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Nuclear DNA phylogeny of the squirrels (Mammalia: Rodentia) and the evolution of arboreality from c-myc and RAG1

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Abstract

Although the family Sciuridae is large and well known, phylogenetic analyses are scarce. We report on a comprehensive molecular phylogeny for the family. Two nuclear genes (c-myc and RAG1) comprising approximately 4500 bp of data (most in exons) are applied for the first time to rodent phylogenetics. Parsimony, likelihood, and Bayesian analyses of the separate gene regions and combined data reveal five major lineages and refute the conventional elevation of the flying squirrels (Pteromyinae) to subfamily status. Instead, flying squirrels are derived from one of the tree squirrel lineages. C-myc indels corroborate the sequence-based topologies. The common ancestor of extant squirrels appears to have been arboreal, confirming the fossil evidence. The results also reveal an unexpected clade of mostly terrestrial squirrels with African and Holarctic centers of diversity. We present a revised classification of squirrels. Our results demonstrate the phylogenetic utility of relatively slowly evolving nuclear exonic data even for relatively recent clades.

1. Introduction

The squirrel family, Sciuridae, is one of the largest families of mammals. It contains an abundant and diverse group of species that have been the subject of numerous and important studies on behavior, ecology, reproductive biology, and morphology; sciurids are among the best understood mammals in these regards. Because most species are diurnal, students of animal behavior have often turned to squirrels for their experiments and observations (Armitage, 1999; Dobson, 1984). Social systems vary from solitary (woodchuck) to highly social (prairie dogs). Moreover, the Sciuridae are found throughout the world (absent only from southern South America, Madagascar, and Australasia), and their diverse habitats, from dense forest to open deserts, make them very useful for study of mammalian adaptation to temperature extremes and resource scarcity and of development of behaviors in different environments. However, the utility of this body of knowledge is compromised by an incomplete understanding of their phylogenetic relationships. Key issues include the early radiation of the group, the relationships of the African and Asian squirrels to other major groups, whether tree squirrels evolved one or more times, whether gliding evolved more than once, and the relationships of flying squirrels to other groups. Until recently (Mercer and Roth, 2003) no comprehensive phylogeny of the family has been published, although a number of morphological systematic studies have been (e.g., Black, 1972; Moore, 1959; Thorington et al., 2002).

1.1. Systematic background

The family Sciuridae comprises 273 species and 50 genera in two subfamilies: Sciurinae, the tree and ground squirrels, and Pteromyinae, the flying squirrels (Hoffmann et al., 1993; Thorington et al., 2002). Cranial morphology (Roth, 1996) and 12S rRNA sequence data (Oshida et al., 1996) suggest, however, that the tree squirrels (Sciurinae) are more closely related to the flying squirrels (Pteromyinae) than they are to the ground squirrels (Sciurinae) and would invalidate this

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classification. Some debate has focused on whether the flying squirrels (consisting of two North American species of *Glaucomys* and 13 Old World genera with 43 species) are monophyletic (Hight et al., 1974; Johnson-Murray, 1977; Thorington, 1984). Previous systematic studies have focused on more accessible Nearctic or Holarctic groups even though the greatest diversity is in the tropics and encompasses separate groups in the Neotropical, Ethiopean, and Oriental realms. In addition, most studies have relied on rapidly evolving mitochondrial genes, primarily cytochrome *b*. They have therefore been relatively successful at resolving relationships within genera (Kruckenhauser et al., 1999; Oshida et al., 2000a; Oshida and Masuda, 2000; Steppan et al., 1999) but had less robust resolution at deeper subfamily levels (Oshida et al., 2000b). Most recently, however, Mercer and Roth (2003) presented a comprehensive phylogeny including nearly all genera, for which they analyzed 2275 bp of mitochondrial 12S and 16S and an exon in nuclear IRBP (see below for discussion). Their results were a significant advance over previous studies.

Within the Sciurinae, 10 tribes are presently recognized (Hoffmann et al., 1993; Moore, 1959): (1) Callosciurini (Oriental tree squirrels; 57 species), (2) Funambulini (Asian striped squirrels, predominantly arboreal or semiarboreal; Oriental and Ethiopean realms; 26 species), (3) Marmotini (marmots, prairie dogs, ground squirrels, chipmunks; Holarctic; 87 species), (4) Microsciurini (dwarf tree squirrels; Neotropical; 5 species), (5) Protoxerini (African tree and bush squirrels; 10 species), (6) Ratufini (giant tree squirrels; southeast Asia; 4 species), (7) Sciurillini (pygmy tree squirrel; Amazonia; 1 species), (8) Sciurini (tree squirrels; greatest diversity in Neotropics but some groups Holarctic; 29 species), (9) Tamiasciurini (red squirrel, rock squirrel; Holarctic; 5 species), and (10) Xerini (Afro-Asian ground squirrels; 6 species).

Molecular studies began with Hight et al. (1974), who measured immunological distances for 17 sciurid genera including 5 flying squirrels. They found that the flying squirrels were closely related to and possibly paraphyletic with respect to tree squirrels (Sciurini and Tamiasciurini). Callahan and Davis (1982) thought *Sciurotamias* a close relative of *Ratufa* based on reproductive-tract characters, although it is commonly thought to be a marmotine (Gromov et al., 1965). McKenna and Bell's (1997) classification placed the pygmy squirrel *Sciurillus* in the Sciurini, separated the chipmunks, Tamiini, from Marmotini, and placed the Indian endemic Ratufini closest to the Nannosciurini (= Callosciurini), Protoxerini, and Funambulini. Here, we follow the taxonomy of Hoffmann et al. (1993) except for recognizing the priority of Pteromyinae over Petauristinae (McKenna and Bell, 1997; Thorington and Hoffmann, in press).

Using mitochondrial and nuclear genes, Mercer and Roth (2003) identified five major lineages (from basal to distal on the pectinate tree): *Sciurillus*; *Ratufa*; Callosciurini + *Funambulus*; a clade formed by Sciurini, Tamiasciurini, and the flying squirrels Pteromyinae; and a widely distributed clade consisting predominantly of terrestrial or semiterrestrial squirrels in Xerini, Marmotini, Protoxerini, and the African members of Funambulini (excluding *Funambulus*). Monophyly of the three polytypic lineages was well supported, but relationships among them varied from moderately to strongly supported. Monophyly of the flying squirrels was affirmed, but they were sister to the clade of Sciurini + Tamisciurini, not to all other squirrels as conventional taxonomy would suggest. The Protoxerini were paraphyletic with respect to the funambulines.

1.2. Hypotheses tested

These proposed relationships may be considered hypotheses to be tested with the molecular data. (1) Consistent with current taxonomy, the Pteromyinae and Sciurinae are each monophyletic and are sister taxa. (2) *Sciurillus* is a member of the Sciurini (Hoffmann et al., 1993; McKenna and Bell, 1997) rather than a basal lineage (Mercer and Roth, 2003). (3) The Callosciurini are monophyletic. (4) *Spermophilopsis* is a member of the Xerini (Moore, 1959). (5) The Tamiasciurini include *Sciurotamias* (Moore, 1959). (6) The Marmotini s. s. are monophyletic. (7) The Protoxerini are derived from the Xerini (Moore, 1959). (8) The Protoxerini are monophyletic. (9) The Marmotini and Xerini are sister taxa (strict terrestrial forms are monophyletic). (10) The southeast Asian Callosciurini and Ratufini are sister taxa. (11) Relationships among the five major clades are as reported in Mercer and Roth (2003). We did not test polyphyly of the Funambulini (Mercer and Roth, 2003) because we lacked a sample for *Funambulus*.

1.3. Ecomorphological reconstruction

A number of additional key questions can be addressed outside a hypothesis-testing framework. The arboreal habits of tree and flying squirrels require many specific morphological features, for example in the wrists, hips, and ankles (Thorington et al., 1997). What were the ecology and morphology of the ancestor(s) to flying squirrels, i.e., arboreal or terrestrial? Did these traits evolve once, or did they evolve convergently in Asia and North America? Is the ground-squirrel lifestyle (and its concomitant adaptations) primitive or derived among sciurids? Has it arisen more than once? We use the phylogenetic results to reconstruct the evolution of these habits.

1.4. Nuclear genes in vertebrate phylogenetics

Nuclear genes are being used much more commonly in vertebrate systematics. Most of the interest has been at relatively deep levels, addressing relationships among classes and orders (Graybeal, 1994; Groth and Barrowclough, 1999; Madsen et al., 2001; Matthee et al., 2001; Murphy et al., 2001; Stanhope et al., 1998). What has been less well evaluated is the utility of nuclear genes, particularly exons, for more recent divergences, where many more nodes are waiting for resolution and where the lower evolutionary rates of nuclear genes may not provide many characters. This more recent, family-level domain is also approximately where the utility of rapidly evolving mitochondrial genes decreases because of saturation. Here we report on the first broad application of two nuclear genes, c-myc and RAG1, to a within-family-level question in mammals.

