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Molecular Phylogeny of the Chipmunk Genus *Tamias* Based on the Mitochondrial Cytochrome Oxidase Subunit II Gene

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Complete sequences of the mitochondrial cytochrome oxidase subunit II gene were used to construct a phylogeny for 21 of the 25 currently recognized chipmunk species. Phylogenetic analyses indicate that *T. striatus* (subgenus *Tamias*, eastern United States) and *T. sibiricus* (subgenus *Eutamias*, Asia) are distantly related to the other species (subgenus *Neotamias*), which constitute a western North American radiation. We discuss and compare our molecular phylogeny to previous taxonomies and present a suggested classification of the species groups for the subgenus *Neotamias*.

KEY WORDS: *Tamias*; phylogenetics; mitochondrial DNA.

INTRODUCTION

The taxonomic status and phylogenetic relationships of the genus *Tamias* have long been a source of debate. The 25 species of *Tamias* (Hoffmann *et al.*, 1993) are difficult to distinguish from each other by either external features or traditional morphological data. Because of this, it has been particularly difficult for taxonomists to resolve the phyletic relationships among these animals. Consequently, species have often been grouped into "species complexes" or "species groups" as a way to deal with animals that are apparently different species, but obviously closely related. Chipmunks are currently considered to belong to one genus *Tamias*, which is divided into three subgenera: *Tamias* (*T. striatus*), *Eutamias* (*T. sibiricus*), and *Neotamias* (rest of the *Tamias* species) (Allen, 1891; Levenson *et al.*, 1985; Nadler *et al.*, 1985). Howell (1929) placed the species of the subgenus *Neotamias* into five species groups based on morphological data: (1) *T. alpinus*, (2) *T. minimus*, (3) *T. amoenus*, (4) *T. quadrivittatus*, and (5) *T. townsendii*. White (1953d) defined three species groups based on bacular morphology: (1) *T. minimus*, 2) *T. quadrivittatus*, and (3) *T. speciosus*. Finally, Levenson *et al.* (1985) and Nadler *et al.* (1985) both split *Neotamias* into five species groups based on electrophoretic data: (1) *T. merriami*, (2) *T. townsendii*, (3) *T. amoenus*, (4) *T. minimus*, and (5) *T. dorsalis*. In addition, these species groups have undergone other minor revisions based on bacular morphology, chromosomes, and karyotypes

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(White, 1953a–d; Nadler and Block, 1962; Nadler, 1964; Nadler *et al.*, 1969, 1977; Sutton and Nadler, 1969; Levenson and Hoffmann, 1984).

Modern methods of molecular sequencing offer some hope of resolving taxonomic relationships that previously have been difficult to elucidate by using more traditional methods. The purpose of this study is to compare and discuss relationships of species in the genus *Tamias* based on cytochrome *c* oxidase subunit II (COII) sequences. Complete sequences of the mitochondrial COII gene were used to evaluate the taxonomic relationships among *Tamias* species. We define species groups based on the molecular phylogeny and compare and discuss our groups with those inferred from other data sets.

METHODS AND MATERIALS

Specimens and DNA Extraction

Total genomic DNA was extracted from the specimens listed in Table I. Specimens were collected in the field either by gun or trapping (see Table I for collection data). Tissue and voucher specimens of collected animals were deposited in the Museum of Southwestern Biology, Albuquerque, New Mexico. Specimens collected in the field were placed on dry ice and then transferred to the lab and the skins and skull were prepared according to Hall (1981). The liver and some thigh muscle were removed for DNA extraction; the remaining tissue was stored in a −80°F freezer. Bacula (male) or baubella (female) were cleaned and preserved in alcohol.

Specimens not collected in the field were obtained through loans of frozen tissue from the following museums or individuals: Museum of Southwestern Biology, Albuquerque, New Mexico; the Museum of Vertebrate Zoology, University of California, Berkeley, California; Dr. Jack Sullivan, University of Idaho; The Burke Museum of Natural History, Seattle, Washington; Zaddock Thompson Natural History Collections, University of Vermont, Burlington, Vermont; and the Carnegie Museum of Natural History, Pittsburgh, Pennsylvania. These specimens, their catalog number, the Genbank accession numbers for the COII sequences, and locality information are included in Table I.

A salt extraction method was used to extract DNA from frozen tissue samples. This extraction required that approximately 3 mm^3 of tissue was ground using a tube-fitting pestle in a 1.5-ml tube containing 450 μl of grinding buffer [400 μl STE, 25 μl 20% SDS, and 25 μl proteinase K (10 mg/ml) per sample]. The resulting solution was incubated for 1 h at 55°C in a water bath. Following incubation, 0.10 KCl was added and the solution was vortexed and then chilled on ice for 1 h. Protein particles were pelleted and the clear supernatant was removed. NaCl (1/2 volume of 50 *M*) and 2 volumes of 95% cold ethanol were mixed with the supernatant to precipitate the DNA. The solution was vortexed and chilled on ice for 15 min. The DNA was pelleted by centrifugation, then it was dried and resuspended in 500 μl of 1 × TE buffer. The resulting solution of pure DNA (2 μl) was used for each 50-μl polymerase chain reaction solution.

Amplification and Sequencing

To obtain double-stranded DNA products, polymerase chain reactions (PCRs) were run in 50-μl reactions. The 50-μl reactions contained optimized amounts of PCR water, 5× buffers C or D (Invitrogen), 5 μl of dNTP (10 m*M*, Invitrogen), 5 μl of each primer

Table I. Specimens Used in this Study[a]

