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EVOLUTIONARY GENETICS AND PLEISTOCENE BIOGEOGRAPHY OF NORTH AMERICAN TREE SQUIRRELS (*TAMIASCIURUS*)

BRIAN S. ARBOGAST,* ROBERT A. BROWNE, AND PETER D. WEIGL

Department of Biology, Wake Forest University, Winston-Salem, NC 27109
Present address of BSA: Burke Museum of Natural History and Culture and Department of Zoology, University of Washington, Seattle, WA 98195-3010

Nucleotide sequence data from the mitochondrial DNA (mtDNA) cytochrome-*b* gene and allozymic data were used to infer the evolutionary and biogeographic histories of New World tree squirrels of the genus *Tamiasciurus.* Phylogenetic analyses of the cytochrome-*b* data support the existence of 3 mtDNA lineages within *Tamiasciurus*: a western lineage consisting of populations of *T. douglasii* from western British Columbia (Canada), Washington, Oregon, and California, and *T. mearnsi* from northern Baja California (Mexico); a southwestern lineage consisting of populations of *T. hudsonicus* from New Mexico and Arizona; and a geographically widespread lineage comprising populations of *T. hudsonicus* from the remainder of the species' range. Levels of mtDNA sequence variation observed within and among populations of *Tamiasciurus* were small (0–2.4%), suggesting that contemporary geographic patterns of genetic variation in *Tamiasciurus* have been established relatively recently (i.e., in the Late Pleistocene). Allozyme analyses also support a close relationship among extant populations of *Tamiasciurus.* No fixed allelic differences were observed among the 3 recognized species and interspecific genetic distances (Nei's D) were substantially less than those typically observed between sibling species. Although differing from the current taxonomy in several respects, geographic patterns of genetic variation observed within *Tamiasciurus* are similar to those observed in a variety of North American boreal forest taxa and most likely reflect effects of forest fragmentation associated with glacial cycles of the Pleistocene.

Key words: biogeography, mtDNA, phylogeography, Pleistocene, *Tamiasciurus*

The genus *Tamiasciurus* contains 3 recognized species, *T. hudsonicus, T. douglasii,* and *T. mearnsi* (Wilson and Reeder 1993), which are distributed throughout montane and boreal forest regions of North America (Fig. 1). The most widely distributed of the 3 species, *T. hudsonicus,* has a geographic distribution that extends from Alaska in the northwest, through the Great Lakes region to the southern Appalachians in the southeast, and southward along the Rocky Mountains into northern Arizona. The geographic distribution of *T. douglasii* is limited to western North America (British Columbia [Canada], Washington, Oregon, and California), primarily west of the Cascades and the Sierra Nevada. The geographic distributions of *T. douglasii* and *T. hudsonicus* are largely exclusive, but the 2 species are sympatric in parts of British Columbia (Smith 1981), Washington (Stevens and Nellis 1974), and Oregon (Hatton and Hoffmann 1979). In general, these 2 species can be distinguished on the basis of pelage coloration (Lindsay 1982). However, individuals exhibiting intermediate pelage coloration occur within these relatively narrow areas of sympatry in the Pacific Northwest, leading Hall (1981) to suggest that *T.*

* *Correspondent: arbogast@u.washington.edu*

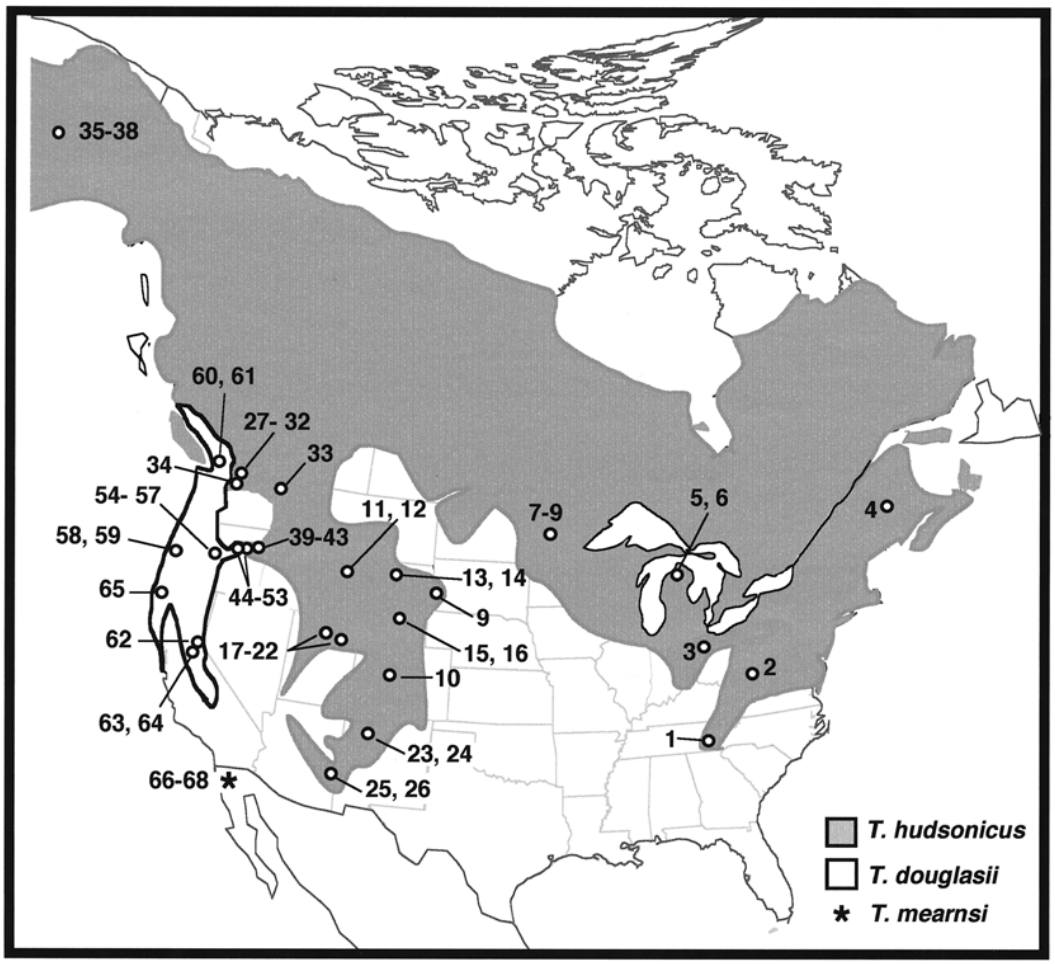

FIG. 1.—Geographic distributions (Hall 1981; Lindsay 1981) and sampling localities for the red squirrel (*Tamiasciurus hudsonicus*), Douglas' squirrel (*T. douglasii*), and Mearns' squirrel (*T. mearnsi*). Numbers correspond to those presented in Appendix I.

hudsonicus and *T. douglasii* may be conspecific.