The proto-oncogene c-myc is a single-copy gene with three exons of approximately 500, 700, and 600 bp, and its structure and function are well known because of its role in tumor development (Braun et al., 1985; Cole, 1986; Prendergast, 1997). It has been identified as most promising for studies at the ordinal and class level (Graybeal, 1994). To date, the gene has proved useful in resolving mammal (Miyamoto et al., 2000; Mohammad-Ali et al., 1995), crocodylian (Harshman et al., 2003), and passerine bird (Irestedt et al., 2001) phylogenies.

Recombination activating gene 1 (RAG1) promotes recombination among the coding segments of T-cell receptors and immunoglobin genes (V(D)J recombination; Fugmann et al., 2000), increasing the diversity and specificity of the immune response. It is a single-copy gene of approximately 3120 bp consisting of a single exon. The N-terminal third forms the divergent region (Fugmann et al., 2000), appears not to be directly involved in the functional interaction with DNA, and has higher substitution rates. Nearly the entire exon has been used to resolve ordinal phylogenetics among birds (Barker et al., 2002; Ericson et al., 2002; Groth and Barrowclough, 1999) and vertebrates (Frippiat et al., 2001), and smaller portions of the conserved region have been applied to deep-level questions in mammals (Madsen et al., 2001; Murphy et al., 2001), bats (Teeling et al., 2002), and sharks (Martin and Burg, 2002). The only application of RAG1 to more recent divergences is in the grouse family (Drovetski, 2002).

2. Materials and methods

2.1. Specimens examined

Forty-four specimens were sequenced in the analysis, representing all 10 recognized tribes (Hoffmann et al., 1993; Moore, 1959) and outgroup taxa (Table 1). Several genera are represented by multiple species (*Callosciurus*, *Heliosciurus*, *Marmota*, *Paraxerus*, *Sciurus*, *Tamias*, *Xerus*). Specimen identification and locality information are listed in Appendix A.

Table 1
Species included in the phylogenetic analyses

Subfamily Pteromyinae	Outgroups
Glaucomys volans	Family Dipodidae
Petaurista petaurista	*Allactaga sibirica*
Subfamily Sciurinae	Family Aplodontidae
Tribe Callosciurini	*Aplodontia rufa*
Callosciurus erythraeus	Family Muridae
Callosciurus prevostii	*Batomys granti*
Dremomys pernyi	*Beamys hindei*
Exilisciurus concinnus	*Deomys ferrugineus*
Sundasciurus philippinensis	*Dendromus mesomelas*
Tamiops swinhoei	*Microtus irene*
Tribe Funambulini	*Microtus pennsylvanicus*
Funisciurus carruthersi	*Mus musculus*
Paraxerus cepapi	*Phyllotis xanthopygus*
Paraxerus ochraceus	*Sigmodon hispidus*
Paraxerus vexillarius	
Tribe Marmotini	
Marmota marmota	
Marmota monax	
Marmota sibirica	
Spermophilus lateralis	
Tamias amoenus	
Tamias ruficaudus	
Tamias sibiricus	
Tribe Microsciurini	
Microsciurus flaviventer	
Tribe Protoxerini	
Heliosciurus ruwenzorii	
Heliosciurus undulatus	
Protoxerus stangeri	
Tribe Ratufini	
Ratufa bicolor	
Ratufa sp.	
Tribe Sciurillini	
Sciurillus pusillus	
Tribe Sciurini	
Sciurus carolinensis	
Sciurus ignitus	
Sciurus stramineus	
Tribe Tamiasciurini	
Sciurotamias davidianus	
Tamiasciurus hudsonicus	
Tribe Xerini	
Spermophilopsis leptodactylus	
Xerus inauris	
Xerus rutilus	

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from liver or muscle by means of PCI/CI "hot" extraction (Sambrook et al., 1989). C-myc exon 2 (transcribed/translated) and a region spanning part of intron 2 and most of exon 3 (entire transcribed region/partial untranslated region) make up 2278 bp and RAG1 (transcribed/translated)

Table 2
List of primers used for PCR and sequencing

Primer	Sequence
C-myc	
Sy2	CYACCACCAGCAGCGACTC
Sy3	YGAGGAGACACCGCCYACC
S7	CTTCCTCATCRTCTTGYTC
S9 (719 rev, DeBry and Seshadri, 2001)	GCGGTGTCTCCTCGTGGAG
S11 (9 fwd, DeBry and Seshadri, 2001)	CAACGTCAGCTTCGCCAACAG
S12	CAATCCACTCCCTCTTACC
S15	CTCWGGGATCTGGTCRCGC
S16	ACTGTCCAACTTRGCCCTC
Sy19	TAGGTAAGAATTGGCATC
S24	AACAGGCTGGAAAAAGGC
S54 (29 fwd, DeBry and Seshadri, 2001)	GGAACTATGACCTCGACTACGACTC
S55 (628 rev, DeBry and Seshadri, 2001)	TACAGGATTTGGGCGAGCTG
S91 (Myc-K, Miyamoto et al., 2000)	CCMAAGACYCAGCCAAGGTTGTGAGGT
S92 (Myc-M, Miyamoto et al., 2000)	RRAGCCTCATTAAGTCTTAGGTAAGAA
S93 (Myc-H, Miyamoto et al., 2000)	TTCCTCCTCTGGCGTTCCAAGACGTTGTG
S125	TGAAACAGATCAGCAACAACCGCA
RAG1	
S70 (modified from R13, Groth and Barrowclough, 1999)	TCCGAGTGGAAATTTAAGMTGTT
S71	TGGCTTCTGGTTATGGAGTGGA
S73	GAGGAAGGTRTTGACACGGATG
S75	AGGAACAAGGTCAAGCACACAG
S76	CTGACAAAGAAGAAGGTGGAG
S77	TCCATGCTTCCCTACTGACCTG
S105	CTCCACRGGGTCAGCCAGAAT
S108	GGGTTTTRGCATCCACRGG
S118	GAAGGGACCATTCAGGTAGTC
S119	GAAGGGACCATTCAGGTAGTC
S120	GTCCATCAAGGCAGACACCAA

makes up the remaining 2199 for a total of 4477 (see Table 2 and Fig. 1 for primers). Initial amplifications were in 25 μl volumes followed by 50–100 μl volumes for sequencing. Typically c-myc was amplified in three overlapping segments with primer pairs S9/S11, S12/S7, and S91/S92 (Fig. 1). RAG1 was typically amplified in two overlapping fragments with primer pairs S70/S73 and S77/S71. For c-myc exon 2, reagent concentrations were 1X Gold reaction buffer (Perkin–Elmer, ABI), 1 mM $MgCl_2$, 0.5 μM DMSO, 0.15 mM dNTP, 0.03 U/μl Amplitaq Gold (Perkin–Elmer, ABI), 1 μM of each primer, and 1 ng/μl whole genomic extract. Cycling conditions for c-myc exon 2 were initial denaturation at 94.0 °C for 15 min; followed by 45 cycles of amplification, 94.0 °C for 45 s, 55.0 °C for 45 s, and 72.0 °C for 1.5 min; followed by a final extension at 72.0 °C for 6 min. Reagent concentrations varied with primer sets for c-myc intron 2, c-myc exon 3, and RAG1; in amount of $MgCl_2$ (2 mM for intron 2, exon 3, RAG1); and primer amount (0.5 μM primer for RAG1). Annealing temperature also varied with gene or gene section: 58 °C for intron 2, 60 °C for exon 3, and 51 °C for RAG1.

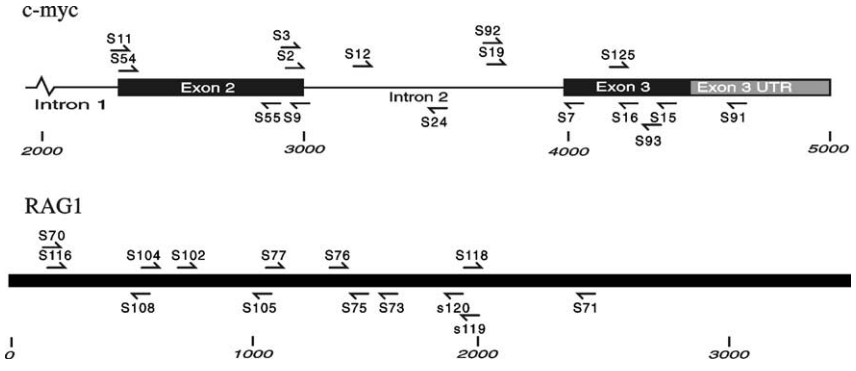

Fig. 1. Maps of primer locations on the two genes sequenced in this study, c-myc and RAG1. Scale bars indicate approximate nucleotide positions measured from the start of each gene.