Species (F/S/E)	Owner (Catalog No.)	GenBank accession No.	Locality
Tamiasciurus hudsonicus (F)	MSB (61555) (NK (4324)	AF147597	New Mexico: Taos Co.
Tamias striatus fisheri (F)	CMNH (105324)	AF147621	Pennsylvania: Beaver Co.
Tamias striatus fisheri (F)	CMNH (105325)	AF147622	Pennsylvania: Beaver Co.
Tamias striatus lysteri (F)	CMNH (105327)	AF147623	Pennsylvania: Bradford Co.
Tamias striatus lysteri (F)	CMNH (105328)	AF147624	Pennsylvania: Bradford Co.
Tamias sibiricus (F)	BMNH (UWBM 39067)	AF147618	Russia: Khabarovskiy Kray
Tamias sibiricus (F)	BMNH (UWBM 39255)	AF147619	Russia: Magdanskaya Oblast
Tamias merriami (F)	MSB (43176) (NK 4669)	AF147598	California: Riverside
Tamias obscurus (F)	MSB (43179) (NK 4646)	AF147604	California: Riverside Co.
Tamias obscurus (F)	MSB (47429) (NK 8069)	AF147605	Mexico: Sierra San Pedro Martir, Baja California
Tamias obscurus (F)	MVZ (148043)	AF147606	Mexico: Baja California
Tamias amoenus (F)	A Piaggio	AF147583	California: Siskiyou Co.
Tamias amoenus (F)	MVZ (152780)	AF147584	California: Nevada Co.
Tamias amoenus (F)	A Piaggio	AF147585	California: Lassen
Tamias amoenus (F)	MSB (72231) (NK 51013)	AF147587	Wyoming: Park Co.
Tamias amoenus (F)	MSB (43427) (NK3137)	AF147586	Washington: Kittatas Co.
Tamias townsendii (F)	MSB (43429) (NK 3136)	AF147626	Washington: Kittatas Co.
Tamias townsendii (F)	MSB (43546) (NK 3252)	AF147627	Washington: Clallam Co.
Tamias townsendii (F)	MSB (53282) (NK 7980)	AF147625	Oregon: Benton Co.
Tamias sonomae (F)	MVZ (152777)	AF147620	California: Marin Co.
Tamias senex (F)	A Piaggio	AF147617	California: Sierra Co.
Tamias senex (F)	MVZ (152779)	AF147616	California: Nevada Co.
Tamias ruficaudus (E)	Jack Sullivan	AF147613	Idaho: Latah Co.
Tamias minimus (F)	A Piaggio	AF147600	California: Sierra Co.
Tamias quadrimaculatus (F)	MSB (83634) (NK 73120)	AF147609	California: Mono Co.
Tamias panamintinus (F)	MVZ (MDM 213)	AF147608	California: San Bernardino Co.
Tamias minimus (F)	MSB (84514) (NK 53727)	AF147601	California: Mono Co.
Tamias minimus (F)	MSB (56781) (NK 4422)	AF147602	Colorado: Dolores, Co.
Tamias minimus (F)	MSB (53280) (NK 7876)	AF147603	Canada: Manitoba
Tamias minimus (F)	MSB (55759) (NK 2113)	AF147599	Canada: Alberta
Tamias durangae (F)	ZTNH (217 CWK1985)	AF147596	Mexico: Durango

Table I. (*Continued*)

Species (F/S/E)	Owner (Catalog No.)	GenBank accession No.	Locality
Tamias bulleri (F)	MSB (48162) (NK 9505)	AF147588	Mexico: Coahuila
Tamias canipes (F)	MSB (57799) (NK 1869)	AF147589	New Mexico: Lincoln Co.
Tamias rufus (F)	MSB (76530) (NK 56201)	AF147614	Colorado: Rio Blanco Co.
Tamias rufus (F)	MSB (76532) (NK 56249)	AF147615	Colorado: Rio Blanco Co.
Tamias quadrivittatus (F)	MSB (56898) (NK 3481)	AF147611	New Mexico: Bernalillo Co.
Tamias quadrivittatus (F)	MSB (61498) (NK 4053)	AF147612	New Mexico: Sandoval Co.
Tamias quadrivittatus (F)	MSB (80142) (NK 56170)	AF147610	Colorado: Costilla Co.
Tamias dorsalis (F)	A Piaggio	AF147595	Arizona: Pima Co.
Tamias palmeri (F)	MSB (59000) (NK 2473)	AF147607	Nevada: Clark Co.
Tamias dorsalis (F)	MSB (76872) (NK 55222)	AF147594	Utah: Beaver Co.
Tamias umbrinus (F)	MSB (76765) (NK 55411)	AF147628	Utah: Beaver Co.
Tamias dorsalis (F)	MSB (70112) (NK 28742)	AF147593	New Mexico: Cibola Co.
Tamias cinereicollis (F)	MSB (53548) (NK 1927)	AF147590	Arizona: Apache Co.
Tamias cinereicollis (F)	MSB (54508) (NK 4225)	AF147591	Arizona: Apache Co.
Tamias cinereicollis (F)	MSB (65041) (NK 19644)	AF147592	New Mexico: Socorro Co.

[a] F, frozen tissue; E, prepared extraction. Museum of Southwestern Biology (MSB), Albuquerque, New Mexico; the University of California Museum of Vertebrate Zoology (MVZ), University of California Berkeley, California; The Burke Museum of Natural History (BMNH) Seattle, Washington; Jack Sullivan (JS), University of Idaho, Moscow, Idaho, Zaddock Thompson Natural History Collections (ZTNH), University of Vermont, Burlington, Vermont; and the Carnegie Museum of Natural History (CMNH), Pittsburgh, Pennsylvania.

(10 pM/μl), *Taq* DNA polymerase (Promega), and 2 μl of genomic DNA. Amplifications of the mitochondrial COII gene required external primer pairs, L7553 with H8320 (Adkins and Honeycutt, 1994), which amplify a segment approximately 700 bp in length. Internal primers were designed specifically to *Tamias* species (Table II). Amplifications were carried out in a P100 thermal cycler (Perkin Elmer) for 33 cycles of denaturation of 94°C for 1 min, annealing at 50 or 52°C for 1 min, and extension at 72°C for 2 min. Amplified PCR products were cleaned prior to sequencing using a polyethylene glycol (PEG) precipitation protocol (Kusukawa *et al.*, 1990).

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. The complete mitochondrial COII gene (684 bp) was sequenced in both directions.

Table II. Cytochrome Oxidase Subunit II Primers and Their Sequences[a]

Primers	Sequence
L7553	5′-AAC CAT TTC ATA ACT TTG TCA A
L7552	5′-ATT ACA TAA CTT TGT CAA
L7556	5′-CAT AAC TTT GTC AAA GTT
L7600	5′-CTT ATC TTA ATT GCT CTT CCC TCA
L7694	5′-ATA ATT GTA TTG TAT TCT TAA TTA GCT CCC
L7786	5′-TTG AAA CTA TCT GAA CTA TC
H7767	5′-GAT AGT TCA GAT AGT TTC AA
H8312	5′-CTT AAA AGG TTA ACG CT
H8316	5′-CTT TAA CTT AAA AGG TTA A
H8320	5′-CTC TTA ATC TTT AAC TTA AAA G

[a] Primers are designed by authors to be *Tamias* specific. The only exceptions are L7553 and H8320, which are universal external primers (Adkins and Honeycutt, 1994). Primer names are based on their alignment in the human mitochondrial genome (Anderson *et at.*, 1981; GenBank No. J01415). Primer labels refer to the strand (L, light; H, heavy) and the position of the 3′ nucleotide. Bold primer names are *Tamias*-specific external primers; others are internal specific primers.

Sequence Analysis

The COII sequences were initially aligned in the Sequencher 3.01 analysis program (Gene Codes Corp., 1995). To determine the beginning and end of the gene, the sequences were translated into the amino acid sequences and were aligned to the human (GenBank No. J01415) and the rat (GenBank No. X14848) sequences. Sequences were trimmed to eliminate any flanking tRNA sequence.

Sequences were evaluated for overall base composition bias. The base composition bias was calculated according to Irwin *et al.* (1991) and ranges in value from zero to one–zero indicating no bias and one showing complete composition bias. An extreme overabundance of one nucleotide can increase the tendency for those sites to become saturated (Irwin *et al.*, 1991). In addition, a skewed bias can violate the main assumption in parsimony analyses, namely, that there is an equal probability of change at all sites (Pena and Kocher, 1995; Spicer, 1995; Yoder *et al.*, 1996).

