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Molecular Phylogeny of the Chipmunks Inferred from Mitochondrial Cytochrome *b* and Cytochrome Oxidase II Gene Sequences

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There are currently 25 recognized species of the chipmunk genus *Tamias*. In this study we sequenced the complete mitochondrial cytochrome *b* (cyt *b*) gene of 23 *Tamias* species. We analyzed the cyt *b* sequence and then analyzed a combined data set of cyt *b* along with a previous data set of cytochrome oxidase subunit II (COII) sequence. Maximum-likelihood was used to further test the fit of models of evolution to the cyt *b* data. Other sciurid cyt *b* sequence was added to examine the evolution of *Tamias* in the context of other sciurids. Relationships among *Tamias* species are discussed, particularly the possibility of a current sorting event among taxa of the southwestern United States and the extreme divergences among the three subgenera (*Neotamias*, *Eutamias*, and *Tamias*). © 2001 Academic Press

***Key Words: Tamias*; *Eutamias*; *Neotamias*; molecular evolution; phylogenetics; mitochondrial DNA; cytochrome *b*; cytochrome oxidase subunit II; maximum-likelihood, molecular clock.**

INTRODUCTION

Chipmunk systematics has been elucidated by means of morphological, karyotypic, immunological, and host–parasite data sets. There have been diverse attempts to determine the patterns of evolution, dispersal, and relatedness among these taxa. The different data sets have generated varying conclusions about the systematics of chipmunks, ranging from the currently recognized single genus (*Tamias*) with three subgenera, to previous designations that included two separate genera with one genus being further subdivided into two subgenera, to the designation of three separate genera (*Tamias, Neotamias*, and *Eutamias*) for all chipmunk species (Allen, 1891; Howell, 1929; Ellerman, 1940; White, 1953a; Nadler, 1964; Nadler *et al.,* 1969, 1977, 1985; Ellis and Maxson, 1979; Hafner, 1984; Levenson *et al.,* 1985; Jameson, 1999). We investigated the taxonomy of this group by generating a molecular phylogeny based on mitochondrial DNA (mtDNA).

Chipmunk species have a remarkable geographical distribution. *Tamias sibiricus* (subgenus *Eutamias*) occurs in Asia, *T. striatus* (subgenus *Tamias*) occurs throughout the eastern United States, and the remaining 23 species (subgenus *Neotamias*) are distributed throughout the western United States and Mexico. The distribution of *Tamias* has led many authors to hypothesize many possible evolutionary events that might have produced this distribution. However, the origin of the ancestral stock is not agreed upon, and we will argue that it is not important for a discussion of the taxonomy of these taxa.

The purpose of this study was to explore the evolution and systematics of the genus *Tamias* as inferred from the complete mitochondrial sequences of the cytochrome *b* (cyt *b*) gene. In addition, we combined cyt *b* sequence with cytochrome oxidase subunit II (COII) gene sequences from an earlier study (Piaggio and Spicer, 2000). The gene phylogenies of COII and cyt *b* were evaluated separately and in combination. We inferred a molecular phylogeny from the combined data to determine whether there was a geographic correlation to the clades on the tree. The cyt *b* data set includes more taxa than did our previous COII data set. Indeed, the cyt *b* phylogeny revealed unexpected relationships and recent speciation events. Therefore, we discuss the taxonomy of the additional taxa based on our cyt *b* molecular phylogeny and in the context of previous morphological analyses. Finally, we use the cyt *b* tree to examine the taxonomic relationships among species in the subgenus *Neotamias*. *Neotamias* represents taxa that have undergone extensive radiation and differentiation across the western United States. The taxonomic relationships inferred from the *Neotamias* clade provide resolution to previous debates on the taxonomy of many species within this group.

METHODS AND MATERIALS

Specimens. Forty-eight specimens representing 23 of the 25 currently recognized species in the genus *Tamias* (Levenson *et al.,* 1985) and the outgroup taxon

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TABLE 1

Specimens Collected and Loaned, Localities, and Catalogue Numbers

Species (F/S/E)	Owner (catalogue)	GenBank Accession No.	Locality
Tamiasciurus hudsonicus (F)	MSB (61555) (NK 4324)	AF147643	New Mexico: Taos Co.
Tamias amoenus (F)	A. Piaggio	AF147629	California: Siskiyou Co.
Tamias amoenus (F)	MVZ (152780)	AF147630	California: Nevada Co.
Tamias amoenus (F)	A. Piaggio	AF147631	California: Lassen Co.
Tamias amoenus (F)	MSB (72231) (NK 51013)	AF147633	Wyoming: Park Co.
Tamias amoenus (F)	MSB (43427) (NK 3137)	AF147632	Washington: Kittatas Co.
Tamias bulleri (F)	MSB (48162) (NK 9505)	AF147634	Mexico: Coahuila
Tamias canipes (F)	MSB (57799) (NK1869)	AF147635	New Mexico: Lincoln Co.
Tamias cinereicollis (F)	MSB (53548) (NK 1927)	AF147636	Arizona: Apache Co.
Tamias cinereicollis (F)	MSB (54508) (NK4225)	AF147637	Arizona: Apache Co.
Tamias cinereicollis (F)	MSB (65041) (NK 19644)	AF147638	New Mexico: Socorro Co.
Tamias dorsalis (F)	A. Piaggio	AF147641	Arizona: Pima Co.
Tamias dorsalis (F)	MSB (76872) (NK 55222)	AF147640	Utah: Beaver Co.
Tamias dorsalis (F)	MSB (70112) (NK 28742)	AF147639	New Mexico: Cibola Co.
Tamias durangae (F)	ZTNH (217 CWK1985)	AF147642	Mexico: Durango
Tamias merriami (F)	MSB (43176) (NK 4669)	AF147644	California: Riverside
Tamias minimus (F)	MSB (84514) (NK53727)	AF147647	California: Mono Co.
Tamias minimus (F)	MSB (77094) (NK 55461)	AF147650	Utah: Summit Co.
Tamias minimus (F)	MSB (56781) (NK4422)	AF147648	Colorado: Dolores, Co.
Tamias minimus (F)	MSB (53280) (NK7876)	AF147649	Canada: Manitoba
Tamias minimus (F)	MSB (55759) (NK2113)	AF147645	Canada: Alberta
Tamias minimus (F)	A. Piaggio	AF147646	California: Sierra Co.
Tamias obscurus (F)	MSB (43179) (NK 4646)	AF147651	California: Riverside Co.
Tamias obscurus (F)	MSB (47429) (NK 8069)	AF147652	Mexico: Sierra San Pedro Martir, Baja California
Tamias obscurus (F)	MVZ (148043)	AF147653	Mexico: Baja California
Tamias ochrogenys (S)	MVZ (151441)	AF147654	California: Sonoma Co.
Tamias palmeri (F)	MSB (59000) (NK 2473)	AF147655	Nevada: Clark Co.
Tamias panamintinus	MVZ (MDM 213)	AF147656	California: San Bernardino Co.
Tamias quadrimaculatus (F)	MSB (83634) (NK 73120)	AF147657	California: Mono Co.
Tamias quadrivittatus (F)	MSB (56898) (NK 3481)	AF147659	New Mexico: Bernalillo Co.
Tamias quadrivittatus (F)	MSB (61498) (NK 4053)	AF147660	New Mexico: Sandoval Co.
Tamias quadrivittatus (F)	MSB (80142) (NK 56170)	AF147658	Colorado: Costilla Co.
Tamias ruficaudus (E)	J.S.	AF147661	Idaho: Latah Co.
Tamias rufus (F)	MSB (76530) (NK56201)	AF147662	Colorado: Rio Blanco Co.
Tamias rufus (F)	MSB (76532) (NK56249)	AF147663	Colorado: Rio Blanco Co.
Tamias senex (F)	A. Piaggio	AF147665	California: Sierra Co.
Tamias senex (F)	MVZ (152779)	AF147664	California: Nevada Co.
Tamias sibiricus	BMNH (UWBM 39067)	AF147666	Russia: Khabarovskiy Kray
Tamias sibiricus	BMNH (UWBM 39255)	AF147667	Russia: Magdanskaya Oblast
Tamias siskiyou (S)	MVZ (182737)	AF147668	Oregon: Jackson Co.
Tamias sonomae (F)	MVZ (152777)	AF147669	California: Marin Co.
Tamias striatus fisheri (F)	CMNH (105324)	AF147670	Pennsylvania: Beaver Co.
Tamias striatus fisheri (F)	CMNH (105325)	AF147671	Pennsylvania: Beaver Co.
Tamias striatus lysteri (F)	CMNH (105327)	AF147672	Pennsylvania: Bradford Co.
Tamias striatus lysteri (F)	CMNH (105328)	AF147673	Pennsylvania: Bradford Co.
Tamias townsendii (F)	MSB (43429) (NK 3136)	AF147675	Washington: Kittatas Co.
Tamias townsendii (F)	MSB (43546) (NK 3252)	AF147676	Washington: Clallam Co.
Tamias townsendii (F)	MSB (53282) (NK 7980)	AF147674	Oregon: Benton Co.
Tamias umbrinus (F)	MSB (76765) (NK 55411)	AF147677	Utah: Beaver Co.