Amplified samples were purified by precipitation with polyethylene glycol (PEG) or Concert Gel Extraction Systems (Gibco BRL). If bands were not bright or multiple bands were present, samples were reamplified from the original extractions or from gel-plug extractions. Products were sequenced directly for both light and heavy strands with amplification and sequencing primers (Table 2) on an ABI 373A or ABI 3100 automated sequencer, with big-dye terminator chemistry (ABI). Sequences for *Marmota marmota* c-myc were acquired from GenBank Accession No. X13232. A small number of taxa did not amplify well enough for sequencing for all genes and were excluded from those subset analyses (e.g., *Marmota sibirica*, *Petaurista petaurista*, and *Tamias amoenus* for c-myc exon 2; *Marmota marmota* for RAG1).

2.3. Phylogeny estimation

Chromatograms were first edited and aligned by Sequencher 3.0 (Gene Codes, Ann Arbor, Michigan). Alignments were then modified by eye for the intron, and minor adjustments were made to exon alignments by reference to the translated protein sequence with MacClade 4.0 (Maddison and Maddison, 2000). C-myc intron 2 could not be aligned reliably across outgroup taxa, so intron data were only included for ingroup taxa. DNA sequences are deposited in GenBank under Accession Nos. AY239470–AY239505 and AY241458–AY241544. Alignment and trees are submitted to TreeBase.

Phylogenetic analyses were conducted under equally weighted maximum-parsimony (MP) and maximum-likelihood (ML) criteria with PAUP* version 4.0b10 (Swofford, 2002) for each gene region separately (c-myc exon 2, c-myc intron 2/exon 3, and RAG1). Because of the small number of characters for c-myc intron 2 and exon 3 UTR, these regions were combined with the contiguous exon 3 for phylogenetic analysis. A sequential optimization approach (Fratti et al., 1997; Swofford et al., 1996) was used to estimate the phylogeny. Initial trees were generated by MP. ML parameter values were estimated under a nested array of substitution models for the MP trees as implemented in Modeltest 3.04 (Posada and Crandall, 1998). The best fit models for the three gene regions by both the likelihood ratio test and Akaike Information Criterion were $GTR + I + \Gamma$ (total, RAG1), $HKY + I + \Gamma$ (c-myc exon 2; Hasegawa et al., 1985), and $K81uf + I + \Gamma$ (1 transition rate, 2 transversion rates; Kimura, 1981; and c-myc intron 2-exon 3). A ML search was then conducted under the preferred model with parameters fixed to the values estimated on the MP tree. Heuristic searches were conducted with 10 (ML total) to 40 (ML individual genes) random-addition replicates and TBR branch-swapping. Model parameters were reestimated from the initial ML tree and the process repeated until the topology remained constant, never requiring more than one iteration. Bootstrapping (Felsenstein, 1985) was performed on all data partitions (100–200 replicates ML, 500 replicates MP) and decay indices estimated using AutoDecay (Eriksson, 1999). Bootstrapping runs under likelihood were limited to 1000 rearrangements, which, although it reduces the likelihood that the optimal tree will be found for each replicate, is a conservative procedure most likely to reduce bootstrap values, not raise them. Exploratory analyses run to completion show that 85% of the time the optimal tree for any given replicate is found by the 1000th rearrangement.

Data sets were analyzed separately, by gene, and combined. Partition-homogeneity tests with 100 replicates showed no significant heterogeneity in signal among gene regions ($P = 0.62$) or genes ($P = 0.26$). Parsimony-informative indels in c-myc intron 2 and untranslated region of exon 3 were coded as presence-absence characters for parsimony analyses.

Bayesian analyses were conducted on the same data sets with MrBayes (Huelsenbeck and Ronquist, 2002). Bayesian analysis on the total data used the $GTR + I + \Gamma$ model as in the ML analyses. Two data partitions were recognized, coding and noncoding, and the model parameters for each partition were estimated separately (''unlinked''). We ran four MCMC chains for 2,600,000 generations, sampling trees every 100 generations. The likelihood converged on a stable value by 50,000 generations, so we excluded the first 100,000 generations from the sampling (as the ''burn-in'' period). For the individual gene regions, the same models were used as in the ML analyses with the addition of partitioning the data by codon position (plus noncoding partition for c-myc intron 2). We ran the analyses with four MCMC chains for 160,000–210,000 generations, sampling trees every 10 generations. Stable likelihoods were reached between 30,000 and 60,000 generations, so we excluded the first 31,000–70,000 generations as the burn-in.

A priori hypotheses were tested with parsimony- and likelihood-based approaches. Tree searches were conducted with constraints enforced to match predicted topologies for each hypothesis. Differences in tree scores between all equally optimal trees from constrained searches and the optimal trees overall were subjected to Templeton's test (Templeton, 1987) under parsimony and one-tailed Shimodaira–Hasegawa (Shimodaira and Hasegawa, 1999) test with restricted likelihood as implemented in PAUP* 4.0b10 (Swofford, 2002). Because the topology of the reference (optimal) tree was not defined a priori (Templeton's test should properly be applied to pairs of a priori hypotheses), we interpret the significance values cautiously.

Species were coded for degree of arboreality as follows: arboreal, proficient in trees and nests in trees, even if some time is spent on the ground (e.g., gray squirrels);

intermediate, spends most of time on the ground but can run into trees (e.g., chipmunks); terrestrial, lives on and nests in the ground, rarely if ever climbs (Kingdon, 1997; Nowak, 1991). Habit of the ancestral squirrel was reconstructed by means of parsimony on the total data ML tree with both delayed and accelerated options. Relative likelihoods for ancestral states were reconstructed on the total data ML tree with branch lengths under one-rate (Mk1) and two-rate (Asym) models using the RecAncestralStates module in Mesquite version 0.994 (Maddison and Maddison, 2003).

3. Results

3.1. Genes

Base composition, transition–transversion ratio, and among-site rate variation (α parameter for the gamma distribution) are summarized for each of the gene regions in Table 3. None of the gene regions shows significant base-composition heterogeneity across taxa, but gene regions differ distinctly in composition. C-myc exon 2 is enriched in C's but low in A's and T's. In contrast, exon 3 is high in A's but still low in T's, intron 2 is high in T's, and exon 3 UTR is moderately high in A's and T's. Differing from all of these c-myc gene regions, RAG1 has nearly equal average base composition.

The amino-acid sequence is highly conserved in c-myc exon 2 among sciurids, having only 18 variable residues and one deletion among 229 residues (8.3%). The gamma distribution shape parameter α can be sensitive to the scope of taxon sampling (Sullivan et al., 1999) when divergences are low because among closely related species very few sites are variable; α estimated from the nucleotides varies from 0.207 (sciuroids plus muroid/dipodoid outgroups) to 0.107 (sciuroids; sciurids plus *Aplodontia*) and 0.014 (sciurids only). Exon 3 shows a similar pattern of variability but with less pronounced among-site rate variation ($\alpha = 0.130$–0.275 vs. 0.014–0.207). Exon 3 thus appears less sensitive to taxon sampling when the gamma distribution is estimated. The untranscribed (intron 2, sciuroids only) and untranslated regions are more variable and have a more uniform pattern of variation across sites ($\alpha = 0.92$ and 1.60, respectively).

In contrast to c-myc exons, RAG1 shows much higher rates of amino-acid substitutions with 161 variable amino-acid residues out of 716 (22.5%). The variable sites are concentrated in the N-terminal divergent region, approximately the first 300 residues in this data set. The three indels are all found within the first 100 residues. Concordantly, the proportion of parsimony informative nucleotide sites is greater than in c-myc (16 vs. 10%). Among-site rate variation ($\alpha = 0.329$–0.480) is intermediate between those of c-myc exons and c-myc intron 2.

A more detailed examination of substitution patterns in RAG1 reveals that the nearly equal base composition is partially due to differing composition bias across codon position and gene region that balance each other (Table 4). Although these among-region biases are not as pronounced as those in c-myc, the divergent region seems enriched in A's and impoverished in T's. The two regions are similar in variation in base frequency across codon position, except that the divergent region shows less variability in G frequencies than does the conserved region. Transversion ratios vary with codon position but less so for the divergent region. Likewise, the gamma shape parameters are greater for the divergent region, indicating more uniform among-site rate variation because of relaxed selective constraints on protein structure. This pattern is particularly reflected at second positions, where the rate of substitutions (all nonsynonymous) is approximately four times that of the conserved region. The very low α value of 0.008 for second positions in the conserved region may reflect strong functional constraint for most of the protein. Nearly one-half of all residues are invariant across a broad

Table 3
Characteristics of gene regions

Region	Base frequencies				No. of sites	Ts/Tv	α
	A	C	G	T			
Exon 2	0.182	0.372	0.269	0.176	724 (71 + 0)	1.96 (2.29)	0.014 (0.207)
Intron 2	0.263	0.203	0.203	0.331	821 (66 + 15)	1.88 (n.a.)	0.92 (n.a.)
Exon 3	0.325	0.261	0.259	0.156	567 (60 + 0)	3.04 (2.95)	0.130 (0.275)
Exon 3 UTR	0.314	0.225	0.214	0.297	164 (31 + 2)	1.48 (1.48)	1.60 (1.04)
RAG1	0.274	0.248	0.255	0.223	2174 (368 + 0)	2.94 (2.61)	0.329 (0.480)

Values calculated for the Sciuridae only, whereas values in parentheses (Ts/Tv, α) are for all taxa including outgroups. Number of sites includes indels. Number of sites in parentheses is the number of parsimony-informative sites within the Sciuridae plus the number of parsimony-informative indels treated as discrete characters regardless of length. Ts/Tv is the transition-to-transversion ratio, and α is the gamma-distribution shape parameter, both calculated on the total-evidence ML tree under a HKY + Γ model. Higher values of α indicate more uniform distribution of variation across sites. Low values, especially those below 0.2, indicate that a small number of sites exhibit many nucleotide substitutions while the majority are invariant.