A variety of techniques was used to infer phylogenetic relationships. Parsimony analyses were performed by using the computer program PAUP 4.0b2 (Swofford, 1999). These analyses were accomplished by using a random stepwise addition option of the heuristic search, for 100 replicates using unordered changes. When several equally parsimonious trees were found, a strict consensus tree (Rohlf, 1982) was produced to summarize the data. In order to assess confidence in the branching patterns, bootstrap (Felsenstein, 1985) and decay analyses (Bremer, 1988) were performed. Bootstrap heuristic searches were set for a closest stepwise addition option, running 300 replicates in PAUP 4.0b2 (Swofford, 1999); decay analyses were performed using the program TreeRot v2 (Sorenson, 1999).

A parsimony analysis does not account for multiple hits, therefore, we performed a Kimura 2-parameter distance analysis (Kimura, 1980). Distance analyses were performed using PAUP 4.0b2 (Swofford, 1999) and were clustered using the heuristic search random stepwise addition option invoking the minimum evolution method with the Kimura

2-parameter distance (Kimura, 1980). Percentage sequence divergence for all pairwise comparisons was corrected for multiple substitutions under the Kimura 2-parameter distance model. These sequence divergences were used to determine the average sequence divergence between and within *Tamias* species.

Maximum likelihood was used to evaluate the fit of the data to the parsimony and distance topologies. The maximum likelihood analyses were performed by using the 2-parameter model for unequal base frequencies of Hasegawa *et al.* (1985), with a discrete approximation to the Γ distribution, and with the transition/transversion (ti/tv) ratio and the shape parameter estimated according to the model. A total of 10 rate categories was used (Yang, 1994), the average rate for each category was represented by the mean (Felsenstein, 1993).

RESULTS

The complete mitochondrial cytochrome oxidase II (COII) data set has 684 aligned base pairs (bp), of which 229 (33%) were variable sites and 140 (20%) were phylogenetically informative. The data represent sequences from 21 *Tamias* species and the outgroup taxa, *Tamiasciurus hudsonicus* and *Sciurus carolinensis* (GenBank No. U18831; Honeycutt *et al.*, 1995). These sequences have been deposited with GenBank under accession numbers AF147583–AF147628 (Table I).

The amount of variation was different among the 229 variable sites and the 140 phylogenetically informative sites, depending on the codon position examined. The first position had 41 variable sites (18%) and 31 (17%) phylogenetically informative sites; the second position had only 2 variable sites (0.9%) and only 1 phylogenetically informative site (0.6%); the third position had 186 variable sites (82%) and 147 phylogenetically informative sites (82%). This trend is expected, since changes in the first and second codon positions would usually result in nonsynonymous changes.

Table III shows overall base composition and bias for the COII data set. As with other mammalian mitochondrial genes, bases are not found in equal proportions. Honeycutt *et al.* (1995) found base composition bias in COII is highest at the third positions and lowest in the first positions in rodents; our results confirm this finding. However,

Table III. Percentage Base Composition and Bias[a] for Cytochrome Oxidase Subunit II

	A	C	G	T	Bias
1st Var.	0.079	0.489	0.073	0.359	0.464
1st All	0.282	0.241	0.236	0.239	0.042
2nd Var.	0.202	0.489	0.298	0.011	0.383
2nd All	0.269	0.246	0.113	0.372	0.188
3rd Var.	0.394	0.253	0.028	0.325	0.296
3rd All	0.466	0.215	0.027	0.292	0.344
Var.	0.336	0.297	0.038	0.328	0.280
All	0.340	0.234	0.125	0.301	0.188

[a] Values are calculated according to coding position (1st, 2nd, and 3rd) for all positions (All) and only variable positions (Var.). The bias is calculated using the formula of Irwin *et al.* (1991).

our data indicate a different trend when considering only the variable positions. In this instance, the finding is reversed, since it is the first position that exhibits the most bias; third positions have the least bias. This is important since it is the variable positions that contribute to the phylogenetic inference. The overall base composition bias in COII (bias = 0.19) is fairly low; however, when considering only the variable sites (bias = 0.28), it is relatively high. Because of this compositional bias in the COII data set, it is necessary to account for this bias in the analyses of the sequence data when building a maximum-likelihood model. Consequently, variable bases have been used for the likelihood analyses, because the overall base composition does not reflect the true base composition of the phylogenetically-important base changes.

The COII sequence was first analyzed by using parsimony. The data set includes 47 specimens representing 23 species. A heuristic search resulted in 476 equally parsimonious trees with a tree length of 626 steps, a consistency index (CI) = 0.479, and a retention index (RI) = 0.774. The strict consensus tree (Fig. 1) of the 476 parsimony trees is presented with bootstrap support and decay analyses at the nodes. Bootstrap support is weak for some of the deep branches and indicates that COII does not provide enough phylogenetically informative characters to resolve relationships in these deeper branches. However, the phylogenetic relationships differentiating most species groups appear to be fairly well resolved.

Minimum evolution trees were constructed by using the standard Kimura 2-parameter (Kimura, 1980) distance to account for multiple hits. We kept the taxa set the same to be able to statistically compare the resulting trees to our parsimony trees. Because identical taxa were maintained, this distance analysis produced 45 equal trees (length 0.88618). Indeed, the multiple distance trees actually represent just one tree, because the only variation among the trees concerns the relationships within the *T. obscurus* and *T. townsendi* specimens. The equivalent parsimony length of the Kimura 2-parameter trees (L = 633, CI = 0.474, RI = 0.767) is similar to the length of the parsimony trees (L = 626), but is longer.

Topological differences between the Kimura 2-parameter trees and the strict consensus parsimony tree indicate there could be biological factors such as ti/tv ratio and among-site rate heterogeneity influencing the data set that should be accounted for. In order to examine if the ti/tv ratio differences, or among-site rate heterogeneity were influencing the data set, we performed maximum-likelihood analyses. These analyses were performed on the parsimony and distance trees to determine which tree most accurately represents a topology that accounts for these influences. A maximum-likelihood evaluation was performed on all trees with the variable base composition data entered (Table III), equal rates of transitions to transversions (ti/tv ratio), and no among-site rate heterogeneity. All Kimura 2-parameter trees had equal scores (-ln Likelihood = 4505.52992) and one parsimony tree had the lowest score (-ln Likelihood = 4472.00551). For the rest of the analyses, we only tested the one parsimony tree that had the lowest score when rates were held equal. Next, we estimated the ti/tv ratio of the data set in order to determine if differing rates of transitional changes versus transversional changes should be taken into account. Again, all the Kimura 2-parameter trees had equal scores (ti/tv = 4.7816, -ln Likelihood = 4163.17128) and the parsimony tree had a lower score than the Kimura 2-parameter trees (ti/tv = 4.6437, -ln Likelihood = 4140.63018). Since these log-likelihood scores were significantly better than when rates of ti/tv are held equal (all K 2-P trees,