Note. F, frozen tissue; E, prepared extraction; S, study skin sample. MSB, Museum of Southwestern Biology, Albuquerque, NM; MVZ, University of California Museum of Vertebrate Zoology, University of California, Berkeley, CA; BMNH, Burke Museum of Natural History, Seattle, WA; J.S, Dr. Jack Sullivan, University of Idaho; ZTNH, Zaddock Thompson Natural History Collections, University of Vermont, Burlington, VT; MNH, Carnegie Museum of Natural History, Pittsburgh, PA.

Tamiasciurus hudsonicus were sequenced for this study (Table 1). Some specimens were collected in the field either by gun or by trap. Tissue and voucher specimens of collected animals were submitted to the Museum of Southwestern Biology in Albuquerque, New Mexico. In the field, specimens were placed on dry ice and then transferred to the lab; the skins and skull were prepared according to Hall (1981). Samples of liver and thigh muscle were removed for DNA extraction; the remaining tissue was stored at −80°F.

TABLE 2

The Cytochrome *b* Primer Sequences

Primers	Sequence
L14724	5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′
L14735	5′-AAT CAT CGT TGT AAT TCA ATA-3′
L14766	5′-TTA ATG ACA AAC ATC CGC AAA AC-3′
L14847	5′-TTC TGC ATG ATG AAA TTT TGG-3′
L15060	5′-GCC GAG GAC TTT ACT ATG G-3′
L15066	5′-GCC GAG GAC TTT ACT ATG GAT CAT A-3′
L15732	5′-ACT AAG ATT CAG AAT A-3′
H15041	5′-TAT GAT CCA TAG TAA AGT CCT CGG C-3′
H15042	5′-CCA TAG TAA AGT CCT CGG C-3′
H15230	5′-GAG AAG CCT CCT CAG ATT CAT TC-3′
H15717	5′-TAT TCT GAA TCT TAG T-3′
H15906	5′-GGT TTA CAA GAC CAG AGT AAT-3′
H15915	5′-AAC TGC AGT CAT CTC CGG TTT ACA AGA C-3′

Note. All primers are designed by authors to be *Tamias* specific. The only exceptions are L14724 and H15915, which are universal external primers (Kocher *et al.*, 1989). Primer names are based on their alignment to the human mitochondrial genome (GenBank Accession No. J01415).

Specimens not collected in the field were obtained through loans of frozen tissue or tissue from study skins from the museums or individuals. The specimens, catalogue numbers, GenBank accession numbers, and locality information are included in Table 1.

DNA extraction, amplification, and sequencing. Total genomic DNA was extracted from frozen tissue samples by standard salt extraction methods (Hillis *et al.*, 1990) with minor modifications. A standard phenol/chloroform method (Werman *et al.*, 1990) with some modifications was used to extract DNA from study skin tissue. To obtain double-stranded DNA products, polymerase chain reactions (PCRs) were run in 50-μl reactions. Amplifications of the mitochondrial cyt *b* gene required external primer pairs, L14724 with H15915 (Kocher *et al.*, 1989), which amplified a segment approximately 1200 bp in length. Internal primers were designed specifically to *Tamias* sequence (cyt *b* primer sequences in Table 2). Amplifications were carried out in a P100 thermal cycler (Perkin–Elmer) for 30 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 1 min, and extension at 72°C for 2 min. Amplified PCR products were cleaned with a polyethylene glycol precipitation protocol (Kusukawa *et al.*, 1990) prior to being sequenced.

All sequencing was done via dye terminator cycle sequencing on a Catalyst 800 Molecular Biology Lab Station and followed the protocol specified by the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Revision B, August 1995; Perkin–Elmer). Primers used for amplification were the same as those used for the single-stranded cycle sequencing reactions.

Sequence analysis and phylogeny estimation. The cyt *b* sequences were initially aligned in Sequencher 3.01 and compared to sequences from *Homo* and *Rattus* (GenBank Accession Nos. J01415 and X14848, respectively). The overall base composition bias was calculated according to Irwin *et al.* (1991) and ranges from zero to one (zero indicating no bias and one indicating complete composition bias). An extreme overabundance of one nucleotide can increase the tendency for sites to become saturated (Irwin *et al.*, 1991). In addition, a skewed bias could violate the assumption of parsimony analyses that there is an equal probability of change to any state among the bases (Pena and Kocher, 1995; Spicer, 1995; Yoder *et al.*, 1996).

A variety of techniques were used to infer phylogenetic relationships with the computer program Paup 4.0b2 (Swofford, 1999). Parsimony analyses were accomplished by use of a random stepwise addition option of the heuristic search; 100 replicates were performed with unordered changes. Also, a step matrix to weight transversional changes was employed to carry out a parsimony analysis. When several equally parsimonious trees were found, a strict consensus tree (Rohlf, 1982) was produced to summarize the data. To assess confidence in the branching patterns, bootstrap analyses were performed (Felsenstein, 1985) with heuristic searches set for a closest stepwise addition option; 500 random iterations were performed.

To generate the most complete data set and to be able to evaluate the evolutionary relationships among other sciurids in relation to *Tamias*, we added other sciurid sequences to the tree. These sequences were obtained from GenBank or other references (see below). Since only cyt *b* data were available for most of the taxa, we used our cyt *b* data alone to compare sciurid divergences, instead of the combined COII and cyt *b* data set. We added *Sciurus aberti* and *Sciurus niger* (Wettenstein *et al.*, 1995) from GenBank (Accession Nos. SAU10171 and SNU10180, respectively), *Sciurus carolinensis* (Thomas and Martin, 1993), and *Tamiasciurus hudsonicus*. For the sister taxa to *Tamias*, we added *Marmota* and *Spermophilus* sequences. These sequences included *Marmota himalayana, Marmota sibirica, Marmota camtschatica, Marmota marmota, Marmota flaviventris, Marmota vancouverensis, Marmota menzbieri*, and *Marmota monax* (GenBank Accession Nos. respectively, AF143928, AF143938, AF143922, AF143929, AF143926, AF143939, AF143931, and AF143932; Steppan *et al.*, 1999), and *Marmota flaviventris* (Thomas and Martin, 1993) for the *Marmota* clade. The *Spermophilus* clade included *Spermophilus richardsonii* (GenBank Accession No. S73150), *Spermophilus columbianus, Spermophilus tridecemlineatus*, and *Spermophilus lateralis* (Thomas and Martin, 1993).

Maximum-likelihood was used to evaluate the fit of the data to the parsimony-based topologies for the cyt *b* data set. We tested hypotheses by log likelihood ratio tests (LRT). The hypotheses were based on models of differing rates of evolutionary changes among sequences; these

models included the Jukes–Cantor model (Jukes and Cantor, 1969), the Kimura two-parameter model (Kimura, 1980), the HKY 85+ estimation of rate heterogeneity (Γ) + estimation of invariable sites (I) (Hasegawa *et al.*, 1985), and the general time reversible model + Γ + I (Lanave *et al.*, 1984; Rodriguez *et al.*, 1990). The LRT results in a likelihood ratio statistic (Δ), which is χ^2 distributed and allows testing of whether one hypothesis is significantly better than another (Yang *et al.*, 1994). When a model of evolutionary change that fits significantly better than that of parsimony was discovered, a maximum-likelihood tree was generated by use of a random stepwise addition option of the heuristic search; 100 replicates were performed. This maximum-likelihood tree and model were used to generate branch lengths among taxa and the branch lengths were then graphed as a distribution of evolutionary change among the taxa. The maximum-likelihood model was also used to test the null hypothesis that the sequences were evolving at constant rates and therefore fit a molecular clock (Felsenstein, 1993).