Tamiasciurus mearnsi occurs only in the forested highlands of northern Baja California (Lindsay 1981; Yensen and Valdés-Alarcón 1999; Fig. 1). Historically, these populations were considered to be conspecific with those of *T. douglasii* (Hall 1981; Townsend 1897), although Allen (1898) proposed that *T. mearnsi* be elevated to the specific level. Recent recognition of *T. mearnsi* as a distinct species (Wilson and Reeder 1993) was based largely on the work of Lindsay (1981), who provided evidence that it is morphologically distinct from populations of *T. douglasii* and *T. hudsonicus* from western North America.

We used mitochondrial DNA (mtDNA) sequence data to infer geographic patterns of genetic variation within the genus *Tamiasciurus* and allozyme data to estimate within-species genetic variation and among-species differentiation. We examined if geographic patterns of genetic variation within the genus were consistent with the current taxonomy and were congruent with those observed in other North American mammals with similar geographic dis-

tributions. We further examined the role of Pleistocene glacial cycles and associated forest fragmentation in shaping geographic patterns of genetic variation within *Tamiasciurus.*

Materials and Methods

Blood or tissue samples were obtained from 68 individuals of *Tamiasciurus* (Appendix I). Cytochrome-*b* sequence data were generated for 43 individuals (32 *T. hudsonicus,* 10 *T. douglasii,* and 1 *T. mearnsi*) and allozyme data were generated for 58 (44 *T. hudsonicus,* 12 *T. douglasii,* and 2 *T. mearnsi*). Both analyses included samples from throughout the geographic distribution of each species (Fig. 1). All tissue and blood samples were stored at −70°C before laboratory analyses.

Analysis of variation in the mtDNA cytochrome-b gene.—Total DNA extractions from either tissues or blood were conducted using either the phenol–chloroform method (Hillis et al. 1996) or the chelex-solution method (Walsh et al. 1991). A 402–base pair fragment of the cytochrome-*b* gene of mtDNA was amplified using the polymerase chain reaction (Mullis and Faloona 1987; Saiki et al. 1988). Amplification and sequencing were performed with the primers L14724 and H15149 (Irwin et al. 1991) as described by Arbogast (1999). Automated sequencing (Wake Forest University School of Medicine, Winston–Salem, North Carolina) was used to sequence the entire 402–base pair fragment of the cytochrome-*b* gene for each individual. To minimize sequencing errors, both strands were sequenced for each individual. Sequences were aligned with Sequencher software (Gene Codes Corp., Ann Arbor, Michigan) and visually inspected for errors. Nucleotide sequences also were translated into amino acid sequences in the computer program MacClade (version 3.07; Maddison and Maddison 1997). All unique cytochrome-*b* nucleotide sequences were deposited in GenBank under accession numbers AF322945–AF322958.

Phylogenetic analyses and estimates of sequence divergence were performed using the computer program PAUP* (Swofford 1999). To provide a basis for comparison with previously published data, sequence divergence for all possible pairwise combinations of unique haplotypes was estimated under the 2-parameter model of Kimura (1980). The computer program MODELTEST (Posada and Crandall 1998), which uses a series of hierarchical likelihood ratio tests to evaluate the fit of 56 nested models of nucleotide substitution, was used to determine which model provided the best fit to the cytochrome-*b* data. The g_1 statistic (Huelsenbeck 1991) was used as an index to evaluate if sufficient levels of phylogenetic signal were present in the data.

Parsimony and maximum-likelihood analyses initially were performed with 3 species of *Sciurus* (*S. niger, S. aberti,* and *S. carolinensis*; GenBank Accession numbers U46178, U10177, and U46167, respectively), simultaneously designated as outgroups; *Sciurus* is the presumptive sister genus to *Tamiasciurus* (Hafner 1984; Hafner et al. 1994). The best-fit model (based on the hierarchical likelihood ratio tests) was then used to infer a cytochrome-*b* gene tree under the optimality criterion of maximum-likelihood, employing the heuristic search option and the tree-bisection-reconstruction branch-swapping algorithm. Maximum-likelihood bootstraps under the best-fit model consisted of 100 iterations, using the fast stepwise-addition-search and strict consensus-tree options.

Parsimony analysis was performed with all character state changes weighted equally and also with transitions down-weighted to reflect the transition : transversion ratio estimated under the best-fit model. The branch-and-bound search option and tree-bisection-reconstruction branch-swapping algorithms were employed. Nodal support was estimated with 1,000 bootstrap replicates, using the fast stepwise-addition-search and strict consensus-tree options. Only unique haplotypes were included in bootstrap analyses, and all sites were used as characters. For parsimony analysis, consistency and retention indices were calculated with all characters included and also with uninformative characters excluded.

Because the most appropriate outgroups for this study (*Sciurus*) are relatively divergent from *Tamiasciurus,* their inclusion in the phylogenetic analyses was likely to introduce a high degree of saturation (Halanych et al. 1999). To address that potential problem, maximum-likelihood and parsimony were also used to examine unrooted networks of the unique haplotypes within *Tamiasciurus.* With the exception of excluding the outgroup taxa, these analyses were conducted in the same manner as described above. Under the assumption of constant evolutionary rates, mid-

point rooting was used to determine the direction of evolution for those networks. Because the midpoint method assumes a molecular clock, a likelihood ratio test was used to test for among-lineage differences in rate of molecular evolution as described in Huelsenbeck and Rannala (1997).

Analysis of allozymic variation.—Tissue or blood samples were analyzed via standard starch-gel electrophoresis (Hillis et al. 1996) with both continuous and discontinuous buffers. Sixteen loci were scored: α-GPDH-1 (EC: 1.1.1.8), G-6PDH-1 (1.1.1.49), IDH-1 (1.1.1.42), LDH-1 (1.1.1.27), PEP-1 (using leu-ala; 3.4.–.–), PGM-1 (5.4.2.2), XDH-1 (1.1.1.204), AAT-1 (2.6.1.1), ACO-1 (4.2.1.3), GPI-1 (5.3.1.9), MDH-1 (1.1.1.37), PGDH-1 (1.1.1.44), SDH-1 (1.3.99.1), SOD-1 (1.15.1.1), and EST-1 and EST-2 (none specified). Allele frequencies for each locus, percent of loci polymorphic (P; using the 95% criterion), and average individual heterozygosity ($\bar{H}$) values were calculated. Genetic distances were calculated as described by Nei (1972).

RESULTS

Phylogenetic analysis of the mtDNA cytochrome-b data.—Fourteen unique haplotypes were observed among the 43 individuals of *Tamiasciurus* examined (Appendix I). Of the 402 base pairs of the cytochrome-*b* gene examined, 19 were variable within *Tamiasciurus.* Levels of sequence divergence among the haplotypes ranged from 0.25% to 2.4% (Table 1). The hierarchical likelihood ratio tests indicated that the TrN model (Tamura and Nei 1993) with among-site rate variation incorporated via a discrete gamma distribution ($\alpha = 0.2625$) provided the best fit to the cytochrome-*b* data set consisting of the 14 unique haplotypes of *Tamiasciurus* plus the outgroups. The estimated transition : transversion ratio under this model was 4.77. When the outgroups were excluded, the hierarchical likelihood ratio tests indicated that the HKY85 model (Hasegawa et al. 1985) provided the best fit to the data. The estimated transition : transversion ratio was 5.70 for the reduced data set. The g_1-statistics for the 2 data sets (with and without the outgroups included) were −2.33 and −0.807, respectively, indicating that the data possessed nonrandom phylogenetic signal (Huelsenbeck 1991).