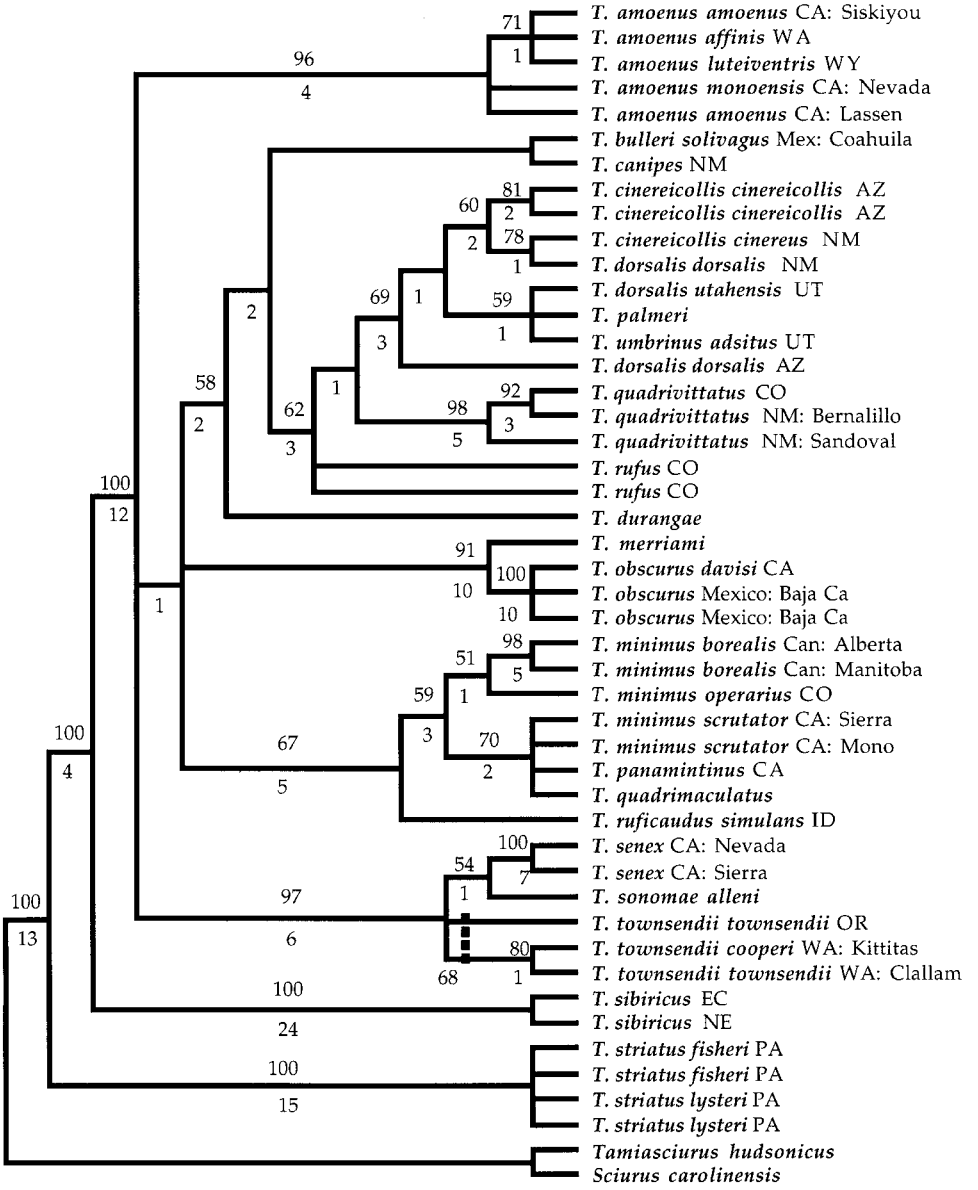

Fig. 1. Strict consensus tree of the 476 equally most parsimonious trees, each with a length of 626 steps. Bootstrap support is indicated on the top of the nodes (only values greater than 50% are presented) and decay analyses are indicated on the bottom of the nodes.

$\chi^2 = 685$, $df = 1$, P, 0.001; parsimony tree, $\chi^2 = 664$, $df = 1$, $P < 0.001$), ti/tv must be accounted for in the model. We then estimated ti/tv and the shape parameter of gamma (α) together because they are interdependent. All the Kimura 2-parameter trees had the same estimations and scores (ti/tv = 13.48, $\alpha = 0.154$, -ln likelihood = 3724.88137). The

parsimony tree had estimations similar to the Kimura 2-parameter trees for ti/tv ratio and the shape parameter, but a lower log-likelihood score (ti/tv = 13.613, $\alpha = 0.154$, -ln likelihood = 3715.35318). Because the log-likelihood score of the parsimony tree is significantly better when ti/tv and shape parameter influences are accounted for ($\chi^2 = 850$, $df = 1$, $P < 0.001$), the estimated parameters of ti/tv and α were used to construct the maximum-likelihood tree, which is considered the best estimate of divergences among the *Tamias* taxa (Fig. 2).

DISCUSSION

Tamias species have proved to be a taxonomic and phylogenetic challenge to mammalogists because they are very difficult to distinguish from each other morphologically. Consequently, these species have often been grouped into "species complexes" or "species groups" by researchers, because phyletic relationships among these closely related taxa have been difficult to resolve. Since it has long been recognized that *T. sibiricus* (subgenus *Eutamias*) and *T. striatus* (subgenus *Tamias*) are distinctly separate species from the diversity of chipmunks in western North America, these species groupings only apply to the western species (subgenus *Neotamias*).

Previous Classifications of the Genus Tamias

Allen (1891) established the earliest compilation of species complexes for *Tamias*. He concluded that the *Tamias* subgenus *Neotamias* could be divided into six species groups (previously all of these species were considered as subspecies of *Tamias asiaticus*) as follows:

1. *Tamias hindsii*—including *T. hindsii* (now considered *T. townsendii*), *T. townsendii*, *T. macrohabdotes* (now considered *T. quadrimaculatus*), *T. quadrimaculatus*, *T. senex*, and *T. merriami*.
2. *Tamias dorsalis*—including *T. dorsalis* and *T. obscurus*.
3. *Tamias umbrinus*—including *T. umbrinus*, *T. cinereicollis*, and *T. bulleri*.
4. *Tamias quadrivittatus*—including *T. quadrivittatus*, *T. luteiventris*, *T. affinis* (both now considered subspecies of *T. amoenus*), *T. neglectus*, *T. borealis* (both now considered subspecies of *T. minimus*), and *T. gracilis* (now considered *T. quadrivittatus*).
5. *Tamias minimus*—including *T. minimus*, *T. consobrinus*, and *T. pictus* (the latter two species now considered as subspecies of *T. minimus*).
6. *Tamias frater*—including *T. frater* (now considered subspecies of *T. speciosus*) and *T. amoenus*.

With relatively few changes, these same species groups are suggested by our molecular data (Fig. 1). By combining Allen's *T. dorsalis*, *T. umbrinus*, and *T. quadrivittatus* species groups, and if the currently recognized subspecies of *T. amoenus* and *T. minimus* are placed in the appropriate groups, and the few species that were yet to be described were added to the appropriate groups, the arrangements would then be equivalent.