RESULTS

The complete cyt *b* was sequenced in both directions, resulting in 1140 aligned base pairs (bp), of which 453 sites were variable, and 393 were phylogenetically informative. The 23 *Tamias* species and *Tamiasciurus hudsonicus* sequences have been deposited with GenBank under Accession Nos. AF147629–AF147677.

Of the 453 variable sites of cyt *b* sequence, there are 77 variable sites in the 1st codon position, 28 in the 2nd position, and 348 in the 3rd position. The combined data set of COII and cyt *b* has a total of 1824 bases; of these there are 648 variable sites. There are 111 variable sites in the 1st codon position, 20 in the 2nd position, and 517 in the 3rd position in the combined data set.

Table 3 shows base composition and base composition bias for the cyt *b* and combined data sets. As with other mammalian mitochondrial genes the bases are not found in equal proportions, but the rates do not vary among the taxa.

The cyt *b* sequences were first analyzed under the maximum-parsimony model. The cyt *b* data set contains 65 specimens, including members of the genera *Tamias, Marmota, Spermophilus, Tamiasciurus*, and *Sciurus*. A heuristic search resulted in 48 equal trees, with tree lengths (L) of 2652 steps, a consistency index (CI) of 0.299, and a retention index (RI) of 0.699. The results of the bootstrap analyses are included on a strict consensus tree of the 48 most parsimonious trees (Fig. 1). The branches near the tips appear to be well supported by bootstrapping, but the relationships among the clades tend to have lower bootstrap values.

To combine the cyt *b* and COII (Piaggio and Spicer, 2000) data sets, we had to determine that they demonstrated equivalent histories. We performed a partition homogeneity test in Paup 4.0b2 (Swofford, 1999), which revealed that the data sets were not significantly heterogeneous ($P = 0.86$), although the tree topologies for cyt *b* and COII that resulted from parsimony analyses were slightly different. To determine whether the topologies were statistically different, a Kishino and Hasegawa (1989) test was performed. The cyt *b* taxa set was reduced to be equivalent to the COII taxa set, to have a valid statistical test. The reduction of taxa in the cyt *b* tree resulted in three most parsimonious trees with 25 taxa. When compared to the 95 most parsimonious COII trees (Piaggio and Spicer, 2000), there was no statistical difference based on the COII data set under a parsimony Kishino–Hasegawa (1989) test (cyt *b* trees one and three: length difference, l.d. = 497; degrees of freedom, $df = 1$; standard deviation, SD = 7.21597; $t = 0.2772$; $P = 0.7817$; cyt *b* tree two: l.d. = 1; $df = 1$; SD = 7.00502; $t = 0.1428$; $P = 0.8865$). Therefore, there were no significant differences between the gene topologies.

The combined data set has 23 taxa, including *Tamias* species and the outgroup *Tamiasciurus hudsonicus* and *Sciurus carolinensis*; this is a reduced-taxa data set because of limited COII data available for sciurids. Taxa representing multiple samples were eliminated so that only one specimen was left to represent each species. The combined data set was analyzed by a maximum-parsimony search that resulted in one tree (L = 1777, CI = 0.490, RI = 0.516). The results of the bootstrap analyses are included on the most parsimonious phylogram (Fig. 2). Transversional parsimony was performed on the com-

TABLE 3

Base Composition Bias for Cytochrome *b* and the Combined Data Set of COII and cyt *b*

	1st	2nd	3rd	Var	All
			Cytochrome *b*		
A	0.272	0.196	0.325	0.244	0.289
C	0.432	0.152	0.369	0.395	0.274
G	0.114	0.076	0.016	0.037	0.121
T	0.182	0.576	0.290	0.324	0.315
Bias	0.272	0.435	0.312	0.292	0.171
			Combined		
A	0.209	0.099	0.335	0.308	0.307
C	0.451	0.197	0.336	0.350	0.261
G	0.087	0.141	0.020	0.035	0.124
T	0.253	0.563	0.309	0.307	0.309
Bias	0.447	0.519	0.453	0.451	0.333

Note. Values are calculated according to codon position (1st, 2nd, and 3rd, all positions (All), and variable positions only (Var). The bias is calculated by the formula of Irwin *et al.* (1991) and ranges in value from zero to one (zero indicating no bias, one indicating complete compositional bias).

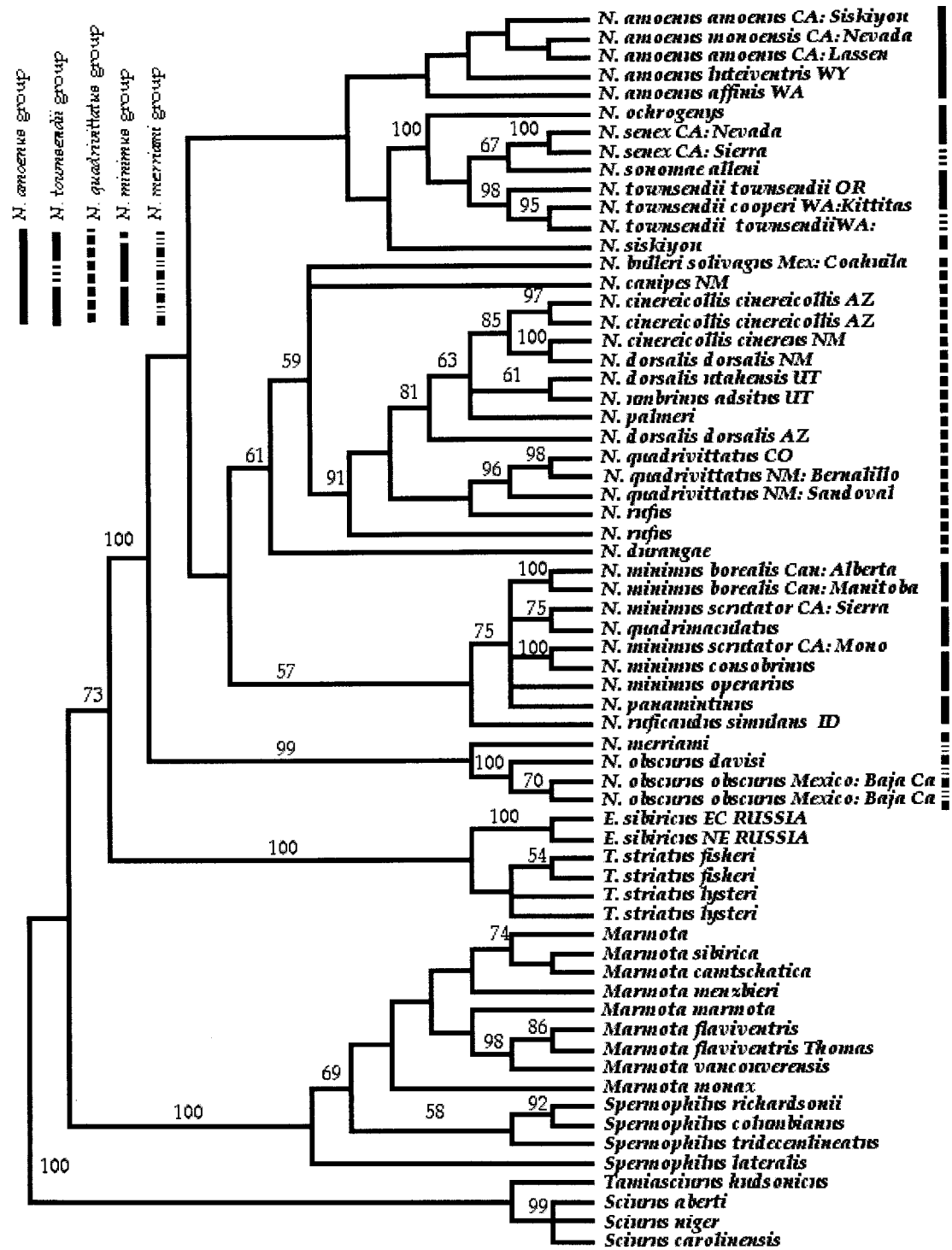

FIG. 1. Mitochondrial cytochrome *b* strict consensus parsimony tree produced by the random stepwise addition branch-swapping algorithm. The search resulted in 48 most parsimonious trees with 2652 steps with a consistency index of 0.299 and a retention index of 0.699. Bootstrap support is indicated on the nodes (only values greater than 50% are presented). Species groups within *Neotamias* are indicated graphically.