In the parsimony analysis using outgroup rooting, both weighting schemes (transitions and transversions weighted equally and transversions weighted 5:1 over transitions) recovered the same 3 equally parsimonious trees. The 3 trees differed only in the extent to which relationships within the western mtDNA clade were resolved. Under the equal-weighting scheme, each tree was 152 steps in length and had consistency and retention indices of 0.875 and 0.846, respectively. When uninformative characters were excluded, the consistency index was 0.873. The 3 trees each had a −ln likelihood score of 1,220.792.

The strict consensus of the 3 most parsimonious trees (Fig. 2A) supported the existence of a distinct ''western'' mtDNA clade (haplotypes VII–XI) within *Tamiasciurus* corresponding to populations of *T. douglasii* (western British Columbia, Washington, Oregon, and California) and *T. mearnsi* (Baja California, Mexico). A geographically widespread ''eastern'' clade (haplotypes I–VI) corresponding to all populations of *T. hudsonicus* except those from New Mexico and Arizona also was recovered. However, bootstrap support for that clade was weak (<50%). The New Mexico and Arizona haplotypes (haplotypes XII–XIV) did not form a monophyletic group in that analysis and were basal to the western and eastern mtDNA clades.

Maximum-likelihood analysis using outgroup rooting and the best-fit TrN + gamma model (Tamura and Nei 1993) produced a single tree (Fig. 2B) with a −ln likelihood score of 1,201.053. Maximum-likelihood analysis also supported the existence of 2 distinct mtDNA lineages within *Tamiasciurus,* 1 of which was the same western clade recovered in the parsimony analysis, and the other a southwestern clade comprised of the New Mexico and Arizona haplotypes. Maximum-likelihood analysis did not recover a monophyletic eastern mtDNA clade. Rather, haplotypes corresponding to

TABLE 1.—Kimura (1980) 2-parameter distances (as percentages) among the 14 unique mtDNA haplotypes (I–XIV) observed within *Tamiasciurus*. Haplotype designations are given in Appendix I, and phylogenetic relationships among the haplotypes are depicted in Figs. 2 and 3. Distances between the *Tamiasciurus* haplotypes and individuals representing 3 species from the putative sister genus, *Sciurus*, are shown for comparison.

	Haplotype													
Haplotype	I	II	III	IV	V	VI	VII	VIII	XI	X	XI	XII	XIII	XIV
I														
II	0.499													
III	0.249	0.249												
IV	0.522	0.514	0.257											
V	1.005	1.003	0.752	1.051										
VI	0.752	0.750	0.500	0.783	0.249									
VII	1.772	1.768	1.515	1.586	1.260	1.005								
VIII	2.031	2.026	1.772	1.855	1.515	1.260	0.249							
IX	1.768	1.765	1.512	1.582	1.257	1.003	0.249	0.499						
X	2.026	2.022	1.768	1.584	1.512	1.257	0.249	0.499	0.449					
XI	2.031	2.026	1.772	2.120	1.515	1.260	0.752	1.005	0.750	1.003				
XII	1.772	1.768	1.515	1.852	1.260	1.005	1.515	1.772	1.512	1.768	1.772			
XIII	2.031	2.026	1.772	2.126	1.515	1.260	1.772	2.031	1.768	2.026	2.031	0.249		
XIV	2.290	2.285	2.031	2.395	1.772	1.515	2.031	2.290	2.026	2.285	2.290	0.500	0.249	
S. carolinensis	17.48	17.45	17.15	16.68	17.81	17.48	17.81	18.15	18.12	23.00	23.04	21.42	21.58	21.23
S. aberti	20.66	20.27	20.31	20.99	20.31	20.66	22.11	22.48	22.06	22.43	21.74	20.66	20.31	19.96
S. niger	20.87	21.19	21.23	21.69	21.94	21.58	22.67	23.04	22.63	18.12	18.82	17.15	16.82	16.50

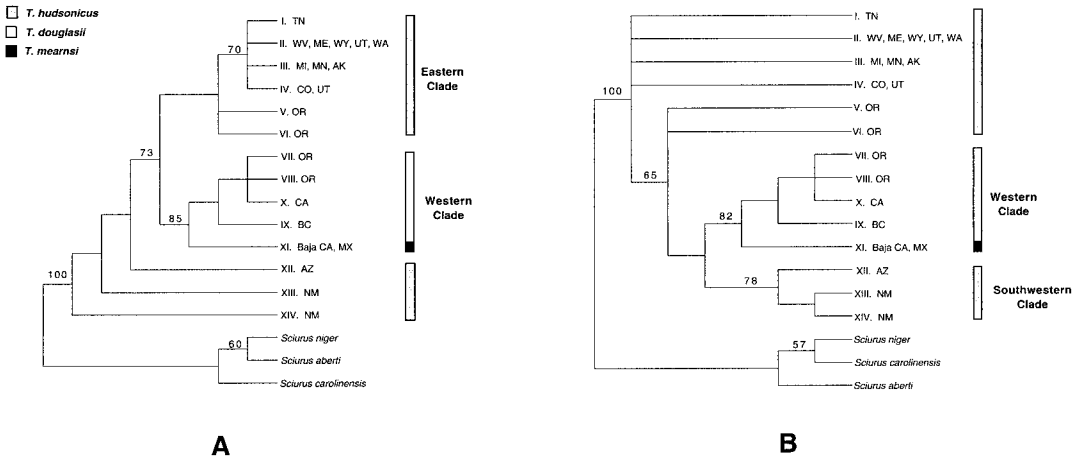

FIG. 2.—Strict consensus of A) the 3 most parsimonious trees and B) maximum-likelihood tree for the 14 haplotypes (I–XIV) of *Tamiasciurus* using outgroup rooting. The 3 most parsimonious trees were identical except for the degree of resolution within the western clade. The maximum-likelihood tree is based on the best-fit model of nucleotide substitution for these data (TrN + gamma). Locality information and haplotype designations are given in Appendix I. Bootstrap values >50% (based on 1,000 replicates for parsimony and 100 replicates for maximum-likelihood) are shown at each node. Bars are used to illustrate the degree of congruence between the cytochrome-*b* gene tree and the current taxonomy of *Tamiasciurus.*

the eastern mtDNA lineage recovered in the parsimony analysis (haplotypes I–VI) were largely unresolved and basal to the 2 recovered clades.