Howell (1929) was the next taxonomist who employed the method of grouping individual *Tamias* species into species groups or complexes. By using cranial morphology and

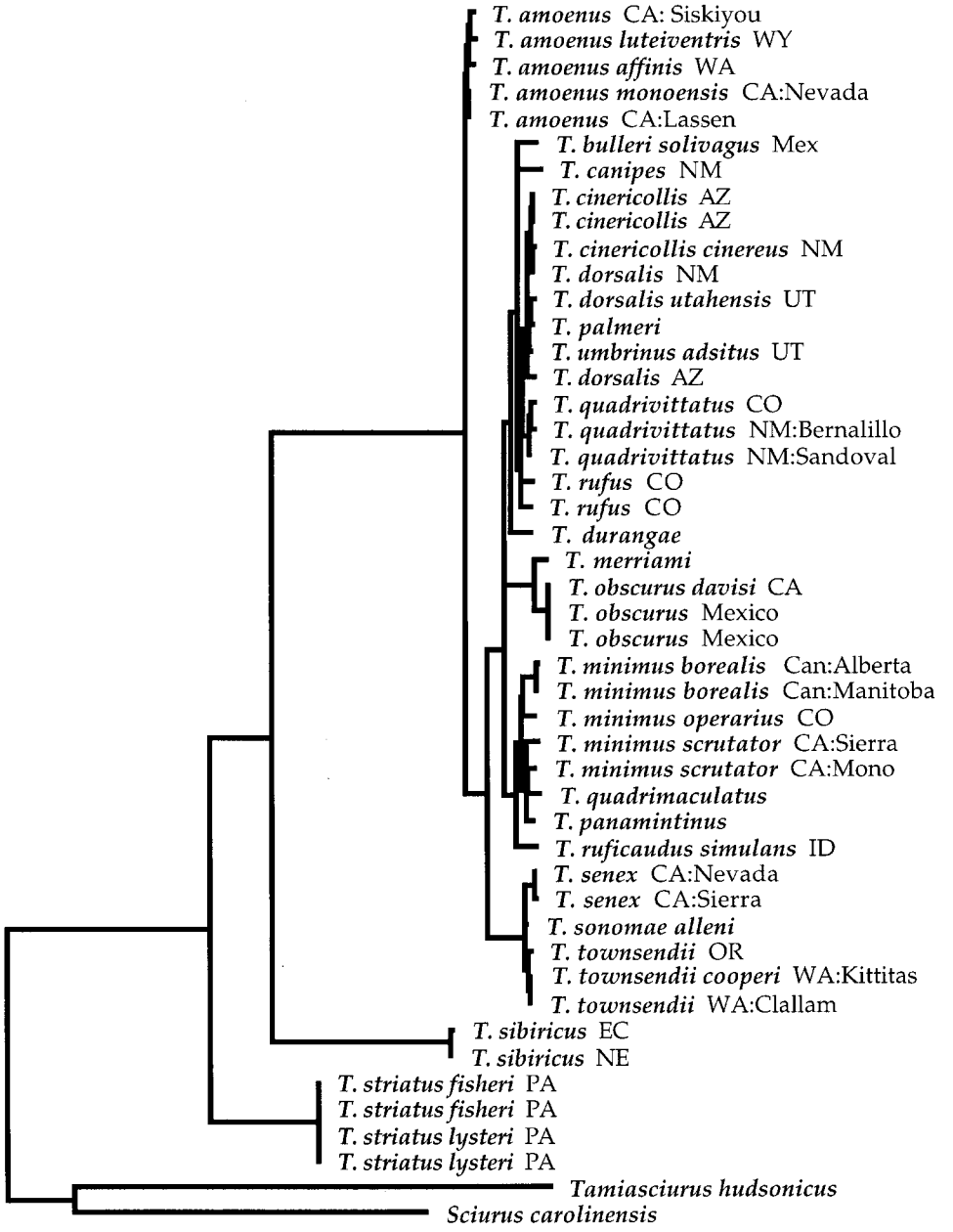

Fig. 2. Maximum-likelihood tree based on the 2-parameter model for unequal base frequencies of Hasegawa *et al.* (1985) and with the transition/transversion (ti/tv) ratio equal to 13.613 and the shape parameter equal to 0.154.

external characters, he attempted to resolve the taxonomy of this group. Previously, Allen (1891) had grouped the western North American chipmunks into a single genus *Tamias* along with *T. sibiricus* and *T. striatus*. Howell (1929) suggested that there were two genera, the genus *Tamias*, which included only *T. striatus*, and the genus *Eutamias*, which had two subgenera, *Eutamias* (*T. sibiricus*) and *Neotamias* (16 western North American species). Howell divided the subgenus *Neotamias* into five species complexes:

1. *Tamias alpinus*—including *T. alpinus* only.
2. *Tamias minimus*—including *T. minimus* only.
3. *Tamias amoenus*—including *T. amoenus* and *T. panamintinus*.
4. *Tamias quadrivittatus*—including *T. quadrivittatus*, *T. speciosus*, *T. hopiensis* (now considered *T. rufus*), *T. palmeri*, *T. umbrinus*, *T. ruficaudus*, *T. cinereicollis*, *T. canipes*, *T. bulleri*, and *T. durangae*.
5. *Tamias townsendii*—including *T. townsendii*, *T. senex*, *T. ochrogenys*, *T. siskiyou*, *T. sonomae*, *T. merriami*, *T. quadrimaculatus*, *T. obscurus*, and *T. dorsalis*.

With some exceptions these species complexes are similar to the species groups of our molecular data (Fig. 1). Howell's (1929) definition of species complexes is that they represent "superspecies" composed of a geographically isolated, but ecologically similar species defined by morphology and external characters, whereas our species groups are defined as the clades of our molecular phylogeny, having genetically similar species grouped together. Although our species groups are defined differently, Howell's (1929) morphological data set reflects almost the same relationships as our molecular phylogeny. There are some differences however, as our data place *T. quadrimaculatus* in a group with *T. minimus*, whereas Howell placed this species in the *T. townsendii* group. Our *T. minimus* group also includes *T. panamintinus*, which would have to be moved from Howell's *T. amoenus* group and *T. ruficaudus* would be moved from Howell's *T. quadrivitattus* group. In addition, *T. merriami* and *T. obscurus* would have to be removed from the *T. townsendii* group and placed in their own species group. This would make six groups under Howell's analyses, since he has *T. alpinus* in its own group, but we cannot confirm or deny this by molecular techniques because we do not have this species in our data set. Finally, *T. dorsalis* should be removed from Howell's *T. townsendii* group. He placed *T. dorsalis* in the *T. townsendii* group based on pelage, but acknowledged that the bacula of *T. dorsalis* resembled *T. cinereicollis* from the *T. quadrivittatus* group, which is where our molecular data place it.