Table 4
Characteristics of RAG1 by functional regions

Region	Base frequencies				No. of sites	Ts/Tv	α
	A	C	G	T			
Divergent							
1st pos.	0.336	0.261	0.261	0.142	240 (36)	1.81	0.637
2nd pos.	0.360	0.221	0.215	0.204	240 (35)	1.73	0.404
3rd pos	0.220	0.316	0.252	0.212	240 (81)	3.02	2.43
Overall	0.305	0.266	0.243	0.186	720 (152)	2.34	0.816
Conserved							
1st pos.	0.286	0.228	0.303	0.183	481 (27)	1.29	0.180
2nd pos.	0.319	0.200	0.185	0.296	481 (16)	2.50	0.008
3rd pos	0.176	0.289	0.288	0.246	481 (185)	3.46	1.81
Overall	0.260	0.239	0.259	0.242	1443 (228)	2.90	0.342

Values calculated are for all taxa including outgroups. Number of sites includes indels. Number of sites in parentheses is the number of parsimony-informative sites within the Sciuroidea plus the number of parsimony-informative indels treated as discrete characters regardless of length. Ts/Tv is the maximum-likelihood estimate of the transition-to-transversion ratio, and α is the gamma-distribution shape parameter calculated on the ML tree under a HKY + Γ model. Higher values of α indicate more uniform distribution of variation across sites. Low values for α, especially those below 0.2, indicate that a small number of sites exhibit many nucleotide substitutions while the large majority are invariant.

sampling of rodents, other mammals, birds, and fishes (Huchon et al., unpubl. ms.). Additional analyses of amino-acid variability in RAG1 across vertebrates are ongoing.

3.2. Phylogenetics

3.2.1. Total data

The single ML tree (Fig. 2; $L = -24{,}061.56$ including outgroups) places *Aplodontia* as the outgroup to the Sciuridae, forming the Sciuroidea (note that, in all figures, muroid and dipodoid outgroups are removed for clarity). Within the Sciuridae, the ML analysis yields five major lineages: (A) the giant squirrel *Ratufa*; (B) the pygmy squirrel *Sciurillus*; (C) a clade consisting of the flying squirrels Pteromyinae, New World tree squirrels Sciurini, and Microsciurini, plus the red squirrel *Tamiasciurus* (= Tamiasciurini s. s.); (D) the Callosciurini; and (E) a clade consisting of the African ground squirrels Xerini, Holarctic ground squirrels Marmotini, African Protoxerini, plus the African members of the Funambulini. The last clade E we will refer to informally as the "ground squirrel" clade because of the habit of most members of the group, in contrast to the exclusively arboreal habits of the other clades. *Ratufa* and *Sciurillus* form a clade and together are the sister group to the other three clades. Parsimony analysis of the total concatenated data set yields 18 trees of 3255 steps that differ among themselves only in the arrangements within *Marmota* and within *Sciurus* and the relationships of the callosciurines *Dremomys* and *Tamiops* to the *Callosciurus-Sundasciurus* clade. The MP consensus tree (not shown) differs from the ML tree only in placing clade C (Pteromyinae + Sciurini s. l., including *Tamiasciurus*) at the root. We will refer to the clade consisting of the Sciurini, Microsciurini, and *Tamiasciurus* as the Sciurini s. l. Support for either rooting is not strong according to bootstrapping (65% ML; 68% MP), but Bayesian posterior probability for the ML rooting is 0.98. Most other nodes are robustly resolved; 67% of nodes have greater than 96% ML bootstrap, and all but three nodes have posterior probabilities greater than 0.95 (within *Sciurus*, within *Marmota*, at the base of the Marmotini).

The giant squirrel *Ratufa* and the pygmy squirrel *Sciurillus* are each considered major lineages because they appear to have split from each other very early in the squirrel radiation. Support is moderate to strong for the following sequence of divergences among the remaining clades: the ancestor of the flying squirrels and the Sciurini (clade C) diverged first, followed by the Callosciurini (clade D) and clade E. Support for the sister-group status of clades D and E is moderately strong, with 83–91% bootstrap percentages and 1.00 posterior probability (pp).

Within clade C, the Pteromyinae (flying squirrels) are strongly supported as a monophyletic group with 100% bootstraps, 1.00 pp, and a decay index of 14. The Sciurini s. l. are also monophyletic (99–100% bootstraps, 1.00 pp) and are the Pteromyinae's closest relatives. The Tamiasciurini s. s. (*Tamiasciurus*) are the basal members of the Sciurini s. l. Neither generic status for *Microsciurus* nor tribal status for the Microsciurini is supported because *Sciurus* is paraphyletic with respect to it.

The fourth major clade (D) among the Sciuridae consists entirely of Callosciurini. It is strongly supported as a monophyletic group (100% bootstraps, 1.00 pp) and separated by a long branch from its sister group, the "ground squirrel" clade E. *Exilisciurus* appears as the sister group to all other callosciurines, then the clade of *Dremomys* and *Tamiops* forms the sister group to a *Callosciurus-Sundasciurus* clade.

The fifth major clade (E), including mostly African groups and the Holarctic Marmotini, is a somewhat unexpected grouping of taxa but is well supported

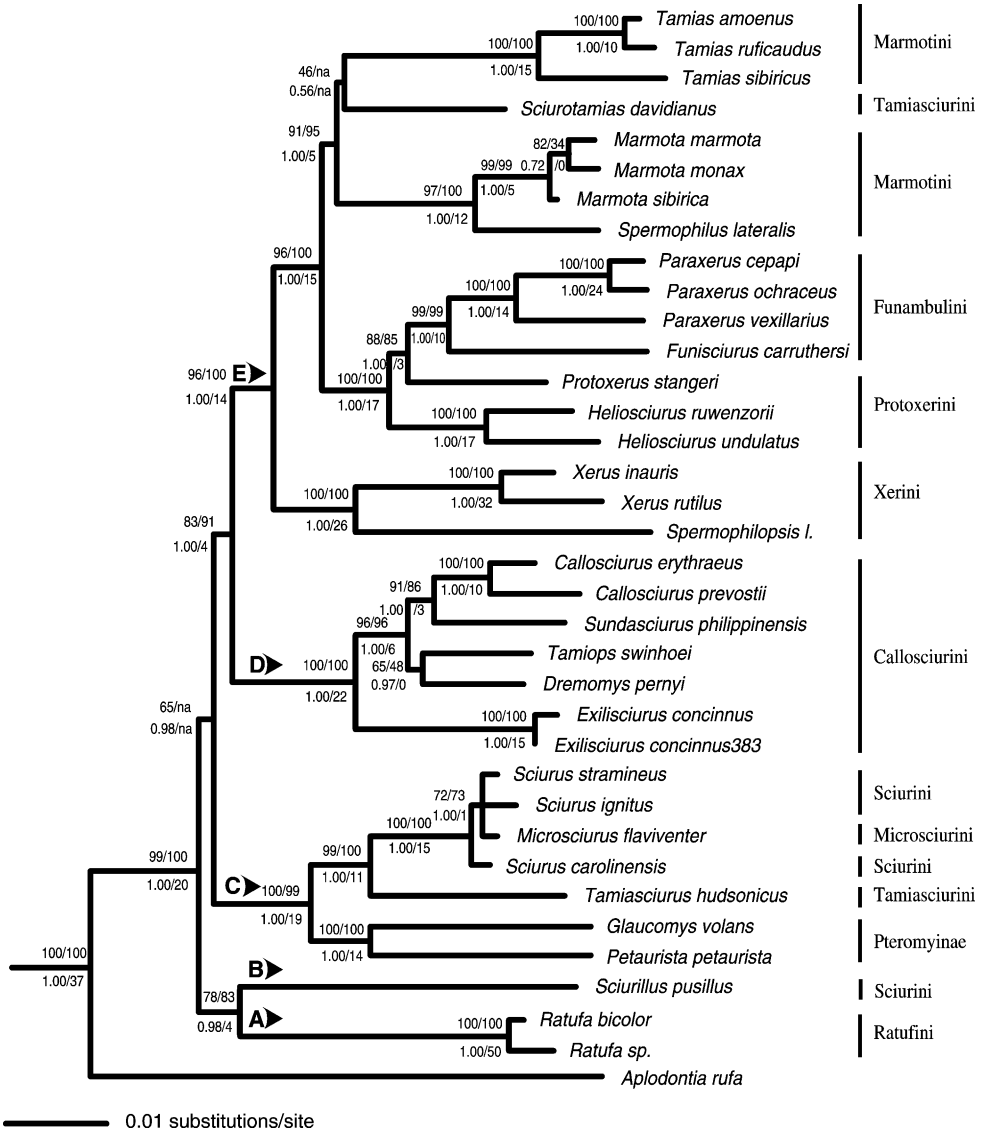

Fig. 2. Phylogram from maximum-likelihood analysis of total-data set (c-myc and RAG1) under the GTR + I + Γ model of evolution. Numbers above branches are likelihood and parsimony bootstrap values, respectively. Numbers below the branches are Bayesian posterior probabilities and decay indices. Large letters with arrowheads indicate the five major clades discussed in the text. Outgroups are pruned for clarity. Taxonomy follows Hoffmann et al. (1993).