Under the best-fit model, the likelihood ratio test of a molecular clock indicated that no significant differences occurred in the rate of cytochrome-*b* evolution among the 14 haplotypes of *Tamiasciurus* (−ln likelihood without molecular clock enforced = 682.131; with molecular clock enforced = 687.820; *d.f.* = 12; $P = 0.30$). Thus, the critical assumption required for using the midpoint method to root the haplotype network of *Tamiasciurus* (that the data are evolving according to a molecular clock) seems to have been met for those data.

Both weighting schemes used in the midpoint-rooted parsimony analysis (transitions and transversions weighted equally and transversions weighted 6:1 over transitions) produced the same 3 trees, which differed only in the extent to which relationships within the western mtDNA clade were resolved. The 3 trees were 20 steps each and had a −ln likelihood score of 682.13. Consistency and retention indices (both with and without uninformative characters excluded) were 1.0 for those trees. Maximum-likelihood analysis produced a single tree that was identical in topology to the strict consensus parsimony tree. The maximum-likelihood tree had a −ln likelihood of 682.11. Thus, when outgroups were excluded and the midpoint method was used to root the network consisting of the 14 unique haplotypes of *Tamiasciurus,* parsimony and maximum-likelihood converged on the identical tree topology (Fig. 3). Both methods recovered the same 3 major mtDNA clades within *Tamiasciurus*: a widespread eastern clade (haplotypes I–VI), a western clade (haplotypes VII–XI), and a southwestern clade (haplotypes XII–XIV). Although a sister relationship between eastern and western clades was recovered in both analyses, bootstrap support for that relationship is weak (<50%). Parsimony and maximum-likelihood bootstrap support for the 3 clades was generally high (>85%),

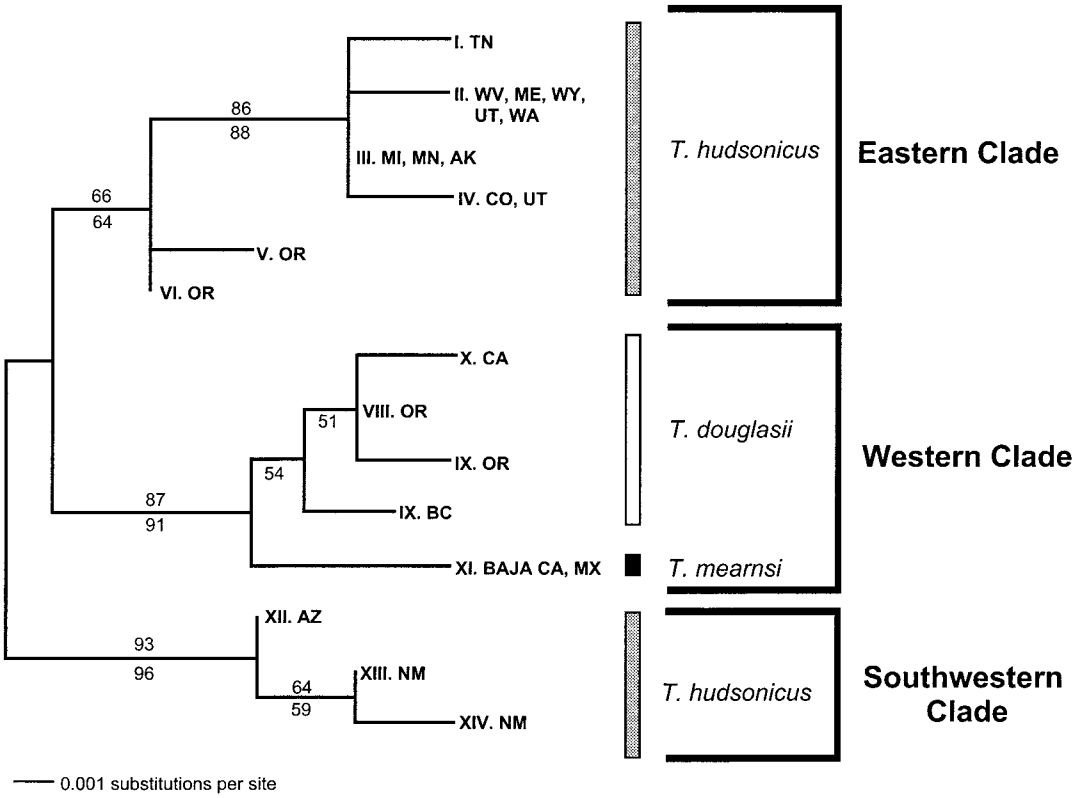

FIG. 3.—Phylogeny recovered by both parsimony and maximum-likelihood for the 14 unique haplotypes (I–XIV) of *Tamiasciurus* using midpoint rooting. Locality information and haplotype designations are given in Appendix I. Parsimony bootstrap values >50% (based on 1,000 replicates) are shown above each node, and maximum-likelihood bootstrap values >50% (based on 100 replicates) are shown below each node. Bars are used to illustrate the degree of congruence between the cytochrome-*b* gene tree and the current taxonomy of *Tamiasciurus.*

except for the node that included the eastern Oregon haplotypes (haplotypes V and VI) within the eastern clade, where bootstrap support was about 65%. The monophyly of the remaining haplotypes corresponding to the eastern clade was well supported.

Allozymic analysis.—Of the 16 loci examined, 8 were nonvariable: EST-1, α-GPDH-1, G-6PDH-1, IDH-1, LDH-1, PEP-1 (using leu-ala), PGM-1, and XDH-1. Based on the 8 variable loci, average individual heterozygosity was 6.7% and 14.6% for *T. hudsonicus* and *T. douglasii,* respectively, and the percentage of loci polymorphic was 37.5% for both species (Table 2). Because allozymic data were available from only 2 individuals of *T. mearnsi,* estimates of heterozygosity and polymorphism for that species were not meaningful and allele frequencies were not interpreted to be realistic. Rather, those values were listed only for comparison with the other 2 species to determine whether alleles were shared or unique. Genetic distance between *T. hudsonicus* and *T. douglasii* was estimated to be 0.015. Because of small sample size, no estimate was made using *T. mearnsi.*

DISCUSSION

Genetic variability and population structure of Tamiasciurus.—Perhaps the most striking result of this study is the relatively small amount of genetic variability found within and among populations of *Tamias-*

TABLE 2.—Allelic frequencies at 8 variable loci and estimates of average individual heterozygosity ($\bar{H}$) and percentage of loci polymorphic (P) for *Tamiasciurus*.