Burgwardt (1967) used allozyme electrophoresis to study relationships of particular *Tamias* species to determine if they belong to the same species group. Burgwardt's electrophoretic results showed no measurable differences between *T. quadrivittatus quadrivittatus* and *T. dorsalis utahensis*. Consequently, Burgwardt concluded that *T. dorsalis* should be included as part of the *T. quadrivittatus* group. Our molecular data support Burgwardt's conclusions and place *T. dorsalis* well within the *T. quadrivittatus* clade. Consequently, with four simple reclassifications, Howell's species complexes would match the species groups as defined by our molecular phylogeny.

Suggested Classification of the Genus *Tamias*

Although modern techniques confirm most taxonomic conclusions of Allen (1891)

and Howell (1929), the intervening period has seen many debates regarding relationships within the *Neotamias* subgenus. We will discuss and compare these different systematic debates in the context of the *Neotamias* species groups as defined by our molecular phylogeny in this current study. Our suggested classification of the subgenus *Neotamias* is as follows:

1. *Tamias amoenus* species group—includes *T. amoenus* alone.
2. *Tamias quadrivittatus* species group—includes *T. quadrivittatus*, *T. rufus*, *T. durangae*, *T. bulleri*, *T. canipes*, *T. dorsalis*, *T. umbrinus*, and *T. cinereicollis*.
3. *Tamias merriami* species group—includes *T. merriami* and *T. obscurus*.
4. *Tamias minimus* species group—includes *T. minimus*, *T. ruficaudus*, *T. panamintinus*, and *T. quadrimaculatus*.
5. *Tamias townsendii* species group—includes *T. townsendii*, *T. senex*, and *T. sonomae*.

Taxonomic Disagreements within the *Tamias townsendii* Species Group

Although the *T. townsendii* species group has remained well defined since Howell (1929), the specific designation of *T. senex* within this group has often been debated. Sutton and Nadler (1974) analyzed bacular morphology among subspecies of *T. townsendii* and, based on their observations, they elevated *T. senex* to its own species. Levenson and Hoffmann (1984) evaluated allozyme electrophoretic data from several *Tamias* species and suggested that *T. senex* should not be elevated to its own species. However, Kain (1985) analyzed morphological and electrophoretic data from the *T. townsendii* group and recommended that *T. senex* should be retained as a separate species. Finally, Sutton (1987) evaluated various data including biogeography and morphology and again concluded that the data supported the classification of *T. senex* as a separate species instead of as a subspecies of *T. townsendii*. Based on the monophyletic grouping of *T. senex* (100% bootstrap) on our strict consensus parsimony tree (Fig. 1), our molecular data is suggestive that the current species designation for this taxon is appropriate.

Grinnell (1915) originally identified and named *T. sonomae* as a separate species, although Howell (1929) later listed it as a subspecies of *T. townsendii*. This listing caused confusion until Johnson (1943) reestablished its classification as a distinct species. Our phylogeny suggests support of the current classification, since the *T. townsendii* taxa and the *T. senex* taxa are monophyletic groupings (supported by low bootstrap) with *T. sonomae* remaining a separate lineage within this species grouping.

Taxonomic Disagreements within the *Tamias merriami* Species Group

In our molecular phylogeny, the *T. merriami*/*T. obscurus* clade is well supported by 91% bootstrap (Fig. 1). Callahan (1975) elevated *Eutamias merriami meriondalis* and *Eutamias merriami obscurus* to *Eutamias obscurus* (today *T. obscurus*) as a separate species from *T. merriami* based on sonogram data, karyotype data, skull morphology, and bacular morphology. Later, Blankenship and Bradley (1984) examined the protein electrophoretic patterns of esterases and found no difference between *T. obscurus* and *T. merriami*. However, *T. obscurus* has a distinct bacular morphology (Callahan, 1975) and bacular data appear to be a valid indicator of species distinctness (White, 1953a–d;

Patterson, 1980, 1981, 1982; Patterson and Thaeler, 1982). Our molecular data indicate *T. obscurus* is a monophyletic group (100% bootstrap support), which suggests support for specific distinction.

Taxonomic Disagreements within the *Tamias minimus* Species Group

The *T. minimus* clade has long been thought to represent two different lineages. White (1953c) recognized two distinct groups among the *T. minimus* subspecies in Wyoming based on cranial and bacular morphology. Karyotype data also indicated two distinct groups among *T. minimus* subspecies, with some subspecies being designated as karyotype A and the rest karyotype B (Nadler *et al.*, 1969; Sutton and Nadler, 1969). These differences seemed to suggest a separation between eastern and western Wyoming subspecies. Along similar lines, Conley (1970) found significant morphological intraspecific variation among subspecies of *T. minimus* in Arizona and New Mexico. In this instance, the subspecies *T. m. atristriatus* appeared to be morphologically and ecologically distinct from the other subspecies, all of which were quite similar to each other. Nadler *et al.* (1977) used the Geimsa banding technique to refine the karyotype groupings they had previously established. Once again, *T. minimus* seemed to represent two separate groups, in particular, the subspecies identified as *T. m. consobrinus* and *T. m. operarius* appeared to have both types of karyotypes. Although they maintain *T. minimus* as monophyletic, they acknowledged that more work was needed to determine if the species represents a monophyletic assemblage. However, based on both electrophoretic and morphological data, Nadler *et al.* (1985) suggest that *T. minimus* does not form a monophyletic group, but instead *T. m. operarius* and *T. m. consobrinus* cluster with the *T. quadrivittatus* species group. Sullivan (1985) examined populations of *T. minimus* from Arizona, Colorado, and New Mexico and discovered three distinct bacular forms of *T. minimus*, which appeared to divide along geographically distinct areas. Sullivan concluded that the Rio Grande Valley in New Mexico divided two distinct lineages of *T. minimus*, with a third to the north in the mountainous part of Colorado. Later, Sullivan and Petersen (1988) determined that although allozyme data might suggest paraphyly in this group (Sullivan and Petersen, 1988; Levenson *et al.*, 1985), morphological data from crania and bacula demonstrated monophyly. Sullivan and Petersen (1988) concluded that the monophyletic relationship based on morphology probably more accurately reflected the phylogenetic relationships than the electrophoretic data.

Our tree, with multiple samples of *T. minimus* subspecies, suggests that *T. minimus* is a monophyletic group (if *T. quadrimaculatus* and *T. panamintinus* are considered part of the group) with low bootstrap support (Fig. 1). Our taxa set includes a specimen of *T. m. operarius* (Colorado), which has been shown in other data sets to form a paraphyletic relationship to the rest of *T. minimus* subspecies (White, 1953c; Nadler *et al.*, 1969, 1977, 1985; Sutton and Nadler, 1969; Levenson *et al.*, 1985). As mentioned previously, *T. m. operarius* has been placed in a group with *T. quadrivittatus* by some authors (Nadler *et al.*, 1977, 1985; Levenson *et al.*, 1985). Our molecular data show a monophyletic grouping among the *T. minimus* subspecies that we examined with bootstrap support of 67% (if *T. quadrimaculatus* and *T. panamintinus* are included as part of the species) and clearly do not support *T. m. operarius* grouping with *T. quadrivittatus*. It is interesting that several investigators have pointed to Wyoming as a place with more than one distinct

T. minimus lineage (White, 1953c; Nadler *et al.*, 1969, 1977, 1985; Sutton and Nadler, 1969; Levenson *et al.*, 1985). We examined one of the subspecies (*T. m. operarius*) that others have indicated is different from the rest, but it is not from Wyoming. Perhaps sampling the areas in Wyoming that White (1953c) and others have identified as having a distinct bacular morphology, and sampling other *T. minimus* populations across their range using molecular phylogenetic techniques, would clarify the unusual relationships of *T. minimus*.

