 $\times$ 10^6 years before present, or middle Pleistocene. Subsequent diversification of the remaining Mesoamerican highland populations from one another appears to have occurred relatively recently, approximately 0.27–0.18 $\times$ 10^6 years ago (late Pleistocene).

Genetic support of Mesoamerican subspecies.—Samples used in this study represented 4 of the 6 Mesoamerican subspecies recognized by Goodwin (1961) and 3 of the 4 recognized by Diersing (1980). The missing subspecies samples are of *G. v. madrensis* from the Sierra Madre

Occidental and *G. v. chontali* from the southern extreme of the Oaxaca highlands. The population of *G. v. madrensis* is known from only 2 specimens, both of which were donated in sun-faded condition to the Smithsonian Institution in 1926. Although a tissue sample was obtained from 1 of the specimens of *G. v. madrensis*, repeated attempts to amplify fragments of the *Cytb* gene from this specimen proved unsuccessful. The other missing sample, *G. v. chontali*, is known and described from a single type specimen and was not available for this study.

Genetic data do not fully support the subspecies designations proposed by Goodwin (1961) or Diersing (1980). A conservative subspecies designation focusing only on the earliest and deepest divergence of Mesoamerican *G. volans* supports 1 of the subspecies proposed by Diersing (1980) and the synonymy of 2 others. Using this conservative approach, the subspecies *G. v. guerreroensis* from the Sierra Madre del Sur is supported, whereas *G. v. oaxacensis* and *G. v. goldmani* would be considered synonymous members of a 2nd subspecies that includes all sampled regions except the Sierra Madre del Sur. Under this scenario the Kimura 2-parameter corrected genetic distance between the 2 subspecies is 2.9–4.4% across all codon positions (Table 3). This is substantially higher than the values observed between North American *G. volans* subspecies (Table 4) and above the threshold for subspecies recognition (>2%) advocated by Bradley and Baker (2001).

At a finer scale genetic support exists for recognizing all geographically isolated populations of Mesoamerican *G. volans* as distinct subspecies. Pairwise Kimura 2-parameter distances observed between geographically isolated Mesoamerican populations ranged from 0.7% to 4.0% (Table 4). These values are generally higher than those observed between recognized subspecies of *G. volans* in North America (0.4–1.6%). Therefore, the classification of each disjunct Mesoamerican population of *G. volans* as a distinct subspecies also could be justified. Such a classification would follow closely the subspecies designations proposed by Goodwin (1961), the only exception being that *G. v. oaxacensis* could be divided further into 2 or 3 additional subspecies based on the genetic data. These subspecies would include populations from both the Oaxaca highlands (*G. v. oaxacensis*) and the Sierra Madre del Sur (*G. v. guerreroensis*; Figs. 2–4). Further division of populations found in the Sierra Madre del Sur into 2 subspecies also may be warranted.

Because of the limited number of specimens available, the results of this study can define only the minimum number of lineages of *G. volans* in Mesoamerica. Although many of the known populations of Mesoamerican flying squirrels were examined in this study, those from the Sierra Madre Occidental, Sierra Madre del Sur in Honduras, the southern extreme of the Oaxaca highlands, and the Trans-Mexican Volcanic Belt were not. The extreme geographic isolation and disjunct nature of the nonsampled population(s) from the Sierra Madre Occidental make it especially likely that they represent 1 or more additional distinct genetic lineages of *G. volans*; as such, future work on this subspecies should be given high priority. Finally, populations of other small mammals, such as pocket gophers from the genera *Cratogeomys* and *Pappogeomys*, are represented by 7 distinct clades within the Trans-Mexican Volcanic Belt (Demastes et al. 2002). This evidence suggests that substantial evolutionary diversity might exist within *G. volans* in the Trans-Mexican Volcanic Belt that has yet to be vouchered.