Species	Loci																		$\bar{H}$ (%)	P (%)
	AAT-1			ACO-1		EST-2				GPI-1		MDH-1		PDGH-1			SDH-1		SOD-1			
	1	2	3	1	2	1	2	3	4	1	2	1	2	1	2	3	1	2	1	2		
T. hudsonicus ($n = 44$)	0.75	0.25		0.05	0.95	0.04	0.38	0.57	0.01	0.14	0.86	0.06	0.94	0.01	0.16	0.83	0.89	0.11	0.99	0.01	6.7	37.5
T. douglasii ($n = 12$)	0.39	0.56	0.05	0.08	0.92		0.18	0.36	0.46	0.12	0.88	0.04	0.96		0.17	0.83	0.92	0.08	1.00		14.6	37.5
T. mearnsi ($n = 2$)	1.00				1.00	0.50	0.50				1.00	0.50	0.50	0.50	0.50		1.00		1.00			

ciurus. For example, levels of estimated mean sequence divergence in the mtDNA cytochrome-*b* gene among the 3 recognized species of *Tamiasciurus* are 1.0–2.4%, or about one-third of those observed within the similarly distributed northern flying squirrel (*Glaucomys sabrinus*—Arbogast 1999; Demboski et al. 1998). Based on the allozyme analyses, the genetic distance value of 0.015 observed between *T. hudsonicus* and *T. douglasii* also is well below that typically observed between sibling species (Avise 1994). Although the sample for *T. mearnsi* is too small for definitive conclusions, the data also are consistent with a high degree of genetic similarity between it and the other 2 species. Overall, allele frequencies, heterozygosity, and polymorphism values (Table 2) are typical of those found in a large outbreeding population (Smith and Wayne 1996).

Although a variety of problems are associated with estimating evolutionary dates from estimates of molecular divergence (Arbogast and Slowinski 1998; Hillis et al. 1996), levels of mtDNA and allozymic variability suggest that the origin of present geographic structuring within *Tamiasciurus* is a recent phenomenon. Based on a gamma-corrected rate of cytochrome-*b* divergence for primates of about 5–6%/million years (Arbogast and Slowinski 1998) and a relative rate of cytochrome-*b* evolution for rodents about 1.5–2 times faster than that of primates (Britten 1986; Dewalt et al. 1993; Li et al. 1987, 1990; Wu and Li 1985), a gamma-corrected rate of cytochrome-*b* evolution for rodents is estimated to be about 7.5–12%/million years. Use of this rate and minimum values of estimated pairwise sequence divergence between the 3 major clades of *Tamiasciurus*, which range from 1.03% to 1.77% (Table 1), produces mean estimated dates of cytochrome-*b* gene divergence of about 80,000–240,000 years ago. Because rates of molecular evolution are lineage specific and because these dates represent only mean estimates and are likely to have large associated standard er-

rors (Arbogast and Slowinski 1998; Hillis et al. 1996), they should be viewed with caution. However, the relatively small levels of mtDNA and allozymic differentiation observed suggest that the majority of geographic structuring of genetic variation within *Tamiasciurus* probably occurred in the Late Pleistocene. Furthermore, given that persistence of ancestral polymorphisms is predicted to lead to estimated dates of gene divergence that predate episodes of actual population separation and speciation (potentially by hundreds of thousands of years—Edwards 1997), divergence of populations corresponding to the 3 major mtDNA clades of *Tamiasciurus* may have occurred as recently as the Wisconsin glaciation or Wisconsin–Holocene transition.

Phylogenetic analysis of the cytochrome-b data.—The relatively few variable characters within *Tamiasciurus* (consisting primarily of 3rd-position transitions), coupled with the availability of only relatively distant outgroups, made phylogenetic analysis of the *Tamiasciurus* sequence data challenging. In particular, difficulty was encountered in rooting the phylogeny of *Tamiasciurus.* This problem is manifested in the failure of the outgroup analyses to resolve the more basal relationships within *Tamiasciurus* and in bootstrap values that are generally low (Fig. 2).

To address the problem of having only relatively divergent taxa with which to root the phylogeny, we reconstructed unrooted networks of the 14 unique haplotypes of *Tamiasciurus* (Fig. 3). Those networks were then rooted using the midpoint method. Several lines of evidence led us to favor this topology as being that which best reflects evolutionary relationships within *Tamiasciurus.* First, theoretical work has shown that the probabilities of obtaining the correct rooted tree are substantially lower than the probabilities of obtaining the correct unrooted tree (Sourdis and Krimbas 1987). Second, because the outgroup taxa are so divergent from the ingroup taxa (about an order of magnitude greater than those within *Tamiasciurus* in terms of cytochrome-*b* sequence divergence; Table 1), inclusion of the outgroups is likely to add a large amount of saturation, homoplasy, and potentially long-branch attraction into the analyses (Halanych et al. 1999; Hendy and Penny 1989; Maddison et al. 1992; Miyamoto and Boyle 1989; Smith et al. 1992; Wheeler 1990). Third, when the outgroups are excluded parsimony and maximum-likelihood methods recover the same topology (Fig. 3), suggesting that this result is robust across a variety of phylogenetic assumptions (Halanych et al. 1999). Finally, when the outgroups are excluded, bootstrap values increase at all major nodes within the phylogeny of *Tamiasciurus,* and consistency and retention indices increase.

Biogeographic implications.—Lindsay (1987) proposed that Pleistocene forest fragmentation played an important role in determining patterns of speciation and geographic variation within *Tamiasciurus.* The genetic data presented here support Pleistocene forest dynamics as an important factor in shaping the biogeographic and evolutionary history of the group. For example, the close relationship of *T. mearnsi* from Baja California with populations of *T. douglasii* to the north supports Lindsay's (1981) biogeographic argument for a Late Pleistocene separation of these 2 populations. Lindsay (1981) proposed that the mechanism responsible for the isolation of *T. mearnsi* on the Baja Peninsula was the fragmentation of appropriate forest habitat for *Tamiasciurus* during Pleistocene interglacials. To the north and west of the Sierra San Pedro Martir, California chaparral (Brown 1994) is currently a barrier between populations of *T. mearnsi* and *T. douglasii* in the Sierra Nevada (Yensen and Valdés-Alarcón 1999). However, during Pleistocene glacial periods, life zones were 600–1,000 m lower than at present, forming a corridor of coniferous forest between the Sierra San Pedro Martir and the Sierra Nevada (Lindsay 1981; Savage 1960). Ancestors of *T. mearnsi* could have invaded the