Levenson *et al.* (1985) examined electrophoretic data and concluded that *T. minimus* has a higher proportion of polymorphic loci than almost any other rodent species. Indeed, our corrected estimates of sequence divergence demonstrate a very high level of sequence divergence among the *T. minimus* subspecies. For example, the COII data set shows an average of 0.7% corrected sequence divergence within species of *Tamias*, with 1.3% being the highest observed within a species other than *T. minimus*. However, *T. minimus* exhibits a within-species sequence divergence that ranges from 0.3 to 3.8%. The two *T. m. borealis* specimens display the least amount of sequence divergence at 0.3%. However, the two *T. m. scrutator* specimens from California show a sequence divergence of 2.0%, which is well above the amount of divergence within other species. However, that is not the greatest amount of observed divergence within *T. minimus*. The corrected sequence divergence between the two subspecies *T. m. scrutator* (California: Sierra) and *T. m. operarius* (Colorado: Dolores) is 3.8%. Perhaps, this indicates unresolved taxonomic relationships within the *T. minimus* species or this sequence divergence could just represent the higher end of within-species divergence. Yet, sequence divergence between two distinct species, *T. quadrivittatus* and *T. cinereicollis*, which are members of the same species group, ranges from 2.0 to 2.9%. In addition, this amount of sequence divergence is shared by only two other species, *T. rufus* and *T. dorsalis*. In this instance, the *T. dorsalis* species have a paraphyletic relationship with other taxa and the *T. rufus* species have long been a subject of a taxonomic debate (see *T. quadrivittatus* clade discussion). Because *T. minimus* is a cosmopolitan species, which appears to adapt more readily to different conditions than any other species, these high values of sequence divergence may just be the high end of a normal range of sequence divergence. On the other hand, the large divergences may indicate that these taxa are in the process of speciation.

The COII data place *T. quadrimaculatus* and *T. ruficaudus* in the *T. minimus* clade. This is a distinctly different grouping than suggested by any previous researcher. However, Layne (1954) found that *T. ruficaudus* is similar to *T. minimus*, while investigating the taxonomic value of the female genital bone (baubella). White (1953d) united *T. quadrimaculatus* and *T. ruficaudus* together in a species group based on bacular morphology and, although this grouping did not include *T. minimus*, it demonstrates morphological similarities between *T. quadrimaculatus* and *T. ruficaudus*. Our phylogenetic results indicate that *T. ruficaudus* is the most basal of the *T. minimus* clade that also includes *T. quadrimaculatus* and *T. panamintinus*, with bootstrap support of 67%.

Taxonomic Disagreements within the *Tamias quadrivittatus* Species Group

This clade has undergone the most debate and taxonomic revision at the species level. We will discuss the work of previous researchers in relation to the species in the *quadrivittatus* group, as defined by our suggested classification, rather than How-

ell's (1929) *T. quadrivittatus* group. In our clade *T. durangae*, *T. canipes*, and *T. bulleri* are most basal to the other taxa within the *T. quadrivittatus* group. Howell (1929) recognized that *T. canipes* approached *T. bulleri* in several characters, including cranial size and coloring, and suggested that *T. canipes* might be a distinct species. Fleharty (1960) elevated *T. canipes* to a species with its own subspecies based on bacular morphology and recognized that the *T. canipes* bacula resemble those of *T. durangae*. The COII data place *T. bulleri*, *T. canipes*, and *T. durangae*, which range in northern Mexico and the southwestern United States, as sister to the rest of the southwestern U.S. taxa with low bootstrap support.

Callahan (1980) extended the range of *T. bulleri bulleri* to occur on both sides of the Rio Mesquital, Durango. This new range closely approached the range of *T. b. durangae*, but Callahan found that *T. b. bulleri* and *T. b. durangae* differed markedly in coloration and bacular morphology, so she elevated *T. b. durangae* to its own species. Based on baubella and pelage, Callahan also placed the subspecies *T. b. solivagus* under *T. durangae*. However, the COII evidence suggests that our *T. d. solivagus* specimen is a distinct species from *T. durangae* and, thus, we refer it to *T. bulleri* (*T. bulleri solivagus*; Howell, 1922).

Baker (1956) analyzed morphological and external features and concluded that *T. durangae* may be derived from *T. cinereicollis*. In fact, *T. durangae* is the most basal branch in our clade that includes *T. cinereicollis*, among other species. Consequently, the molecular data appear to indicate the reverse of Baker's conclusion; however, he was the first to illustrate the evolutionary link between these two taxa.

Hoffmeister and Ellis (1979) designated *T. quadrivittatus rufus* as a newly assigned subspecies of *T. quadrivittatus*. They found a clear distinction between *T. q. rufus* and *T. q. quadrivitattus* based on cranial and external characters, except in the very narrow zone of intergradation, although this work did not address bacular morphology of these two taxa. However, Patterson (1984) demonstrated contrasting bacular and cranial data showing *T. rufus* to be very distinct from *T. quadrivittatus*. Consequently, Patterson elevated the status of *T. rufus* to its own species and the molecular data support the sister taxon relationship of the distinct species *T. quadrivittatus* and *T. rufus*.

As mentioned previously, among the *Tamias* taxa we found an average corrected sequence divergence of 0.7% within a species, with 1.3% the highest value detected among monophyletic taxa. However, the two *T. rufus* specimens show a very high level of sequence divergence at 2.2%. Clearly, this species needs further research to determine the reason for this unusually high rate of sequence divergence, particularly since these animals were collected in the same locality (Colorado: Rio Blanco).

Originally, Hardy (1945) considered *T. umbrinus* as a subspecies of *T. quadrivittatus*, but White (1953b) elevated it to a species based on differences in shaft length of the bacula, although the overall form of the bacula is very similar between these two species. Other character differences that led White to designate *T. umbrinus* as a separate species included the smaller cranial breadth and much darker pelage in *T. umbrinus* relative to that of *T. quadrivittatus*. Later, Long and Cronkite (1970) confirmed the specific distinctness between *T. umbrinus* and *T. quadrivittatus* based on bacular morphology. However, Nadler and Block (1962) found no differences in the karyotype between *T. quadrivittatus* and *T. umbrinus*. Bergstrom and Hoffmann (1991) explored the relationships among three chipmunk species in the front range of Colorado. Among these three species were *T.*

umbrinus and *T. quadrivittatus*, which showed no evidence of hybridization, since cranial measurements all seemed to show relative separation between these species and did not seem to converge in the zone of overlap. In addition, bacular and baubellar characters were conservative within species and appeared to be reliable diagnostic features among the species. The molecular phylogeny places both these species in the same clade, but not as sister taxa. Thus it appears that *T. quadrivittatus* is basal to other southwestern taxa that are involved in a paraphyletic relationship.