bined data set with 23 taxa (Brown *et al.*, 1982; Swofford and Olsen, 1990) and compared to the equally weighted maximum-parsimony tree. If this tree has a topology distinctly different from that of the equally weighted parsimony tree, then it is possible that the equally weighted tree might not accurately represent the real

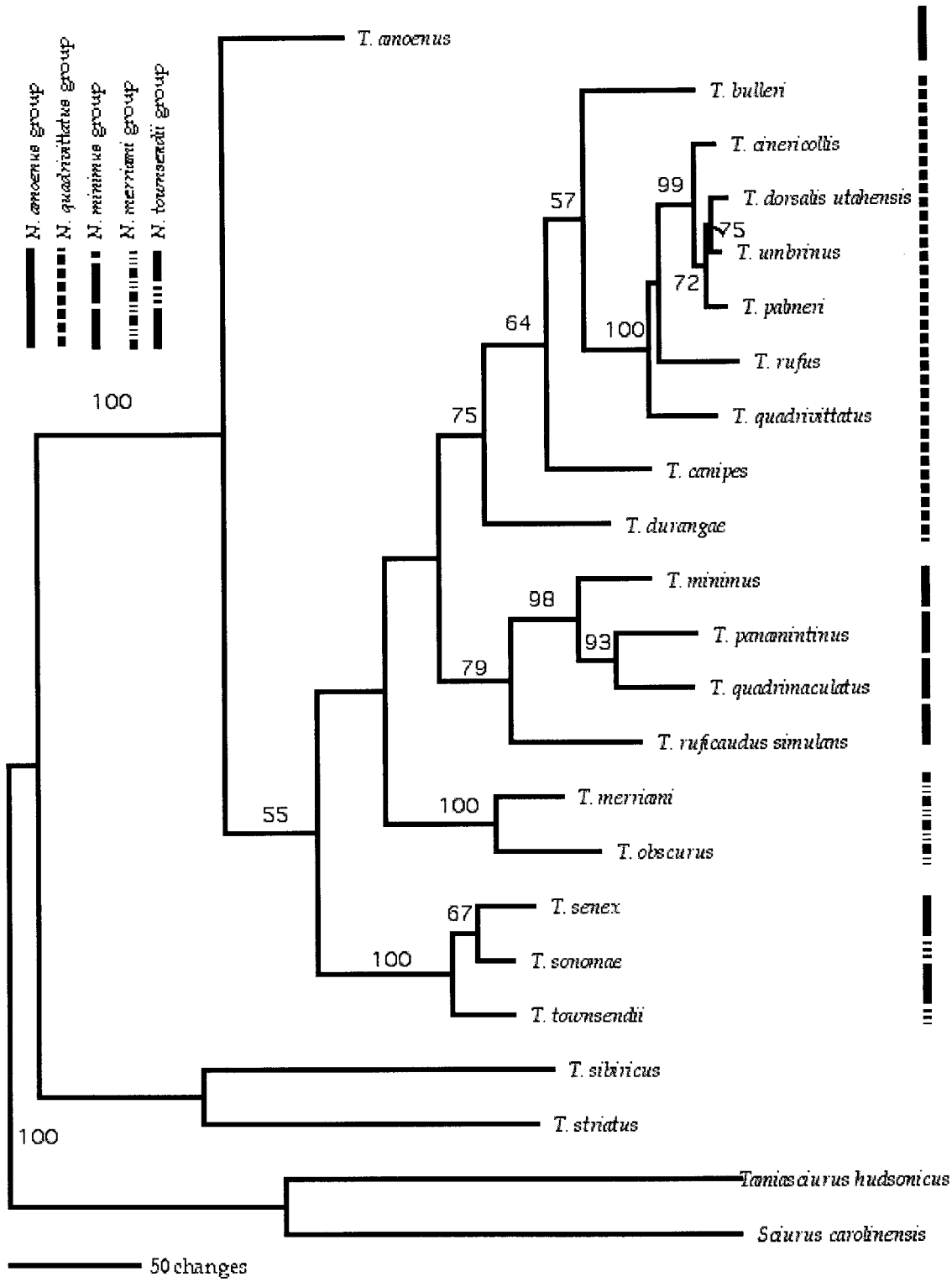

FIG. 2. Mitochondrial cytochrome oxidase subunit II and cytochrome *b* combined most parsimonious phylogram tree produced by the random stepwise addition branch-swapping algorithm. The search resulted in one most parsimonious tree with 1824 steps with a consistency index of 0.480 and a retention index of 0.518. Bootstrap support is indicated on the nodes (only values greater than 50% are presented). Species groups within *Neotamias* are indicated graphically.

TABLE 4

Maximum-Likelihood Analysis of Hierarchical Substitution Models for the cyt *b* Sequence Data

H_0 vs H_1	-LnL_0	-LnL_1	-2lnΛ	*df*	*P*
JC vs K2P	14407.075	13180.61	2452.93	2	<0.0001*
K2P vs HKY85	13180.61	12865.442	630.34	1	<0.0001*
HKY85 vs HKY85+Γ	12865.442	11225.756	3279.37	1	<0.0001*
HKY85+Γ vs HKY85+Γ+I	11225.756	11210.568	30.38	1	<0.0001*
GTR vs GTR+Γ	12667.989	11206.742	2922.49	1	<0.0001*
GTR + Γ vs GTR+Γ+I+base freq.	11206.742	11192.937	27.61	5	<0.0001*
GTR+$\Gamma\Gamma$+I+base freq. (clock enforced)	11192.937	11221.209	56.54	4 1	0.054
Maximum-likelihood GTR+Γ+I+base freq.	n/a	11187.906	n/a	n/a	n/a
Maximum-likelihood GTR+Γ+I (clock enforced)	11187.906	11271.230	166.65	4 1	<0.0001*

Note. Likelihoods were evaluated with the likelihood ratio test as described under Methods and Materials. JC, Jukes–Cantor (1969); K2P, Kimura (1980); HKY85, Hasegawa *et al.* (1985); GTR, general time-reversible model (Lanave *et al.*, 1984; Rodriguez *et al.*, 1990); Γ, shape parameter of the gamma distribution estimated with 10 rate categories; I, proportion of invariable sites. Degrees of freedom when the hypothesis of a molecular clock is tested equal $n - 2$, where n = the number of taxa sampled (Felsenstein, 1993).

* Hypothesis rejected.

evolutionary history, which might be reflected in the transversional changes alone. A total of nine weighted transversional parsimony trees (L = 376; unweighted parsimony equivalent L = 1857) were produced. The topology of these trees was not different from the topology of the unweighted parsimony tree (L = 1777). The transversional parsimony does add length to the most parsimonious tree. Furthermore, log likelihood scores were also compared for the transversional parsimony data set (-ln likelihood = 12005.048) and the unweighted parsimony combined data set (-ln likelihood = 11789.318); again, the unweighted parsimony had a lower score, indicating a better fit to the data. Therefore, the unweighted parsimony tree appears to reflect the best estimate of the evolutionary history for these species.

We used the cyt *b* data with a reduced taxa set to examine whether other factors (e.g., transition/transversion rates or among-site rate heterogeneity) influenced the data set. For these analyses we used various maximum-likelihood methods. We used only the cyt *b* data so we could include other sciurid sequences, which were not available for the COII gene. To reduce the data set to make the maximum-likelihood iterations complete in a reasonable amount of time, we used only one representative for taxa that demonstrated monophyletic relationships with others of the same species in the parsimony analysis (Fig. 1). Therefore, only *T. dorsalis* and *T. cinereicollis* have more than one specimen in the maximum-likelihood tree because these taxa appear paraphyletic in the parsimony analysis (Fig. 1). The reduced taxa set contained 43 taxa including other sciurids obtained from sources described previously. This reduced data set resulted in nine most parsimonious trees (L = 2438, CI = 0.318, RI = 0.604).