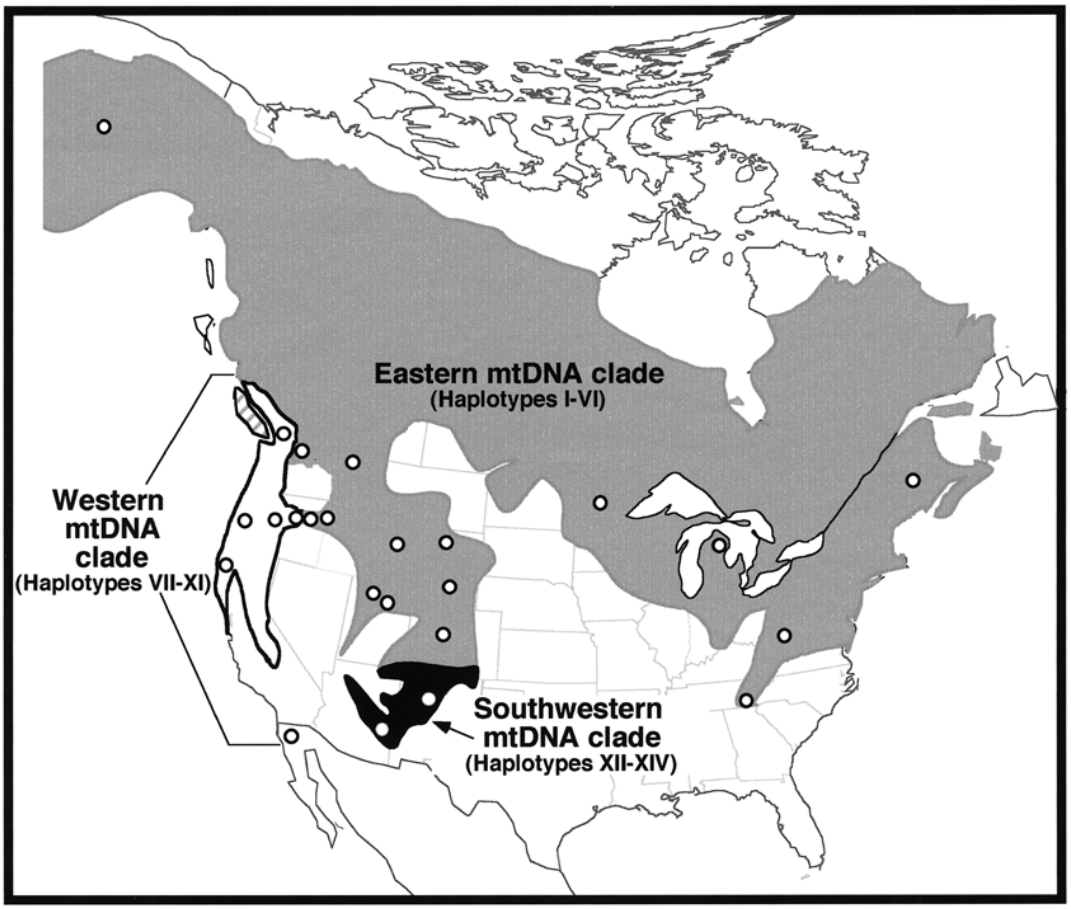

FIG. 4.—Geographic distributions of eastern (gray), western (white), and southwestern (black) mitochondrial DNA clades of *Tamiasciurus* based on midpoint-rooted parsimony and maximum-likelihood trees (Fig. 3). Open circles indicate collection localities for individuals from which mtDNA cytochrome-*b* data were obtained. Complete locality information is given in Appendix I.

Baja Peninsula during 1 or more of the mesic cycles that began about 700,000 years ago (Hafner and Riddle 1997). *T. mearnsi* likely would have become isolated from *T. douglasii* most recently about 12,000 years ago (Lindsay 1981; Yensen and Valdés-Alarcón 1999).

Analysis of the mtDNA data also reveals the presence of a distinct lineage of *Tamiasciurus* in the southern Rocky Mountains (Figs. 2–4). Lindsay (1987) noted morphologic differences in the southwestern populations of *Tamiasciurus* and suggested those differences were the result of strong influences imposed by the vicariant biogeography of the southwestern United States and factors related to Allen's rule (an ecogeographic relationship between temperature and extremity length). Historical fragmentation of coniferous forests in the southern Rocky Mountains almost certainly isolated southwestern populations of *Tamiasciurus* from more northern populations at various times in the Pleistocene (Harris 1990; Lindsay 1987), most recently about 6,000–9,000 years ago (Hafner and Sullivan 1995). During the Wisconsin glacial maximum, the combination of the Colorado River drainage and continuous montane glaciers south of the river would have ef-

fectively divided the boreal forest habitat of northern Colorado from that of New Mexico and Arizona (Hafner and Sullivan 1995). As a result, populations of *Tamiasciurus* persisting in the Southwest would have become isolated from those in the northern Rockies. Subsequently, as the Wisconsin glacial period came to a close and the climate warmed, populations in New Mexico and Arizona would have become more fragmented, isolated from one another on montane islands separated by lowland areas of nonboreal habitat.

Evidence for the historical isolation of coniferous forests in the southern Rocky Mountains is supported by molecular data of Douglas-fir (*Pseudotsuga menziesii*), which has been shown to possess a pronounced phylogeographic discontinuity in the southwestern United Sates (Aagaard et al. 1995). This discontinuity within Douglas-fir seems to be highly congruent with the southwestern mtDNA discontinuity observed in *Tamiasciurus.* Similar phylogeographic discontinuities observed in the American pika (*Ochotona princeps*—Hafner and Sullivan 1995), the long-tailed vole (*Microtus longicaudus*—Conroy and Cook 2000), and the tassel-eared squirrel (*Sciurus aberti*—Lamb et al. 1997), also may have been produced or reinforced by similar historical fragmentation of boreal forest and high-altitude montane habitats during the Late Pleistocene.

The southeastern United States and the northern Rocky Mountains are the most likely locations for forest refugia for populations corresponding to the eastern mtDNA clade of *Tamiasciurus* during the most recent glacial maximum. Existence of Pleistocene boreal forest refugia in the southeastern United States is supported by floristic reconstructions of the region and a variety of mammalian fossils (Davis 1983; Kurtén and Anderson 1980; Ritchie 1987). However, because fossil material from *Tamiasciurus* dating to the Wisconsin glacial maximum has not been found further south than Pennsylvania (Kurtén and Anderson 1980), the extent of the distribution of *Tamiasciurus* in the southeastern United States during this period is unclear. The mtDNA haplotypes of *Tamiasciurus* found only in the Blue Mountains of eastern Oregon (haplotypes V and VI) may have persisted during the most recent glaciation in 1 or more boreal refugia in the northern Rocky Mountains. Molecular evidence from several plant taxa from western North America suggests that this area may have been an important refugia during glacial maxima (Soltis et al. 1997).

Despite having an extensive geographic distribution (Fig. 4), the eastern mtDNA clade of *Tamiasciurus* possesses little mtDNA variation (Table 1). A similar pattern has been observed in a variety of taxa (Arbogast 1999; Avise et al. 1984, 1987; Gill et al. 1993; Green et al. 1996; Zink 1996; Zink and Dittmann 1993) that are presumed to have undergone rapid, postglacial, northward dispersal from a limited population source (Ball and Avise 1992; Gaines et al. 1997; Gill et al. 1993). Palynologic evidence indicates that after the most recent glacial retreat, boreal forests quickly recolonized most of northern North America, with spruce (*Picea*) populations previously confined to the southeastern United States recolonizing Alaska by about 8,000 years ago (Ritchie 1987). A rapid northward and westward recolonization of boreal forest such as this would have provided an opportunity for a similarly rapid recolonization of northern North America by southeastern populations of *Tamiasciurus* and the eventual coalescence of these populations with those that had persisted in the northern Rocky Mountains.