Biogeographic history of G. volans in Mesoamerica and eastern North America.—Traditionally, it has been widely accepted that a historical connection between the now-separated populations of *G. volans* in Mesoamerica and eastern North America occurred in the form of a forested corridor that linked northeastern Mexico and Texas during a late-Pleistocene glacial maximum when cooler, wetter climatic conditions prevailed in the region (Braun 1988; Martin and Harrell 1957; Muul 1968; Weigl 1969). Although such a corridor likely did exist, our estimates suggest that populations of *G. volans* from eastern North America diverged from those in Mesoamerica in the middle Pleistocene (approximately $0.75–0.50 \times 10^6$ years ago), much earlier than predicted by a late-Pleistocene dispersal hypothesis. Additionally, based on our phylogenetic analyses, no clear evidence can be found for multiple invasions of *G. volans* from eastern North America into Mesoamerica, or vice versa. Thus, a single divergence of Mesoamerican and eastern North American populations of *G. volans* in the middle Pleistocene is the most parsimonious conclusion based on our mtDNA data. Further insights into the biogeographic history of *G. volans* can be inferred from mismatch distributions, Fu's *F*-statistic, and relative levels of genetic variation. For example, populations of *G. volans* from eastern North America exhibit a largely unimodal mismatch distribution (not shown) and a significantly negative value of Fu's *F*-statistic, both indicative of a recent population expansion. The disjunct and highly structured nature of the Mesoamerican populations we sampled precluded us from treating them as a single population; as a result, we could not calculate a mismatch distribution or estimate Fu's *F*-statistic. However, despite our small sample size, Mesoamerican populations of *G. volans* exhibited higher levels of nucleotide diversity than those from eastern North America. The higher nucleotide diversity of the Mesoamerican populations occurs despite the much more extensive geographic sampling (i.e., from Texas to Florida and Florida to Nova Scotia) of eastern North American populations. Thus, increased sampling is likely to uncover additional genetic diversity within Mesoamerican populations of *G. volans*, whereas further sampling in eastern North America is not.

Taken together, the greater level of genetic diversity in the Mesoamerican populations and the evidence for a recent population expansion in the populations from eastern North America are inconsistent with a late-Pleistocene dispersal hypothesis wherein Mesoamerica was colonized recently (late Pleistocene) from eastern North America. Instead, examination of our data suggests a biogeographic scenario in which the vicariant, dispersal, or founder event(s) that led to the current

disjunct geographic distribution of *G. volans* occurred before the late Pleistocene. Because of the lack of shared haplotypes between samples of *G. volans* from eastern North America and Mesoamerica (Figs. 3 and 4) the direction of past migration(s) or colonization(s), or both, cannot be inferred from our data. The relatively high levels of genetic variability observed in the Mesoamerican populations of *G. volans* and the strong signal of a recent population expansion observed in the eastern North American populations could be produced under several biogeographic scenarios, including a founder event from Mesoamerica or repeated episodes of intense bottlenecking in the eastern North America populations followed by rapid population expansion associated with the glacial–interglacial periods of the late Pleistocene, or both.

The biogeographic histories of plants and animals in Mesoamerica clearly have been shaped by the complex topology of the region in combination with climatic changes associated with glacial cycles of the Quaternary (Dawson 2005; Luna et al. 1999). Several previous studies have used phylogenetic relationships of small mammals to elucidate biogeographic patterns in the mountain and highland regions of Mesoamerica (Arellano et al. 2005; Carleton et al. 2002; León-Paniagua et al. 2007; Sullivan et al. 1997, 2000). The widespread, yet disjunct high-elevation distribution of Mesoamerican flying squirrels makes them an excellent species to test the generality of previously observed phylogeographic patterns and to increase our understanding of the biogeography of the region.