Using these nine parsimony trees, we tested hypotheses of differing rates of evolutionary changes utilizing the log likelihood ratio tests. The resulting likelihood ratio statistic Δ and the χ^2 statistic between the models (Table 4) demonstrated that the GTR+G+I+estimated base frequencies model was the best model under maximum-likelihood. The tree with the lowest -In likelihood, generated by application of this maximum-likelihood model to the parsimony trees, is presented with bootstrap results (Fig. 3). We also generated a maximum-likelihood tree with the same model (Fig. 4). The trees have different topologies, but both are presented to demonstrate that membership within the *Tamias* clades do not change, regardless of which model or algorithm is applied. Only the relationships among the clades in the parsimony and the likelihood trees change. In fact, the difference between the parsimony tree with the GTR+I+G+estimated base frequencies likelihood model (−ln likelihood = 11192.937) and the likelihood tree with the same model (−ln likelihood = 11187.906) is only -lnΛ = 5 (Table 4).

Both the parsimony tree (with the GTR+I+G+ estimated base frequencies likelihood model) and the likelihood tree with the same model were tested under a molecular clock hypothesis of constant rates of evolutionary change (Table 4), and the hypothesis was rejected ($P < 0.001$). Therefore, we cannot apply a molecular clock to the data to estimate divergences. To compare divergences among taxa, branch lengths were generated from the maximum-likelihood tree (with the GTR+I+G+estimated base frequencies likelihood model). These branch lengths were used to generate a distribution of genetic distances within and between genera (Fig. 5). These branch lengths and the phylogenies presented are estimates of evolutionary relationships among *Tamias* taxa and allow evaluation and discussion of the evolution and systematics of this genus.

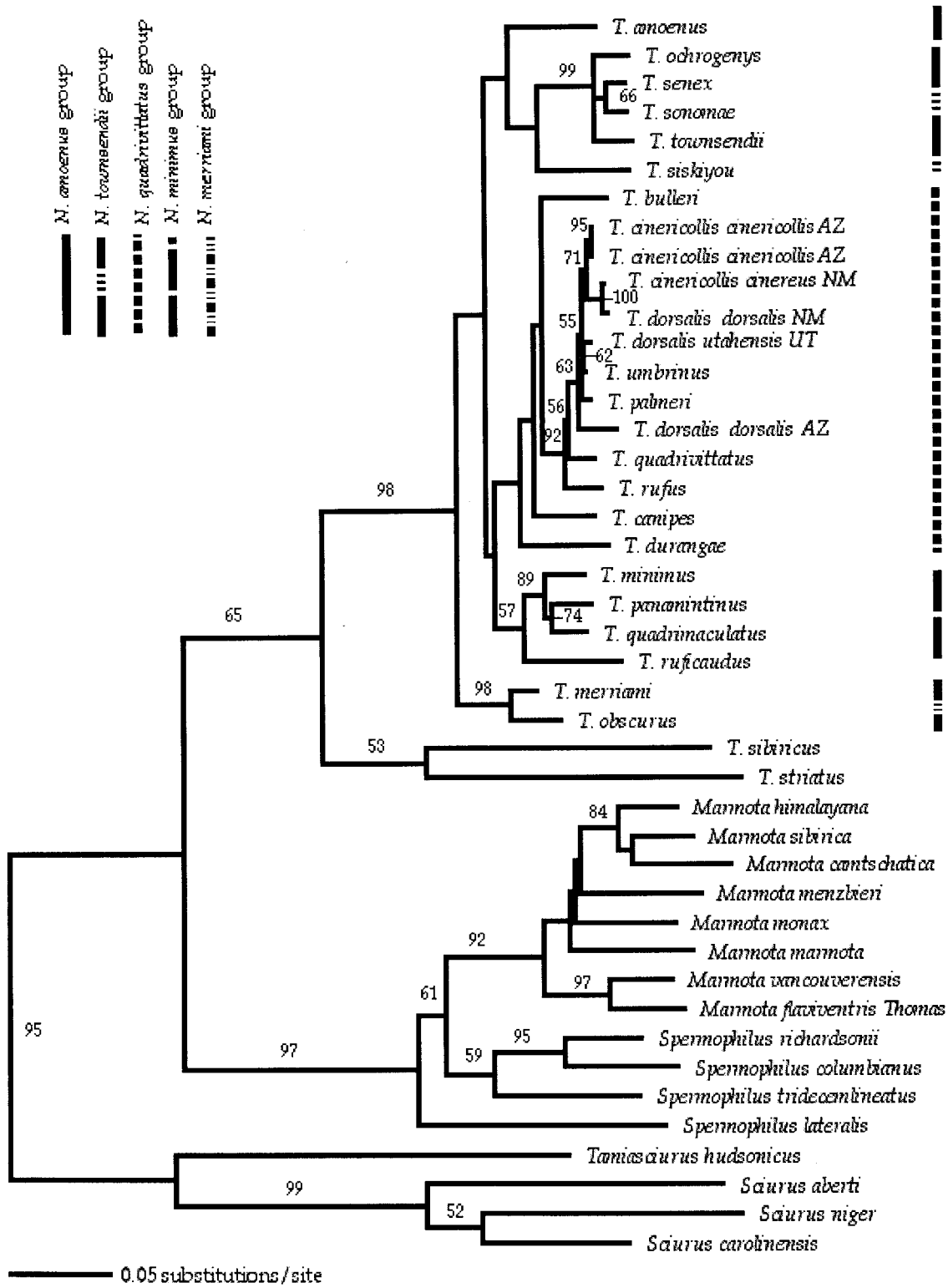

FIG. 3. Mitochondrial cytochrome *b* parsimony phylogram inferred from likelihood estimations of the GTR+I+G+estimated base frequencies model. This includes 43 taxa; all taxa that represented a second sample of a monophyletic group of the same species were pruned from the initial cyt *b* tree. The parsimony heuristic search resulted in nine trees with 2438 steps, a consistency index of 0.318, and a retention index of 0.604. Bootstrap support is indicated on the nodes (only values greater than 50% are presented). The parsimony tree presented has the lowest -1n likelihood score when the GTR+I+G+estimated base frequencies model is applied. Species groups within *Neotamias* are indicated graphically.