The most likely locations of Late Pleistocene refugia for the southwestern and western clades of *Tamiasciurus* are the American Southwest and the Pacific coastal region of the United States, respectively. In contrast to the eastern mtDNA lineage of *Tamiasciurus,* which seems to have undergone an extensive postglacial range expansion, the southwestern and western mtDNA

lineages of *Tamiasciurus* appear to have expanded their ranges only slightly northward after glacial retreats. Asymmetrical patterns of postglacial range expansion and secondary contact between populations previously isolated in separate refugia may explain geographic locations of major phylogeographic discontinuities observed in *Tamiasciurus* (Fig. 4).

The phylogeographic discontinuity observed in *Tamiasciurus* in the Pacific Northwest (Fig. 4) aligns closely with genetic and distributional discontinuities in a variety of boreally distributed vertebrates. These include the mule deer (*Odocoileus hemionus*—Cronin 1992), black bear (*Ursus americanus*—Byun et al. 1997; Cronin et al. 1991; Demboski et al. 2000; Wooding and Ward 1997), marten (*Martes americanus*) and dusky shrew (*Sorex monticolus*—Demboski et al. 2000), northern flying squirrel (*G. sabrinus*—Arbogast 1999); leopard frog (*Rana pretiosa*—Green et al. 1996), giant salamanders (*Dicamptodon*—Good 1989), common yellowthroat (*Geothlypus trichas*) and rufous-sided towhee (*Pipilo erythrophthalmus*—Ball and Avise 1992), chickadee (*Parus*—Gill et al. 1993), and fox sparrow (*Passerella iliaca*—Zink 1996). A particularly high level of concordance seems to exist between the phylogeographic patterns of *Tamiasciurus* and *G. sabrinus* (Arbogast 1999). The major differences between the phylogeographic patterns of these 2 taxa are that the western clade of *Tamiasciurus* seems to extend slightly further to the east than does that of the western clade of *G. sabrinus* and that the current distribution of *G. sabrinus* does not extend southward into regions of the United Sates occupied by the southwestern clade of *Tamiasciurus.* The genetic discontinuity shared by *Tamiasciurus* and a wide variety of boreally distributed vertebrates in the Pacific Northwest supports an evolutionary scenario wherein at least 2 previously separated boreal forest biotas have come into recent secondary contact in this region (Arbogast 1999) forming a pronounced biogeographic ''suture zone'' (Remington 1968:322).

Taxonomic implications.—The nature of species boundaries within *Tamiasciurus* has remained somewhat enigmatic to date, largely because of an incomplete understanding of whether the recognized species are reproductively isolated. For example, Smith (1968) and Stevens and Nellis (1974) collected specimens with intermediate pelage coloration from British Columbia and Washington, respectively, which they proposed to be hybrids. Intermediate pelage also has been observed in the Blue Mountains of Oregon (Hatton and Hoffmann 1979) and on Vancouver Island (Lindsay 1982), suggesting that hybridization also may occur in these areas. To address taxonomic problems posed by the existence of intermediately colored squirrels, Lindsay (1982) evaluated the morphology of 20 cranial characters in populations of *T. douglasii* and *T. hudsonicus* from the Pacific Northwest. He concluded that the 2 species are morphologically distinct and that squirrels exhibiting intermediate coloration consistently clustered with individuals of 1 species or the other. Thus, Lindsay (1982) argued that *T. douglasii* and *T. hudsonicus* are isolated reproductively and represent separate species. However, Verts and Carraway (1998) found a large number of *Tamiasciurus* from central Oregon that could not be classified as either *T. douglasii* or *T. hudsonicus* based on the characters used by Lindsay (1982). The presence of unique mtDNA haplotypes (haplotypes V and VI) in this region indicates that populations of *Tamiasciurus* currently inhabiting the Blue Mountains of north-central and northeastern Oregon have become genetically differentiated from both western and more eastern populations of *Tamiasciurus,* although apparently to a lesser degree in the latter case. This may explain difficulties in classifying individuals from this region as either *T. hudsonicus* or *T. douglasii* (Verts and Carraway 1998).

Because mtDNA typically is inherited in

a matrilineal fashion in mammals (Avise et al. 1987), the cytochrome-*b* data presented here bear only circumstantially on species boundaries within *Tamiasciurus*. However, from a taxonomic perspective, it is important that the mtDNA and allozymic data agree in suggesting that all populations of *Tamiasciurus* examined in this study are closely interrelated. Furthermore, absence of fixed allelic differences among the 3 recognized species of *Tamiasciurus* suggests that they may not be isolated reproductively. Although samples from the Baja California populations of *Tamiasciurus* (currently assigned to *T. mearnsi*) are small, phylogenetic analysis of the mtDNA sequence data consistently included *T. mearnsi* from Baja California within the western clade along with individuals assigned to *T. douglasii* (Figs. 2 and 3). Bootstrap values supporting this relationship are high, and levels of sequence divergence between *T. mearnsi* and *T. douglasii* are quite low (Table 1). Thus, Hall's (1981) inclusion of *T. mearnsi* as a subspecies within *T. douglasii* seems to be a more accurate reflection of species boundaries within the genus than is the current taxonomy (Lindsay 1981; Wilson and Reeder 1993).

The mtDNA data also support the presence of a distinct southwestern clade within *Tamiasciurus* (Fig. 3). Populations of *Tamiasciurus* in the southwestern Rocky Mountains show sufficient morphologic distinctness from more northern populations of *T. hudsonicus* to have warranted the earlier specific status of *T. fremonti* before the discovery of apparent introgression between the 2 groups (Hardy 1950). Recent morphologic studies also support the presence of a distinct group of *Tamiasciurus* in the southwestern United States (Lindsay 1987). As such, the combined mtDNA and morphologic evidence suggest that *T. hudsonicus* is comprised of 2 distinct evolutionary lineages.

With the exception of those populations comprising the southwestern clade, 3 of the 4 methods of phylogenetic reconstruction, including the phylogeny presented in Fig. 3, support the presence of a widespread clade consisting of the remaining populations of *T. hudsonicus* (Figs. 2–4); the exception (Fig. 2B) most likely reflects problems associated with the inclusion of distant outgroups in the phylogenetic analyses. The geographic boundary between the eastern clade and the western *T. douglasii–T. mearnsi* clade seems to be coincident with the John Day River in Oregon. This result also is supported by mtDNA sequence data from the more rapidly evolving control region (B. S. Arbogast, in litt.). Analysis of vocalization data also suggests that the John Day River may be an important geographic boundary between populations of *Tamiasciurus*. C. C. Smith communicated to Hatton and Hoffmann (1979) that squirrels south and west of the John Day produced calls characteristic of *T. douglasii*, whereas those east of the river gave calls characteristic of *T. hudsonicus* (Verts and Carraway 1998). Thus, evidence exists that 2 previously separated, but closely related, evolutionary lineages of *Tamiasciurus* come into contact, or nearly into contact, in this region.