(96–100% bootstraps, 1.00 pp, decay index of 14). Within clade E, the Xerini are the sister group to a well-supported clade consisting of Marmotini + Protoxerini + African funambulines. Within the latter group, the African members form a clade that, like the Xerini, is also strongly supported (100% bootstraps). The Protoxerini are paraphyletic with respect to African Funambulini because *Protoxerus* is more closely related to funambulines (Funisciurina) than to *Heliosciurus* (88% ML bootstrap, 1.00 pp). Monophyly of the Marmotini is weaker than that of the nodes below it but still strong, with 91% ML bootstrap and 1.00 pp. The Chinese rock squirrel *Sciurotamias*, sometimes associated with *Tamiasciurus*, appears to be a member of the Marmotini. The MP tree differs slightly from the ML tree in placing *Sciurotamias* at the base of the Marmotini rather than sister to the chipmunk *Tamias*, but support for either arrangement is weak (0.56 posterior probability, 68% MP bootstrap). The data indicate that *Tamias* diverged early in marmotine evolution from the true ground squirrels *Spermophilus* and the marmots *Marmota*.

3.2.2. Individual gene regions

Each the three gene regions analyzed separately produced phylogenies largely congruent with the total-data tree and those of the other gene regions. For example, the ML phylogeny from analysis of c-myc intron 2–exon 3 is illustrated (Fig. 3; $L = -3357.66$). The only areas of disagreement between intron 2 and exon 3 and the total-data tree are the placement of *Sciurotamias*

and, more importantly, the rooting of the Sciuridae. Under parsimony, the root is placed between clade C and all other squirrels. There were 240 most-parsimonious trees of 843 steps (including outgroups). Because of the very long branches leading from the myodont outgroups (due to apparent rate acceleration in myodonts, Steppan et al., unpubl. ms.) and the inability to align the intron between outgroups and sciuroids, we rooted the intron 2–exon 3 ML tree with *Aplodontia* alone.

For c-myc exon 2, parsimony analysis found 361 trees of 426 steps (tree not shown). The root within Sciuridae is imprecisely determined by this gene region because of a polytomy consisting of three clades: *Ratufa Sciurillus* (A + B, weakly supported), Petauristinae + Sciurini s. l. (clade C), and all remaining squirrels (clades D + E). Monophyly of the Marmotini and Protoxerini is undetermined because several members of each are part of a large polytomy forming clade E. Maximum likelihood yields a single tree (not shown, $L = -3355.81$) that is consistent with the MP consensus tree in most respects. The root is placed between the Pteromyinae + Sciurini s. l. and all other squirrels. The giant squirrel *Ratufa* and the pygmy squirrel *Sciurillus* are not sister taxa here, in contrast to the MP tree, but are instead two basal lineages. However, most internal branches are very short and not well supported.

RAG1 provides better-supported phylogenies (trees not shown) that are in full agreement with the total-data tree with the exception of the rooting. Therefore, the primary area of disagreement among all the analyses is the rooting of the Sciuridae (all recover the same five primary lineages, and all support flying-squirrel monophyly). Four root placements are represented among the analyses: between clades A + B and C + D + E (total data ML, RAG1 MP, consistent with c-myc exon 2 MP),

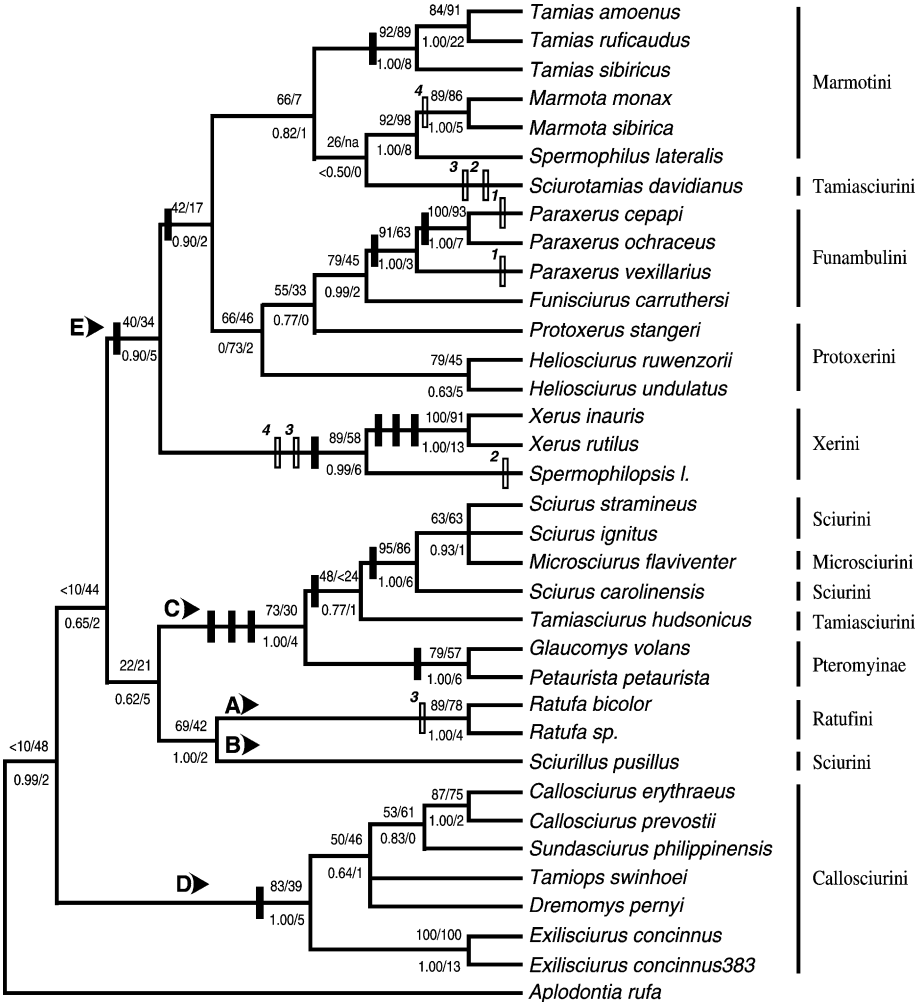

Fig. 3. Cladogram from maximum-likelihood analysis of c-myc exon 3 and portions of intron 2 and untranslated region of exon 3 under the K81uf + I + Γ model of evolution. Numbers above branches are likelihood and parsimony bootstrap values, respectively. Numbers below branches are Bayesian posterior probabilities and decay indices. Outgroups are pruned away for clarity. Solid vertical bars indicate unique indels, open bars indicate homoplasious indels. Numbers above open bars identify the four different indels, two of which (2, 3) are length variants in a long string of repeat bases. Large letters with arrowheads indicate the five major clades discussed in the text. Taxonomy follows Hoffmann et al. (1993).

between clade C and all others (total data MP, c-myc exon 2 ML, and c-myc exon 3 ML), on the branch leading to clade A (RAG1 ML), and between clade D and all others (c-myc exon 3 ML). Only the total-data ML analyses provides moderately strong support for their rooting (65–78% ML bootstrap, 0.98 pp on relevant nodes).

3.3. Indels

Of the 19 parsimony-informative indels, 15 were perfectly congruent with all the trees reported here (Fig. 3). The clades supported by these indels were (with numbers of indels if more than (1): Sciurini + Pteromyinae (3), Pteromyinae, Sciurini, *Sciurus* + *Microsciurus*, Callosciurini, the ground squirrel clade, Xerini, *Xerus* (3), Marmotini + Protoxerini + Funambulini, Paraxerus, and *Paraxerus cepapi* + *P. ochraceus*. Of the four indels that appeared homoplasious, two involved length variation in c-myc intron 2 for a string of more than five identical nucleotides for which homoplasy is not unexpected. Of the remaining two, one was found in the Xerini and Marmotini but not the Protoxerini, and the other was shared by *Paraxerus cepapi* and *P. vexillarius* but not *P. ochraceus*. Insertions and deletions therefore appear not to be prone to convergence or reversal and thus are highly informative regarding phylogeny when at low frequency. The indels observed here provide good support for the key results from nucleotide-substitution phylogenies, most significantly monophyly of the flying squirrels and their association with the sciurine tree squirrels.