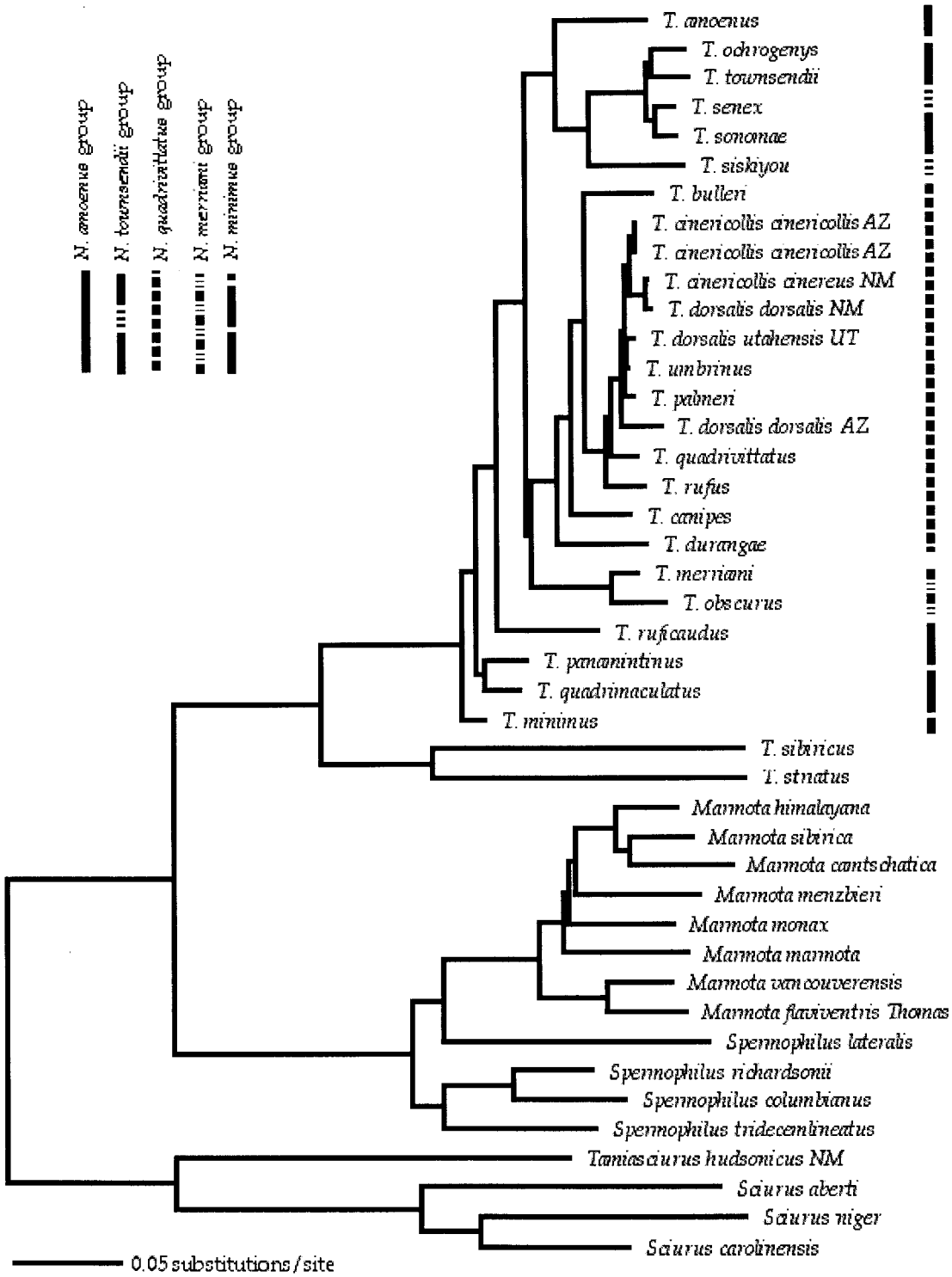

FIG. 4. Mitochondrial cytochrome *b* maximum-likelihood phylogram inferred from a GTR+I+G+estimated base frequencies model. This includes 43 taxa; all taxa that represented a second sample of a monophyletic group of the same species were pruned from the initial cyt *b* tree. The heuristic search for this tree resulted in a tree with -ln likelihood 11187.906. Species groups within *Neotamias* are indicated graphically.

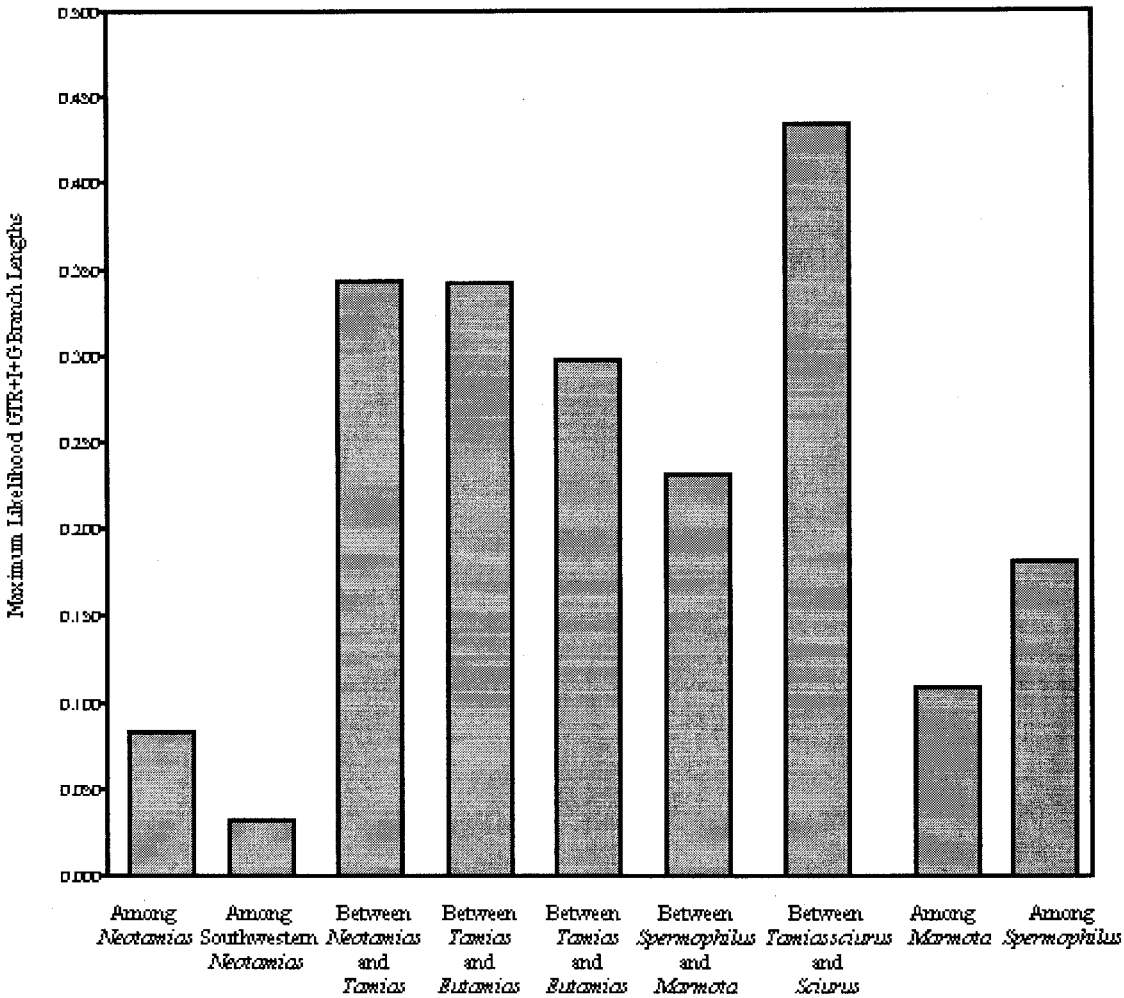

FIG. 5. Graph showing the distribution of branch lengths (generated from the maximum-likelihood tree inferred from a GTR+I+G+estimated base frequencies model) within and between *Tamias* subgenera and other sciurid genera. This distribution demonstrates that the divergences between *Tamias* subgenera are equivalent to divergences between other sciurid genera, supporting the elevation of *Tamias* subgenera to three genera, *Neotamias*, *Tamias*, and *Eutamias* (Jameson, 1999).

DISCUSSION

Ancestral taxon of chipmunks. The systematics of chipmunks has undergone many revisions based on bacular, morphological, allozyme, chromosomal, and host–ectoparasite data sets (White, 1953a; Nadler and Block, 1962; Nadler, 1964; Sutton and Nadler, 1969; Nadler *et al.*, 1977; Levenson and Hoffmann, 1984; Levenson *et al.*, 1985; Oshida and Yoshida, 1994; Jameson, 1999). The results of these studies have focused debate mainly over which species is most ancestral, where this ancestor arose, and how it dispersed. Some authors support an idea that an ancestral stock arose in Asia and spread to North America (Moore, 1961; Nadler, 1964; Nadler *et al.*, 1969, 1977; Sutton and Nadler, 1969; Ellis and Maxson, 1979; Jameson, 1999). Other authors cite evidence supporting a dispersal of ancestral stock from North America into Asia (Black, 1963, 1972; Nadler *et al.*, 1985). Additionally, some authors indicate that it is possible that the ancestral stock arose in the Holarctic mesophytic forests and differentiated across Asia and North America (Levenson *et al.*, 1985).

The molecular data appear to show that *T. sibiricus* and *T. striatus* are sister taxa to the rest of the *Tamias* species, but do not distinguish which evolved first or the direction of migration. On some level this is not easily resolved or important to the overall evolution of the *Tamias* species in western North America. As Allen (1891) stated, "from the extreme susceptibility of this plastic group (chipmunks) to the influences of the environment, it is one of the most instructive and fascinating groups among North American mammals. Whether the type originated at some point in North America, or in the northern part of Eurasia, it is perhaps idle to speculate, but that it has increased, multiplied, spread and become differentiated to a wonderful degree in North America is

beyond question. . .Probably a more striking illustration of evolution by environment cannot be cited." What can be resolved is how divergent the taxa have become and how many genera are represented within the chipmunks.

Generic debates. The geographic distribution of *Tamias* has led many authors to raise questions about the generic status of this group. Based on the geographic distribution and morphology of chipmunk species, Howell (1922) divided *Tamias* into two genera, *Eutamias* (including *T. sibiricus* and the western North American species) and *Tamias* (*T. striatus*). Howell (1929) expanded the two-genus model by further dividing *Eutamias* into two subgenera, *Eutamias* (*T. sibiricus*) and *Neotamias* (western North American species).