Our results do not support the current taxonomy for *Tamiasciurus*. The lack of observed genetic differentiation of the Baja California populations (currently recognized as *T. mearnsi*) suggests that these populations do not represent a distinct species. Furthermore, the low levels of genetic differentiation between *T. douglasii* and *T. hudsoni*cus, coupled with the existence of apparent hybrids and individuals that cannot be classified to species based on morphology where the 2 species come into contact in the Pacific Northwest (Verts and Carraway 1998), suggest that *T. douglasii* and *T. hudsonicus* are probably conspecific. Thus, recognition of a single, phenotypically variable species, *T. hudsonicus*, comprised of 3 subspecies, *T. h. hudsonicus* (eastern clade), *T. h. douglasii* (western clade, including *T. mearnsi*), and *T. h. mogollonensis* (southwestern clade), would

best reflect the currently available genetic and morphologic data. Alternatively, each of the 3 proposed subspecies could be recognized as separate phylogenetic species (Cracraft 1983). Although differing from the current taxonomy in several respects, geographic patterns of genetic variation observed within *Tamiasciurus* are consistent with those observed in a variety of North American boreal forest taxa. These common phylogeographic patterns most likely were generated as a result of changes in the distribution of boreal forests associated with glacial–interglacial cycles of the Quaternary.

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APPENDIX I

Sampling localities, type of data collected, and mitochondrial (mtDNA) haplotype designations for individuals of *Tamiasciurus hudsonicus*, *T. douglasii*, and *T. mearnsi* examined in this study. Assignments of species is based on distributional data presented in Hall (1981) and Lindsay (1981). Localities are depicted in Fig. 1. ''X'' indicates type of data (mtDNA, allozymic, or both) collected for a particular individual. Roman numerals designate which of the 14 unique mtDNA haplotypes (I–XIV) was observed in a particular individual.

Taxon	State or province	County	Museum tissue, GenBank accession number[a]	Allozymes	mtDNA	mtDNA haplotype designation
T. hudsonicus	Tennessee	Carter	BSA226		X	I
	West Virginia	Randolph	BSA225		X	II
	Ohio	Summit	MSB:NK63048	X		
	Maine	Hancock	MSB:NK62126	X	X	II
	Michigan	Mackinac	UMMZ:162427	X	X	III
	Michigan	Alger	UMMZ:167068	X	X	III
	Minnesota	Station	MSB:NK63189	X	X	III
	Minnesota	Station	MSB:NK63201	X		
	South Dakota	Lawrence	PDS477	X		
	Colorado	Jackson	MSB:NK56591	X	X	IV
	Wyoming	Teton	PDS463	X	X	II
	Wyoming	Teton	PDS464		X	II
	Wyoming	Big Horn	PDS474	X	X	II
	Wyoming	Big Horn	PDS476	X		
	Wyoming	Albany	PDS483	X		
	Wyoming	Albany	PDS484	X	X	II
	Utah	San Juan	DSR3073	X		
	Utah	Wasatch	DSR3077	X	X	IV
	Utah	Wasatch	DSR3078	X	X	IV
	Utah	Wasatch	DSR3079	X		
	Utah	Wasatch	DSR3080	X	X	II
	Utah	Wasatch	DSR3081	X	X	II
	New Mexico	Sandoval	NMMNH:1767	X	X	XIII
	New Mexico	Sandoval	NMMNH:1768	X	X	XIV
	Arizona	Apache	MSB:NK17871	X	X	XII
	Arizona	Apache	MSB:NK17872	X	X	XII
	Washington	Okanogan	UWBM:BSA181	X	X	II
	Washington	Okanogan	UWBM:BSA184	X		
	Washington	Okanogan	MSB:NK8541	X		
	Washington	Okanogan	MSB:NK8542	X		
	Washington	Okanogan	MSB:NK8560	X		
	Washington	Okanogan	MSB:NK8561	X		
	Washington	Pend Oreille	SGM4		X	II
	Washington	Chelan	MSB:NK8571	X		
	Alaska	Fairbanks[b]	AF17840		X	III
	Alaska	Hill Island[b]	AF17873		X	III
	Alaska	—	MSB:NK20629	X	X	III
	Alaska	—	MSB:NK21987	X		
	Oregon	Union	UWBM:BSA201	X		
	Oregon	Union	UWBM:BSA202	X	X	V
	Oregon	Union	UWBM:BSA203	X	X	V
	Oregon	Union	UWBM:BSA204	X	X	V
	Oregon	Union	UWBM:BSA205	X		
	Oregon	Grant	UWBM:BSA206	X	X	V
	Oregon	Grant	UWBM:BSA207	X		

APPENDIX I—Continued.

Taxon	State or province	County	Museum tissue, GenBank accession number[a]	Allozymes	mtDNA	mtDNA haplotype designation
	Oregon	Grant	UWBM:BSA208	X	X	V
	Oregon	Grant	UWBM:BSA209	X	X	V
	Oregon	Grant	UWBM:BSA210	X	X	VI
	Oregon	Grant	UWBM:BSA211	X		
	Oregon	Grant	UWBM:BSA212	X	X	VI
T. douglasii	Oregon	Grant	UWBM:BSA213	X	X	VII
	Oregon	Grant	UWBM:BSA214	X		
	Oregon	Grant	UWBM:BSA215	X		
	Oregon	Klamath	UWBM:BSA216	X	X	VII
	Oregon	Klamath	UWBM:BSA217	X	X	VII
	Oregon	Klamath	UWBM:BSA218	X	X	VII
	Oregon	Klamath	UWBM:BSA219	X	X	VII
	Oregon	Lane	UWBM:BSA220	X	X	VIII
	Oregon	Lane	UWBM:BSA221	X	X	VIII
	British Columbia	Vancouver[b]	BSA:BC423		X	IX
	British Columbia	Vancouver[b]	BSA:BC3062		X	IX
	California	Alpine	MSB:NK8586	X		
	California	Calaveras	MSB:NK8582	X		
	California	Calaveras	MSB:NK8583	X		
	California	Humboldt	MSB:NK8579		X	X
T. mearnsi	Mexico	Baja California[b]	BSA235		X	XI
	Mexico	Baja California[b]	MSB:NK8062	X		
	Mexico	Baja California[b]	MSB:NK8068	X		

[a] Tissues and corresponding voucher specimens are housed in the following institutions: University of Alaska Museum, Fairbanks (AF); Museum of Zoology, University of Michigan, Ann Arbor (UMMZ); Museum of Southwestern Biology, University of New Mexico, Albuquerque (MSB); New Mexico Museum of Natural History, Albuquerque (NMMNH); and Burke Museum of Natural History and Culture, University of Washington, Seattle (UWBM). Tissues provided by private individuals (S. G. Mech, B. R. Riddle, D. S. Rogers, and P. D. Sudman) are designated by their initials or the catalog number of the senior author (BSA).

[b] Noncounty locality.