We observed the deepest genetic divergence within Mesoamerican flying squirrels to be between the western Sierra Madre del Sur and all other Mesoamerican populations. A similar biogeographic pattern is seen in 2 small mammal species, *Neotoma mexicana* and *Peromyscus winkelmanni*, each of which is endemic to the western Sierra Madre del Sur (Edwards and Bradley 2002; Sullivan et al. 1997). Habitat expansion–contraction cycles of the Pleistocene are likely responsible for the isolation and subsequent speciation of these endemic taxa. Similarly, Pleistocene expansion–contraction cycles likely are responsible for the isolation and subsequent divergence of populations of *G. volans* in the western Sierra Madre del Sur from those found elsewhere in Mesoamerica. A related biogeographic pattern seen in Mesoamerica is that isolated mountain and highland regions often reveal monophyletic assemblages based on phylogenetic analysis of genetic data. This biogeographic pattern is observed in birds and mammals (García-Moreno et al. 2004; Sullivan et al. 2000). The small sample size of Mesoamerican specimens of *G. volans* prevents rigorous testing of this biogeographic pattern. However, examination of the data presented here suggests that the montane regions we sampled in Mesoamerica contain mtDNA lineages of *G. volans* that have sorted geographically to a considerable degree.

Finally, one of the most notable biogeographic patterns observed in Mesoamerican small mammals is a relatively deep phylogeographic divergence across the Isthmus de Tehuantepec. This divergence has been documented in the genera *Neotoma*, *Peromyscus*, *Habromys*, and *Reithrodontomys* (Carleton et al. 2002; Edwards and Bradley 2002; Sullivan et al. 1997, 2000). In a thorough review, Carleton et al. (2002) suggested that divergence at the Isthmus should be considered the expected model for Mesoamerican mammals restricted to middle and upper montane humid forests. However, Mesoamerican populations of *G. volans* do not exhibit the predicted deep phylogenetic divergence at the Isthmus. We found the level of divergence between populations of *G. volans* on either side of the Isthmus to be similar to those observed between other geographically isolated populations in Mesoamerica (i.e., those found on separate mountaintops; Table 4). The most likely hypothesis to explain this phenomenon is that *G. volans* was not present in Mesoamerica, or at least not south of the Isthmus, before it became a major biogeographic barrier for many other small mammals. This hypothesis is consistent with the suggestion by Sullivan et al. (2000) that vicariance at the Isthmus was associated with the last marine transgression in the late Pliocene–early Pleistocene. A vicariant event at this time likely would predate the presence of *G. volans* in Mesoamerica based on the divergence dates (middle Pleistocene) estimated in this study. As such, this easily could explain why *G. volans* does not exhibit the phylogeographic discontinuity at the Isthmus de Tehuantepec observed in many other taxa.

Future work should focus on clarifying the distributions, phylogenetic relationships, and conservation status of Mesoamerican flying squirrels. Obtaining more specimens and localities for *G. volans* throughout the highlands of Mesoamerica is crucial to understanding the contemporary geographic distribution of this species. Such efforts are very likely to reveal additional genetically or morphologically unique populations, or both. This seems especially true for the Sierra Madre Occidental and Trans-Mexican Volcanic Belt. The most recent collections of Mesoamerican flying squirrels confirm presence at new localities, highlighting the limitations of the known distribution of flying squirrels in Mesoamerica (Ceballos and Galindo 1983; Ceballos and Miranda 1985). This underrepresentation of localities and associated studies of genetic diversity warrant designating the Mesoamerican populations of *G. volans* as data-deficient under the 2001 International Union for the Conservation of Nature and Natural Resources categories and criteria guidelines (International Union for the Conservation of Nature and Natural Resources 2001). Furthermore, given that many high-elevation areas of Mesoamerica are experiencing severe deforestation (i.e., $>1\%$ per year in Mexico—Sánchez-Cordero et al. 2005), a situation likely to be accelerated by global climate change, future studies may reveal that many populations warrant protected status. Thus, obtaining detailed data on the conservation status of Mesoamerican flying squirrels should be given high priority.

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