3.4. Comparing genes

RAG1 yields a better-supported estimate of phylogeny than the comparably sized c-myc data set (coding and noncoding, tree not shown). Of all concordant clades, RAG1 has greater parsimony bootstrap percentages than c-myc for 11 (average 15% greater), equal percentages for seven (most at 100%), lower percentages for five clades (11.6%), and greater percentage for the one root-determining node where the trees differ (11%). However, c-myc intron 2 and exon 3 UTR contain a significant number of insertions and deletions that appear highly informative phylogenetically. Weighting of indels is problematic, but their greater frequency in noncoding regions may provide an advantage over RAG1 and other exons by providing strong support (even in small numbers) for some nodes that are not well supported by nucleotide transformations alone. Analyzed separately, c-myc exon 2 yields a phylogeny in general agreement with the total-data tree but with much reduced resolution (65% of nodes resolved in strict consensus) and reliability measures. Exon 2 of c-myc appears either to evolve too slowly or to have too few characters to resolve many parts of the tree simultaneously.

3.5. Hypothesis testing

The results of the tests based on topological constraints are summarized in Table 5. On the basis of the optimal ML topology from the total data analysis, we reject the following hypotheses: (1) the Pteromyinae and Sciurinae are each monophyletic and are sister groups, (2) *Sciurillus* is a member of the Sciurini, (5) *Sciurotamias* and *Tamiasciurus* are sister taxa, (7) Protoxerini is derived from the Xerini to the exclusion of other tribes, (9) the Marmotini and Xerini are sister groups, (10) the southeast Asian tree-squirrel taxa Callosciurini and *Ratufa* form a monophyletic group, and (11) relationships conform to the results of Mercer and Roth (2003) in the following topology: (*Sciurillus* (*Ratufa* (Callosciurini (Pteromyinae + Sciurini s. l. (Xerini, Marmotini, *Sciurotamias*, Protoxerini, African funambulines))))).

Table 5
Tests of a priori hypotheses

Constraint	ΔMP score	*P* (Templeton)	ΔML score	*P* (S–H)	Conclusion
1: Pteromyinae/Sciurinae	24	<0.0001*	54.98	<0.0001*	Reject
2: *Sciurillus* is a sciurine	61	<0.0001*	161.49	<0.0001*	Reject
3: Callosciurini monophyletic	(21)	(0.0033*)	(77.22)	(<0.002*)	Accept
4: *Spermophilopis* is a xerine	(21)	(0.0004*)	(55.68)	(<0.0001*)	Accept
5: *Sciurotamias* is a tamiasciurine	72	<0.0001*	206.36	<0.0001*	Reject
6: *Sciurotamias* is a marmotine	(3)	(0.257)	(4.94)	(0.134)	Accept
7: Xerine origin of Protoxerini	39	0.0001*	134.91	<0.0001*	Reject
8: Protoxerini monophyletic	5	0.059	7.2	0.094	Accept
9: Marmotini plus Xerini	16	0.0001–0.0047*	40.87	0.004*	Reject
10: Callosciurini plus Ratufini	15	0.051–0.055	24.23	0.033*	Reject
11: Mercer and Roth backbone	18	0.004*	15.20	0.022*	Reject

MP and ML scores are the difference in the number of steps or log-likelihood units between the optimal trees and the constrained trees for the total data set. *P* values are the probabilities calculated with Templeton's test for parsimony and the Shimodaira–Hasegawa test for likelihood. Values in parentheses indicate that the optimal tree matches the a priori hypothesis and thus that the values reported are for the alternative constraint where the a priori hypotheses were excluded from the search. Asterisks indicate significant differences at the 0.05 level, one-tailed test.

Although it is not supported by the optimal trees, we cannot reject the hypothesis that the Protoxerini are monophyletic. In addition, trees in which *Sciurotamias* is not a marmotine (although still a member of the ground-squirrel clade) are not significantly different from the optimal trees. Although the a priori hypotheses of callosciurine monophyly and xerine membership of *Spermophilopsis* cannot be rejected by the data because those hypotheses match the optimal trees, the a posteriori alternative hypotheses are notably less parsimonious or likely.

3.6. Arboreal or terrestrial ancestor?

Parsimony reconstruction of the common ancestor of living squirrels onto the total data ML tree yields an estimate of arboreality (Fig. 4), but this conclusion is sensitive to character coding in *Exilisciurus* because of its position relative to other callosciurines at the base of the tribe. The reconstruction for the most recent common ancestor (MRCA) of squirrels becomes equivocal under DELTRANS optimization if *Exilisciurus*, which spends much of its time close to the ground, is coded as intermediate (reconstruction remains arboreal under ACCTRANS optimization). Most callosciurine genera, including both arboreal and intermediate forms, were not sampled for this study, so additional sampling could alter the parsimony conclusion regarding the ancestral squirrel.

Likelihood reconstruction is more definitive than parsimony in this case and less sensitive to coding of *Exilisciurus*. Under a one-rate Mk1 model, the marginal

Fig. 4. Evolution of arboreal and terrestrial habits optimized onto the maximum-likelihood phylogeny for the total data set. Shading represents all equally parsimonious optimizations, indicating that the most recent common ancestor of living squirrels was arboreal. The subsequent sequence of transitions to and from terrestrial habits is equivocal.

probability that the MRCA of squirrels is arboreal is 98%, 50-fold greater than that it was terrestrial, even though all outgroups are terrestrial. Coding *Exilisciurus* as intermediate in habit lowers the relative likelihood to only 30-fold greater (96.5% marginal probability of arboreal). Under a two-rate model, the MRCA is 300 times more likely to be arboreal than terrestrial, under both codings of *Exilisciurus*. The high probability of arboreality appears to be a consequence of four major arboreal lineages diverging very close to the MRCA. We did not integrate the likelihoods over all probable trees using Bayesian approaches because trees wherein the key basal relationships are not present represent less than 1% of sampled trees and thus would not significantly change the integrated relative likelihoods.

4. Discussion

4.1. Sciurid phylogenetics

The molecular data do not support the traditional division of squirrels into flying squirrels (Pteromyinae) and all other squirrels (Sciurinae). Instead, all three gene regions provide moderate to strong support for the hypothesis that flying squirrels are derived tree squirrels, specifically related to New World tree squirrels (Sciurini). The data allow a strong rejection of alternative hypotheses (Table 5). The fundamental phylogenetic divisions within the Sciuridae are not between flying squirrels and all others but among four to five major lineages: the giant tree squirrels *Ratufa*, the pygmy squirrel *Sciurillus*, the New World tree squirrels plus flying squirrels, the southeast Asian callosciurine tree squirrels, and the predominantly terrestrial remaining groups. Some aspects of the branching order among these five groups appear to be well supported by these data despite the short internal branches. For example, the data allow rejection of the proposal that two of those basal branches, *Ratufa* and the Callosciurini, are closely related (Table 5). These short branches indicate a relatively rapid divergence. *Ratufa* and *Sciurillus* appear either to be the basal members of the Sciuridae or, more probably, to form a monophyletic sister group to all remaining squirrels. Further, the Callosciurini (clade D) and the ground squirrel clade (E) also appear to form a monophyletic group and together are the sister group to the Neotropical tree squirrel-flying squirrel clade C. Given the concordance between our results and those of Mercer and Roth (2003) for the major conclusions, a revision of sciurid taxonomy is needed, and we discuss this issue below.

One of the outstanding questions in squirrel phylogenetics is whether or not the flying squirrels form a monophyletic group. Albumin immunological distances indicated that they may not be because the Asian genus *Iomys* lay outside a clade formed by other members of the group (Hight et al., 1974). Doubts regarding monophyly were also based on the geography of the group, which includes one North American genus (*Glaucomys*) and many Asian genera, but nuclear and mitochondrial sequence data strongly supported monophyly (Mercer and Roth, 2003). Although we were not able to include *Iomys* in our sample, our data also show convincingly that the American and at least one Asian genus form a clade. The sequence-based phylogenies support both the conventional assumption that gliding evolved only once in this group and the more recent comparative morphological studies (Thorington, 1984; Thorington et al., 1997, 2002).

Parsimony reconstruction based on the phylogenetic results presented here does not yield any insight into the geographic origins of squirrels. *Ratufa* and the Callosciurinae are southeast Asian, *Sciurillus* and most sciurine tree squirrels are Neotropical (although the basal members of the tree–flying squirrel clade are mostly Holarctic), and the ground squirrel clade appears basally Afro-Asian but includes major North American and Palearctic groups.