Ellerman (1940) in his study of rodent genera did not accept *Eutamias* as a valid genus, because he did not consider the characters used to elevate it to a generic rank to be phylogenetically informative. These characters include the presence/absence of the P3 upper premolar, which Ellerman pointed out were previously shown to have no importance in demonstrating evolutionary relationships. Ellerman (1940) suggested that color pattern is too influenced by the environment and that geographical distribution is not an acceptable phylogenetic character.

Bryant (1945) examined this taxonomic question on the basis of fossil evidence. The earliest fossil that Bryant records is from the late Miocene collected by Hall in Barstow, California in 1930, which is described as *Tamias (Neotamias) ateles*. Bryant demonstrated that the primitive dentition includes the upper P3 tooth and concluded that the absence of the upper P3 is merely the final phase of an evolutionary trend and has no supraspecific significance. Bryant concluded that Ellerman's (1940) grouping of all chipmunk species into one genus, *Tamias*, was correct.

White (1953a) evaluated the *Tamias* species on the basis of bacular morphology, cranial morphology, malleus, hyoid process, dentition, and external features. White believed that P3 was a significant taxonomic character since it is a primitive dentition retained in squirrels, and any change should be considered significant. White examined many morphological and external characters and designated those that he considered phylogenetically significant and those that were shared or not shared among *Tamias, Eutamias*, and *Neotamias*. White (1953a) found 10 characters that *Eutamias* and *Neotamias* shared and that neither shared with *Tamias*. White placed *Neotamias* as a subgenus with the subgenus *Eutamias* under the genus *Eutamias* and placed *Tamias* as its own genus, which agrees with Howell (1929). White resolved that *Neotamias* was more closely related to *Eutamias*, based on morphology and color, and that *Tamias* and *Eutamias* should be considered different genera based on presence/absence of P3 and cranial and bacular characters. Finally, White concluded that the genera *Tamias* and *Eutamias* probably evolved from distinct lines of Sciurids, *Eutamias* under the tribe Callosciurini and *Tamias* under the tribe Marmotini.

Based on karyotypic data, Nadler *et al.*, (1969, 1977) placed all the chipmunk taxa into one genus, *Tamias*, divided into three subgenera, *Eutamias, Tamias*, and *Neotamias*. Ellis and Maxson (1979) examined various data, including data generated from the immunological technique of micro-complement fixation, morphology, and chromosomes, and suggested that *Tamias* and *Eutamias* should be maintained as distinct genera. Hafner (1984) analyzed allozyme data and supported the classification into two distinct genera. Most recently, Levenson *et al.* (1985) analyzed electrophoretic data, cranial morphology, and external characters and concluded that there should be only one genus, *Tamias*, with three subgenera, *Eutamias* (*T. sibiricus*), *Tamias* (*T. striatus*), and *Neotamias* (rest of the species).

Jameson (1999) examined ectoparasites, specifically fleas and sucking lice of chipmunks. He discovered that the taxa living on *Neotamias* are confined to *Neotamias* and furthermore that these parasite complexes are not related to the parasite complex found on *T. striatus* or *T. sibiricus*. Furthermore, the fleas on *T. striatus* are most closely allied with a genus of fleas from eastern Asia. Jameson (1999) concluded that *Neotamias* species must be very closely related, must be recently diverged, and must have a "history quite separate from that of *T. striatus*." Jameson (1999) concluded that, based on the evolutionary relationships of chipmunk ectoparasites, the subgenera *Neotamias, Tamias*, and *Eutamias* should be elevated to three separate genera. Jameson states that this is the best taxonomic arrangement based on the relationships and apparent history of these taxa.

Generic classification is often subjective and based on an individual's concept of the features that define a genus. However, we can use the maximum-likelihood branch lengths to compare the divergences of chipmunks to the divergences of other squirrel genera. For example, *Marmota* and *Spermophilus* are considered distinct genera, and they diverged considerably later than the subgenera *Eutamias, Tamias*, and *Neotamias* (Fig. 4). A distribution of the branch lengths within and between groups (Fig. 5) clearly illustrates that the divergences between the *Tamias* subgenera are comparable to the divergences between the other sciurid genera. Consequently, we support Jameson's (1999) conclusion that each chipmunk subgenus should be elevated to its own genus. Further, since clades within the *Neotamias* are stable regardless of which analysis is applied to the data, we propose that these clades are species groups that replace the species groups suggested by previous authors. The following discussion will consider in detail this classification, examine geo-

TABLE 5

Revised Classification of Chipmunks Based on cyt *b* Molecular Sequences

Genus *Tamias*—includes only *Tamias striatus*
Genus *Eutamias*—includes only *Eutamias sibiricus*
Genus *Neotamias*—includes five species groups
- *N. amoenus species group*—includes only *N. amoenus*
- *N. quadrivittatus species group*—includes *N. quadrivittatus, N. rufus, N. durangae, N. bulleri, N. canipes, N. dorsalis, N. umbrinus* (syn. *N. palmeri*), and *N. cinereicollis*
- *N. merriami species group*—includes *N. merriami* and *N. obscurus*
- *N. minimus species group*—includes *N. minimus, N. ruficaudus, N. panamintinus*, and *N. quadrimaculatus*
- *N. townsendii species group*—includes *N. townsendii, N. senex, N. sonomae, N. siskiyou*, and *N. ochrogenys*

graphic distributions of clades, and specify the taxa that belong in each genus and species group (Table 5).

Systematics of species groups. The genus *Neotamias* represents an amazing example of adaptive radiation in the western United States and many authors have sought to untangle the systematics of these taxa. The COII phylogeny (Piaggio and Spicer, 2000) resolved some of the taxonomic problems, but several still remained within this group. The COII data set did not include all the taxa that are included in the current analysis of the mitochondrial cyt *b* gene, in particular, *N. ochrogenys* and *N. siskiyou* of the *N. townsendii* clade and *N. minimus consobrinus* of the *N. minimus* clade. These taxa help to clarify taxonomic relationships that have previously been debated. Although our analyses demonstrated that the relationships among the clades are not resolved, the clades (species groups; Table 5) remain intact regardless of the model applied to the data. Therefore, it is important to examine these species groups in detail and in regard to species groups designated previously by other authors.

The cyt *b* sequences of *N. ochrogenys* and *N. siskiyou* were added to the cyt *b* data set to provide resolution of the relationships of these species to the *N. townsendii* species group. Adams and Sutton (1968) concluded that *N. townsendii ochrogenys* had a baculum distinct from that of *N. townsendii* and suggested that this distinction warranted species differentiation. Sutton and Nadler (1974) analyzed bacular morphology of three subspecies of *N. townsendii: N. t. ochrogenys, N. t. senex*, and *N. t. siskiyou*. They concluded that these subspecies should be elevated to their own species. Levenson and Hoffmann (1984) analyzed electrophoretic data and determined that the species *N. ochrogenys, N. senex*, and *N. siskiyou* should not be elevated to species status, despite the findings of Sutton and Nadler (1974). A year later, Kain (1985) analyzed morphological and biochemical data of the *N. townsendii* group and decided that *N. ochrogenys* and *N. senex* should be retained as separate species. Finally, Sutton (1987) analyzed various data, including biogeography and morphology, and once again concluded that the data supported the classification of *N. ochrogenys, N. senex*, and *N. siskiyou* as distinct species. Our cyt *b* molecular data indicate that *N. ochrogenys* is a distinct lineage (Fig. 4) within the *N. townsendii* group. *N. senex* also appears to be a distinct lineage (Fig. 4) and, finally, *N. siskiyou* groups with the *N. townsendii* group as a distinct lineage and is the most basal taxon in this clade. Therefore, our analyses support the designation of these taxa as species.

Within the *N. minimus* clade, previous phylogenetic analyses have indicated paraphyletic relationships among the subspecies. In particular, in some data sets *N. m. operarius* and *N. m. consobrinus* have appeared to group outside of the rest of the *N. minimus* taxa (White, 1953b; Nadler *et al.*, 1969, 1977, 1985; Sutton and Nadler, 1969; Levenson *et al.*, 1985). We demonstrated in our COII phylogeny that *N. m. operarius* (Colorado) formed a monophyletic relationship with the rest of the *N. minimus* species plus *N. panamintinus* and *N. quadrimaculatus* (Piaggio and Spicer, 2000). In the current analysis of cyt *b*, we have also included *T. m. consobrinus* (Utah) and again we find a monophyletic relationship among the six *N. minimus* taxa, *N. panamintinus*, and *N. quadrimaculatus* (Fig. 1).