This paraphyletic relationship includes *T. umbrinus* as a sister taxon to both *T. palmeri* and *T. dorsalis utahensis*, and *T. dorsalis dorsalis* (New Mexico) appears to be more closely related to *T. cinereicollis cinereus* (New Mexico) than it is to any other *T. dorsalis*. Also, *T. cinereicollis cinereus* is more closely related to *T. dorsalis dorsalis* (New Mexico) than it is to any other *T. cinereicollis* taxon. Finally, *T. dorsalis dorsalis* (Arizona) is a sister taxon to *T. quadrivittatus* and also to the *T. palmeri*, *T. umbrinus*, and *T. dorsalis utahensis* grouping, but does not form a monophyletic grouping with any *T. dorsalis* specimens. Accordingly, the phylogeny (Fig. 1) reveals paraphyletic relationships among four species, *T. cinereicollis*, *T. dorsalis*, *T. umbrinus*, and *T. palmeri*.

This paraphyletic relationship demonstrates either that these taxa are not unique species because they are exchanging genetic material or that these are recently speciated taxa still undergoing a sorting event. It has been shown that both males and females of *T. dorsalis*, *T. umbrinus*, and *T. cinereicollis* have unique genital bone morphology (White, 1953b; Sutton, 1982). Thus, based on these characters, *T. umbrinus*, *T. dorsalis*, and *T. cinereicollis* appear to be distinct species.

Although *T. umbrinus*, *T. palmeri*, and *T. dorsalis utahensis* appear to be genetically identical in our data, Burgwardt (1967) examined data of the blood serum electrophoresis that indicated *T. dorsalis* was very different from *T. palmeri*. Also, morphologically, *T. dorsalis utahensis* has been considered a separate species from *T. umbrinus* and *T. palmeri* (White, 1953b; Sutton, 1982). However, there do not appear to be any differences between *T. umbrinus* and *T. palmeri*. White (1953b) found that *T. umbrinus* has a distinguishable baculum from all other species except *T. palmeri*. Sutton (1982) found no differences in the os clitoris (baubella) of *T. umbrinus* and *T. palmeri*, suggesting again that *T. palmeri* should be considered a subspecies of *T. umbrinus*. Stanley (1991) performed a bacular morphological analysis on several populations of *T. palmeri* and *T. umbrinus* and also concluded that *T. palmeri* should be a subspecies of *T. umbrinus*. Indeed, our corrected sequence divergence between these two species (0.6%) is well within the range found for within *Tamias* species comparisons (0.1–1.3%). It appears from our data that *T. umbrinus* and *T. palmeri* are the same species, so we suggest that *T. palmeri* should be recognized as *T. umbrinus palmeri*.

The taxa *T. dorsalis*, *T. cinereicollis*, and *T. umbrinus* are clearly different species morphologically, yet our molecular phylogeny appears to show them to be genetically very similar. However, we conclude that it is quite possible that these taxa are distinct species and that they are currently undergoing speciation or a sorting event.

Other Taxonomist's Species Groupings

White (1953d) surveyed *Tamias* species using only bacular morphology and concluded that this was a valid taxonomic indicator. He defined three species groups based

on bacular morphology:

1. *Tamias minimus group*—including *T. alpinus*, *T. minimus*, *T. townsendii*, *T. sonomae*, *T. amoenus*, *T. dorsalis*, and *T. merriami*.
2. *Tamias quadrivittatus* group–including *T. quadrivittatus*, *T. ruficaudus*, *T. cinereicollis*, and *T. quadrimaculatus*.
3. *Tamias speciosus* group—including *T. speciosus*, *T. panamintinus*, *T. umbrinus*, *T. palmeri*, and *T. bulleri*.

Based on our molecular phylogeny, it appears that, although the bacula seems to be a valid taxonomic tool to differentiate among species (Howell, 1938; Wade and Gilbert, 1940; Moore, 1959; Burt, 1960; Adams and Sutton, 1968; Sutton, 1995), it is not a good character to determine phylogenetic relationships. Patterson (1982) ascertained that bacula could exaggerate phyletic relationships because they may serve as a "lock and key" function and would exaggerate relationships between recently speciated forms. Patterson and Thaeler (1982) concluded that bacular variation may not correspond to phyletic divergence if the baculum serves as a reproductive isolating mechanism for sympatric or parapatric species. In this case, the relationships deduced from bacular morphology between recently diverged species would be exaggerated because of the reproductive function. Sullivan (1985) demonstrated homoplasy in bacular characters of *T. canipes*, which brings into the question the utility of bacula in phylogenetic evaluations. The paraphyletic grouping of *T. dorsalis*, *T. umbrinus*, and *T. cinereicollis* appears to support the idea that bacula serve a function in reproductive isolation, although though these species are very closely related. Thus, bacula may show a species distinctness, but misrepresent the evolutionary relationships between species.

Nadler *et al.* (1969, 1977) analyzed chromosomal data and divided *Neotamias* into two species groups: (1) karyotype A—*T. cinereicollis* and *T. minimus* from mainly the central and southern Rocky Mountains; (2) karyotype B—*T. minimus* from the eastern foothills of the Rockies, the adjacent plains, and also on the eastern and western sides of the Great Basin, and the remaining species. However, Nadler *et al.* (1969) suggested that chromosomes are of little help in solving taxonomic problems in this group. The molecular data support this position.

More recently, Levenson *et al.* (1985) analyzed electrophoretic data, cranial morphology, and external characters and concluded that there should be only one genus of chipmunk, the genus *Tamias*, with three subgenera, *Eutamias* (*T. sibiricus*), *Tamias* (*T. striatus*), and *Neotamias* (rest of the species). The Levenson *et al.* (1985) classification is the one in common use today. They also revised the *Neotamias* species groups and placed them into five provisional species groups:

1. *Tamias merriami* species group—*T. merriami* and *T. obscurus* (possibly *T. bulleri* and *T. palmeri*).
2. *Tamias townsendii* species group—*T. sonomae*, *T. siskiyou*, *T. senex*, and *T. townsendii* (possibly *T. quadrimaculatus*).
3. *Tamias amoenus* species group—*T. amoenus* and *T. umbrinus* (possibly *T. cinereicollis*, *T. canipes*, and *T. durangae*).
4. *Tamias dorsalis* species group—*T. dorsalis*, *T. quadrivittatus*, and *T. panamintinus* (possibly *T. rufus*).

5. *Tamias minimus* species group—*T. minimus* and *T. ruficaudus* (possibly *T. alpinus* and *T. speciosus*).

These species groups appear initially quite different from ours. Levenson *et al.* (1985) found branching inconsistencies that occur in their results with the computer program (Polymorphism Parsimony Program) used. The differences between our data set and theirs are most likely due largely to these inconsistencies and the differences in the number of characters in each data set. The Levenson *et al.* (1985) electrophoretic and morphological data set is significantly smaller than our COII data set. Therefore, we have more resolution and support within our resulting phylogeny.

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