Mercer and Roth's (2003) phylogeny of the Sciuridae combined data from mitochondrial 12S and 16S (732 and 472 aligned sites, respectively) and nuclear IRBP (1171 aligned sites). The results of the present study and theirs are in broad agreement. The pygmy squirrel *Sciurillus* and the giant tree squirrel *Ratufa* are basal branches, flying squirrels (Pteromyinae) are monophyletic and the sister group to Sciurini + Tamiasciurini (together forming their clade V and our clade C), and the xerine ground squirrels of Africa and Asia form a clade along with the Marmotini, Protoxerini, and African funambulines (their clade IV and our clade E). Other key areas of agreement include nesting of *Microsciurus* within a paraphyletic *Sciurus*, that the red squirrel *Tamiasciurus* is the sister group to the Sciurini s. s., that *Exilisciurus* is basal within the Callosciurini, and that the Protoxerini are paraphyletic with respect to African funambulines.

Conflict between the studies remains, however, with respect to two aspects of the branching order among the five major clades and the position of the rock squirrel *Sciurotamias*. Mercer and Roth (2003) place *Sciurillus* (clade I, B) as the first branch diverging from all other squirrels, a position never seen in any of our individual gene-region analyses or in the total-data analyses. Our results either place *Ratufa* (clade II, A) basal or *Ratufa* and *Sciurillus* as basal sister taxa (Fig. 5). Their rooting is supported by 0.73 pp compared to our 0.98 pp. The lower posterior probability for the 12S/16S/IRBP tree may reflect inclusion in their data set of half as many aligned characters as in ours or possible conflict between their nuclear and mitochondrial genes. In the second region of conflict, Mercer and Roth place the Callosci-

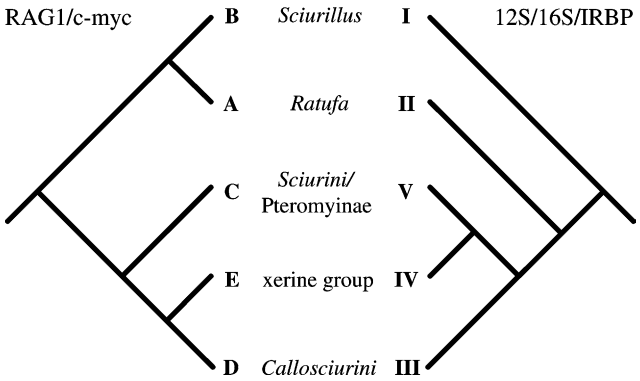

Fig. 5. Comparison between the results of two studies, the one reported here (RAG1/c-myc) and that of Mercer and Roth (2003; 12S/16S/IRBP), for the five major lineages found in both studies. Nearly all other relationships are identical in the two studies. Lineage names are as used in the respective studies.

urini (clade III, D) basal among the remaining three major clades, whereas our analyses place the Pteromyinae–Sciurini s. l. (clade V, C) basally. Both data sets provide strong Bayesian support for their respective groupings (0.99, 1.00 pp). Our data also show strong support from bootstrapping (96% ML, 100% MP), but no other measures of support are reported by Mercer and Roth. Finally, *Sciurotamias* lies outside the Marmotini/Protoxerini s. l. clade in Mercer and Roth (2003) but within the Marmotini in our results. Mercer and Roth report 0.56 pp for their result, whereas our data yield 1.00 pp.

To assess the different results further, we conducted tree searches constrained to match the Mercer and Roth (2003) branching pattern among the five major clades. Despite the short internal branches, the c-myc and RAG1 data are sufficient to reject the alternative hypothesis (Table 5; total data, $p = 0.004$ MP, $p = 0.022$ ML). In contrast, the IRBP data (pruned to the genera sampled in this study) do not support rejection of the RAG1/c-myc topology ($p = 0.200$, MP and ML). Whether this difference is due to lineage sorting among independent loci or to differences in the amount of data is unclear. Mercer and Roth (2003) did not report results from analyzing their genes separately, and alignments were not publicly available for the ribosomal genes.

The results of the present study show that the ground squirrel clade E consists of three major lineages: the Old World Xerini (including Asian *Spermophilopsis* and African *Xerus*), the Holarctic Marmotini (including Asian *Sciurotamias*), and an African clade formed by the African members of the Funambulini (subtribe Funisciurina) and a paraphyletic Protoxerini. Although the data do not allow rejection of monophyly of the Protoxerini, they do allow rejection of a xerine origin of the Protoxerini (Table 5).

4.2. Systematic recommendation

The results of our study support (consistent with Mercer and Roth, 2003) at least five changes from conventional taxonomy. Subfamilial status of the Pteromyinae is not warranted unless four–six other tribes of roughly equal or greater age are also elevated to subfamily status (sister taxa must be of the same rank). The tribe Sciurini does not include *Sciurillus* (subtribe Sciurillina; McKenna and Bell, 1997), but is closely related to *Tamisciurus* and thus could include the Tamiasciurini. The Tamiasciurini do not include *Sciurotamias* (Table 5), a genus that instead appears to be a marmotine. The Tamiini nest within the Marmotini. The Protoxerini are paraphyletic with respect to the Funisciurina. Because the results from Mercer and Roth (2003) (also supported by immunological distances, Hight et al., 1974) provide strong support for the placement of *Funambulus* as the sister group to Callosciurini, both groups being from Southern Asia, the African Funisciurina must be removed from the Funambulini. We recommend placing the Funisciurina (Moore, 1959) within the Protoxerini (Moore, 1959). In the following classification we strive to maximize stability of names but have chosen to elevate several tribes to subfamily level to avoid leaving too many tribes within a single subfamily. The Xerinae Osborn (1910) is the oldest available subfamily-level name for the clade that includes the Xerini, Marmotini, and Protoxerini.

Classification:

Family Sciuridae
- Subfamily Ratufinae, new rank
- Subfamily Sciurillinae, new rank
- Subfamily Sciurinae
  - Tribe Pteromyini, new rank
  - Tribe Sciurini
- Subfamily Callosciurinae
  - Tribe Callosciurini
  - Tribe Funambulini
- Subfamily Xerinae, new contents
  - Tribe Xerini
  - Tribe Marmotini
  - Tribe Protoxerini

Subfamily Sciurinae is redefined as the most recent common ancestor of Sciurini and Pteromyini and all of its descendants. Subfamily Xerinae is defined as the most recent common ancestor of the Xerini, Marmotini, and Protoxerini (including Funisciurina) and all of their descendants.

4.3. Ecological adaptations of squirrels

The earliest direct ancestors of squirrels appear to be the ischyromyid rodents (including paramyids), which

first appeared in the fossil record in the earliest Eocene about 54 million years ago and which are represented in the modern rodent fauna by *Aplodontia rufa* (Simpson, 1945). These rodents were terrestrial, and modern *Aplodontia* is a specialized burrower. In contrast, the earliest known fossil squirrel, *Douglassisciurus* (= *Protosciurus*) *jeffersoni*, from the middle Chadronian (= early Oligocene) in Montana about 35 million years ago, was clearly a specialized tree squirrel comparable to the modern *Sciurus niger* (Emry and Korth, 1996; Emry and Korth, 2001; Emry and Thorington, 1982). Our phylogenetic results are consistent with the fossil evidence in identifying the earliest true squirrels as arboreal (Fig. 4). Arboreality may be a key adaptation at the base of the squirrel lineage that aided the divergence of that group from its terrestrial/fossorial ancestors.

Fossil evidence suggests the center of sciurid diversification was in North America, but our data do not provide an answer. Parsimony optimization of areas as characters is equivocal on the distribution of the MRCA of squirrels. Although Mercer and Roth (2003) reconstructed the geography to indicate that the protoxerine lineage must represent a colonization of Africa, it is equally parsimonious to reconstruct the colonization of Africa to have occurred earlier (at the origin of clade E) followed by expansion of the Marmotini out of Africa. The molecular evidence suggests that habitat diversification occurred relatively early, with divergence of now-terrestrial from now-arboreal lineages not long after the basal radiation of squirrels (Fig. 2), perhaps within 3 My (Mercer and Roth, 2003; Fig. 2). The fossil record of squirrels is "woefully inadequate" (Black, 1972), but by the late Oligocene the first ground squirrel appeared, the xerine *Heteroxerus*. Several lineages of xerines are traceable through the Miocene and Pliocene in Europe, western Asia and Africa (Black, 1972; Gromov and Baranova, 1981). In North America, several lineages of ground squirrels, probably all marmotines, appeared in the late Oligocene and flourished throughout the Miocene, including *Protospermophilus* and *Miospermophilus*. A calibrated molecular clock tree is consistent with the fossil record on the timing of marmotine and xerine diversification (Mercer and Roth, 2003). Our molecular data agree with morphology (Black, 1972) in placing the split between chipmunks (*Tamias*) and ground squirrels early in marmotine evolution.

From our data it is clear that the MRCA of modern squirrels was arboreal. A reversal to a terrestrial habit may then have evolved once, leading to the "ground squirrel" clade, followed by reevolution of arboreality in the ancestor to some protoxerines, but both parsimony and likelihood approaches are equivocal on the last point (e.g., Fig. 4). A closer look at the functional anatomy of protoxerines (e.g., Thorington et al., 1997), could therefore be fruitful should those features be independently derived. Independent origins of terrestrial habit in xerines and marmotines cannot be rejected in likelihood reconstructions. The data are clear that gliding evolved once, from an arboreal ancestor.