The *N. minimus* clade reveals other surprises. Our cyt *b* (Figs. 1 and 3) and COII (Piaggio and Spicer, 2000) data place *N. ruficaudus* and *N. quadrimaculatus* in the *N. minimus* clade. It is unexpected to find *N. quadrimaculatus* grouping in this clade because it has always been placed in the *N. townsendii* species group. It is also rather surprising that the larger-sized chipmunks *N. ruficaudus* and *N. quadrimaculatus* are closely related to the diminutive *N. minimus*. However, morphological and external characters in *Neotamias* appear to reflect environmental conditions rather than phyletic relationships in chipmunks. Patterson (1980b, 1981, 1982) found correlation between morphological shifts and niche shifts and concluded that there is convergence of morphological and external characters, which is driven by competition and environment. Later, Patterson (1983) found correlation between cranial and mandibular characters; mandibular characters are known to be influenced by environmental factors. Therefore, if traditional morphological characters do not approximate the evolutionary history among these taxa, then the sizes of these animals may be due to their ecological niches and to convergence.

Distribution patterns. Our molecular phylogenies (Figs. 1–4) suggest five distinct clades within the genus *Neotamias*. We consider these clades equivalent to species groups. These species groups appear to correspond to the geographical ranges of the taxa (Fig. 6).

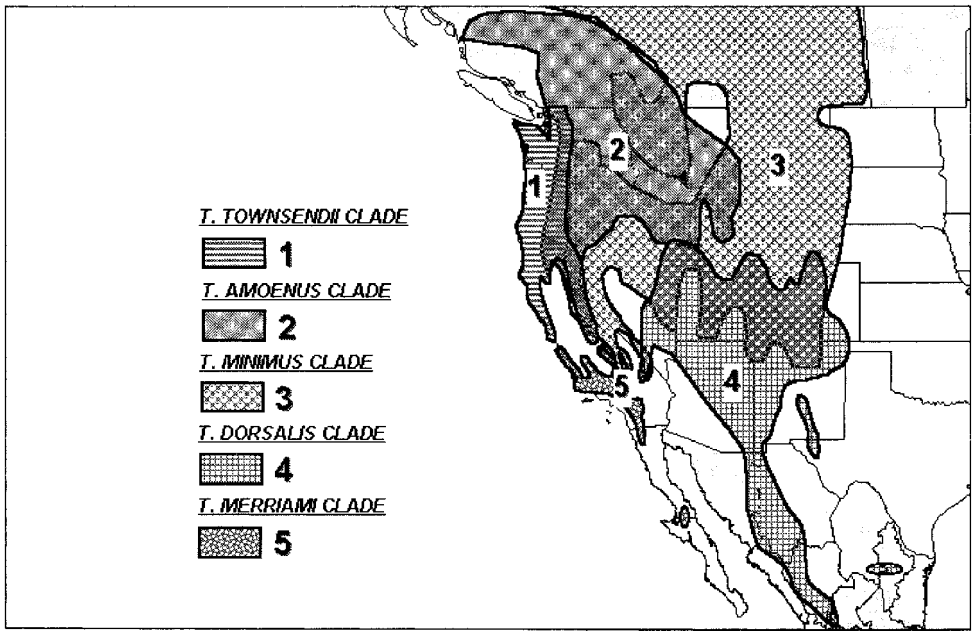

FIG. 6. Map of *Neotamias* clades; (1) *T. townsendii* clade; (2) *T.amoenus* clade; (3) *T. minimus* clade; (4) *T. dorsalis* clade; (5) *T. merriami* clade. The map suggests that clades occupy particular geographical areas, indicating phylogeographic patterns among the *Neotamias* taxa.

We have mapped the ranges of all the taxa based on Hall (1981) and labeled each of the five *Neotamias* clades (Fig. 6). Our map suggests that the taxa within each clade occupy particular geographical areas, indicating phylogeographic patterns among the *Neotamias* taxa. The *N. townsendii* clade (Fig. 6; clade 1) represents all of the Pacific Coast taxa, the *N. dorsalis* clade (Fig 6; clade 4) has taxa that range exclusively throughout the southwestern United States, and the *N. merriami* clade (Fig. 6; clade 5) includes taxa uniquely from southern California and Baja California. These three clades maintain discrete geographical boundaries, but there are two clades, *N. amoenus* (Fig. 6; clade 2) and *N. minimus* (Fig. 6; clade 3), which appear to exhibit overlapping boundaries with each other and among other clades. This overlap may be because both of these clades appear to have evolved generalist species, which have been able to extend their ranges into the ranges of other more distantly related taxa because of their ability to adapt to a wide array of habitats.

Speciation within the N. quadrivittatus species group. The paraphyletic relationships of *N. dorsalis, N. palmeri, N. umbrinus*, and *N. cinereicollis* were first revealed and discussed in our COII analysis (Piaggio and Spicer, 2000). That discussion focused mainly on the taxonomic literature and what it revealed about this group. We will now focus the discussion on the evolutionary processes involved in this paraphyletic grouping with the information provided by the cyt *b* data set (Eq. 4).

N. dorsalis and *N. cinereicollis* appear to exclude each other from habitats through competition (Findley, 1969; Patterson, 1980a, 1981, 1982; Klingel, 1996). Both species occupy most mountain habitats in the absence of the other. When their ranges overlap, *N. cinereicollis* rarely descends below the higher mesic forests and it is common up to the timberline, whereas *N. dorsalis* usually occupies the lower zones.

Brown (1971) determined that, in Nevada, *N. dorsalis* and *N. umbrinus* appear to exclude each other from certain habitats. It seems that *N. dorsalis* is limited to the lower-elevation pinyon–juniper habitats in the presence of *N. umbrinus* and, likewise, *N. umbrinus* seems to be limited in the presence of *N. dorsalis* to the higher-elevation forests. On mountain ranges in Nevada where there is one of these species without the other, the remaining species occupies the entire range of habitats (Brown, 1971). These animals are about the same size; however, they have distinct pelages and can be distinguished by an observer. Brown observed interactions at bait stations and determined that *N. dorsalis* is more aggressive than *N. umbrinus*. The concept of competitive exclusion was put forward by Brown to explain the pattern of distribution where these two species ranges overlap. He presumed that the force driving this competition was food. Since these animals exclude each other and because they are morphologically distinct from one another, it may be assumed that these animals are separate species. Therefore, the paraphyletic relationship of these taxa indicates that there may be a current sorting event among these taxa.

It is quite possible that *N. dorsalis, N. umbrinus*, and *N. cinereicollis* are all currently participating in

this sorting event. The maximum-likelihood branch lengths indicate a recent separation (Fig. 4). These divergences support the possibility that these animals have recently speciated and have yet to become entirely genetically unique.

N. palmeri is also part of the paraphyly; however, we have previously suggested that *N. umbrinus* and *N. palmeri* should be recognized as *N. umbrinus umbrinus* and *N. umbrinus palmeri* (Piaggio and Spicer, 2000). All the rest of the taxa in the paraphyly, however, appear to be distinct species based on bacular morphology and ecological differentiation in each other's presence.

Sorting is evidenced by the tendency of these species to exclude each other from habitats through competition, indicating that each species in the presence of another is specializing in a particular niche. This is one way that speciation occurs in sympatry (Schiliewen *et al.,* 1994) or is expressed after two allopatrically speciated forms are reunited in sympatry (Rice and Hostert, 1993; Losos *et al.*, 1997, 1998; Orr and Smith, 1998). Competition for habitats and specialization in separate habitats in the presence of the other species is common among chipmunk species (Heller, 1971; Sheppard, 1971; Chappell, 1978; Sharples, 1983; Bergstrom, 1992). Ecological differentiation could be a factor that led to prezygotic isolation and to morphological shifts of the reproductive morphology (i.e., bacula and baubella) resulting in postzygotic isolation and speciation. This idea is supported by White's (1953c) evidence that each species has distinct bacular morphology. More research on the ecology and reproduction of these species in areas of overlap and areas of isolation may test the validity of the idea that these species represent recent or current speciation events.

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