4.4. Utility of RAG1 and c-myc for within-family questions

Our results show that the nuclear genes RAG1 and c-myc yield strongly supported phylogenetic results even for fairly recent divergences, such as those within genera. Two-thirds of all nodes are resolved with greater than 95% ML bootstrap support, and only two nodes have posterior probabilities less than 95%. The individual gene regions are also effective, although RAG1 provides more strongly supported results on average than the individual c-myc regions or the combined c-myc data. Most importantly, some nodes deep in the phylogeny and separated by very short branches are well supported, a region of the tree that may be difficult for mitochondrial genes to resolve given saturation at that level in rodents. For example, relationships among or even within murid rodent subfamilies are poorly resolved by mitochondrial protein-coding genes (Engel et al., 1998; Jansa et al., 1999; Smith and Patton, 1999). The alignment of nuclear genes at this level is trivial or relatively unambiguous; nuclear genes thus provide a significant advantage over mitochondrial ribosomal genes, which, although they have lower evolutionary rates than mitochondrial protein-coding genes, can be much more difficult to align (e.g., Mercer and Roth, 2003; excluded at least 170 ambiguously aligned 12S and 16S sites from their analyses). The genes we sequenced, particularly the faster-evolving 5′ region of RAG1, should be effective for similar phylogenetic questions in vertebrates.

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Appendix A

List of specimens sequenced. Abbreviations: Appalachian State University (ASU); Carnegie Museum of Natural History (CMNH); Field Museum of Natural History (FMNH); Institute of Developmental Biology, Russian Academy of Science (IDB); Institute of Zoology, Chinese Academy of Science, Kunming (IZ); University of Kansas (KU); Laboratory of Molecular Systematics, Smithsonian Institution (LMS); Louisiana State University Museum of Zoology (LSUMZ); Museum of Vertebrate Zoology, Berkeley (MVZ); Texas Cooperative Wildlife Collection (TCWC); University of Vermont (UV). Tissues collected by R.S. Hoffmann (RSH) are housed in the collections of the U.S. National Museum (USNM). The collector numbers EAR and LRH refer to uncataloged specimens housed at FMNH and collected by Eric Rickart and Lawrence Heaney, respectively. Collector numbers JMG refer to uncataloged specimens collected by Jeff Good and housed at the University of Idaho.

Dipodidae: *Alactaga sibirica*. USNM 449152. China, Qinghai Prov., Hainan State, Gonghe Co., Daotanghe, Hudong, E. shore, Qinghai Lake.

Muridae: *Batomys granti*. EAR 1822. Philippines, Luzon Is., Camarines Sur Prov., Mt. Isarog, 1750 m. *Beamys hindei*. FMNH 166652. Tanzania, Morogoro Region, Kilosa District, Ukaguru Mts., Mamiwa-Kisara Forest Reserve, 1 km E, 0.75 km S Mt Munyera. *Dendromus mesomelas*. FMNH 153931. Tanzania, Kilimanjaro Region, Same Dist., South Pare Mts, Chome Forest Reserve, 3 km E, 0.7 km N Mhero. *Deomys ferrugineus*. FMNH 149427. Zaire, Haute Zaire, Ituri, Epulu, 2 km W, Wpulu R, rt bank. *Microtus irene*. USNM 444173. China, Qinghai Prov., Yushu State, Nangqeng Co., Bei Zha Forestry Station. *Microtus pennsylvanicus*. USA, Massachusetts. *Mus musculus*. Lab colony, strain BALB/C. *Phyllotis xanthopygus*. LSUMZ M1440. Peru, Arequipa Dept., Ca 53 rd km E Arequipa. *Sigmodon hispidus*. TCWC AK9175.

Aplodontidae: *Aplodontia rufa*. MVZ 185228. USA, California.

Sciuridae:

Pteromyinae: *Glaucomys volans*. LSUMZ M5762. USA, W.Virginia, Kanawha Co. *Petaurista petaurista*. UV 593. Pakistan, NWFP, Mansehra Dist., Sharan, 2440 m.

Sciurinae:

Callosciurini: *Callosciurus erythraeus*. IZ 98627. China,Yunnan, Jingdong, Wuliangshan, Raomalu, 2230 m. *Callosciurus prevostii*. LSUMZ M6027. Zoo captive. *Dremomys pernyi*. MSWC 217. China, Sichuan, Xian, Shimian, 17 km SSE Dahonggu. *Exilisciurus concinnus*. FMNH 166485. Philippines, Mindanao Is., Bukidnon Prov., Sumilao Municipality, 10.6 km S, 2.8 km W Sumilao. FMNH 167383. Philippines, Mindanao Is., Bukidnon Prov., Sumilao Municipality. *Sundasciurus philippinensis*. LRH 3136, *Tamiops swinhoei*. IZ 98626. China, Yunnan, Jingdong, Wuliangshan, Raomalu, 2100 m.

Funambulini: *Funisciurus carruthersi*. FMNH 144318. Uganda, Western, Kasese Dist., Rwenzori Mts., Mubuku R., L bank, Ruboni. *Paraxerus cepapi*. CMNH 105770. South Africa. Transvaal Prov., Farm Greefswald 37, Shashi/Limpopo Confluence, 67 km W Messina, 22°13′S, 29°22′E. *Paraxerus ochraceus*. FMNH 151220. Tanzania, Kilimanjaro Region, Same Dist., South Pare Mts., Chome Forest Reserve, 5.5 km S Bombo, near Kanza Village. *Paraxerus vexillarius*. FMNH 147237. Tanzania, Tanga Region, Korogwe Dist., West Usambara Mts., 12.5 km NW Korogwe, Ambangulu Tea Estate.

Microsciurini: *Microsciurus flaviventer*. LSUMZ M1567. Peru, Loreto Dept., Quebrada Orán, ca. 5 km N Rio Amazona, 85 km NE Iquitos.

Marmotini: *Marmota marmota*. LMS M00018. Italy, Modena, Monte Cimone. *Marmota monax*. ASU 16756. USA, North Carolina, no exact locality. 35°30′N 82°30′W. *Marmota sibirica*. IDB 9324. Russia, Chitinsk Obl.Ononsk rai., Pobeda; 57°30′N, 116°E. *Spermophilus lateralis*. KU 141076. USA. Colorado, Boulder. *Tamias amoenus*. JMG030. USA, Idaho, Clearwater Co., Orofino, 46°29′N 116°18′W. *Tamias ruficaudus*. JMG001. USA, Idaho, Boundary Co., 17 mi N Sandpoint. *Tamias sibiricus*. IZ 98714. China, Inner Mongolia, precise locality unknown.

Protexerini: *Heliosciurus ruwenzorii*. FMNH 149004. Burundi, Cibitoke Prov., Bukinanyana Commune, Kibira Nat. Pk., Ndora Zone, Giserama Col, Gatare Stream, 1.9 km N, 1.1 km E Kiru. *Heliosciurus undulatus*. FMNH 151215. Tanzania, Kilimanjaro Region, Same Dist., South Pare Mts., Chome Forest Reserve, 7 km S Bombo. *Protoxerus stangeri*. FMNH 149005. Burundi, Cibitoke Prov., Bukinanyana Commune, Kibira Nat. Pk., Ndora Zone, Giserama Col, Gatare Stream, 1.9 km N, 1.1 km E Kiru.

Ratufini: *Ratufa bicolor*. LSUMZ M2884. Houston Zoo, Houston, Texas. *Ratufa sp*. LSUMZ M3476. Houston Zoo, Houston, Texas.

Sciurini: *Sciurillus pusillus*. LSUMZ M1561. Peru, Loreto Dept., Quebrada Orán, ca. 5 km N Rio Amazona, 85 km NE Iquitos. *Sciurus carolinensis*. USNM 588785. USA, Virgina, Fairfax Co., Oakton. *Sciurus ignitus*. LSUMZ M4471. Bolivia, La Paz Dept., Prov. B Saavedra, 83 km by road E Charazani, Cerro Asunta Pata. *Sciurus stramineus*. LSUMZ M936. Peru, Piura Dept., Parinas, 7 km N, 15 km E Talara.

Tamiasciurini: *Sciurotamias davidianus*. USNM 588782. China, Shan mountains, N of Beijing. *Tamiasciurus hudsonicus*. KU 140379. Canada, Yukon Territory, Coyote Creek, 55 km. N, Johnson Crossing.

Xerini: *Spermophilopsis leptodactylus*. IDB 24044. Turkmenistan, Karakumi, Seidi. *Xerus inauris*. USNM 588784. San Diego Zoo, original locality, South Africa. *Xerus rutilus*. CMNH 102306. Kenya, Eastern Prov., Machakos Dist., 3 km E Kathekani, 700 m.

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