Journal of Mammalian Evolution, Vol. 4, No. 4, 1997

DNA–DNA Hybridization Evidence for the Recent Origin of Marmots and Ground Squirrels (Rodentia: Sciuridae)

Olivier Giboulet,[1,3] Pascale Chevret,[2] Raymond Ramousse,[1] and François Catzeflis[2]

A molecular analysis, based on DNA/DNA hybridization experiments, examined seven species of the sciurid tribe Marmotini, in order to evaluate their evolutionary relationships. The branching pattern obtained from a neighbor-joining analysis and least-squares methods indicates that spermophiles and marmots are closely related. This result is in agreement with previous studies based on biochemical data which have suggested a *Miospermophilus* origin of *Marmota* (like *Spermophilus*), instead of the *Protospermophilus* origin usually proposed by paleontology. Using a molecular time scale calibrated by the fossil record of *Sciurus* (used here as an outgroup), we estimate the marmot/ground squirrel divergence at 6 My, at the same time as the major lineages of ground squirrels diverged from each other. Consequently, the species of *Marmota* appear as one of the numerous specialized forms derived from the explosive radiation of the *Spermophilus* lineage in the late Miocene.

KEY WORDS: molecular phylogeny; DNA/DNA hybridization; Sciuridae, ground squirrels; *Marmota*.

INTRODUCTION

The tribe Marmotini (Sciuridae: Rodentia) encompasses the ground squirrels and marmot species of the Holarctic region, where they are present from the Arctic tundra to the tropical desert. This taxon is traditionally subdivided into five genera (Moore, 1959): *Spermophilus*, *Ammospermophilus*, *Spermophilopsis*, *Marmota*, and *Cynomys*. Ground squirrels are common model organisms for studies in physiology, circannual rhythms (hibernation), medicine (hepatitus virus), and ethology; numerous species are, or have been, the subject of research (Murie and Michener, 1984; Carey *et al.*, 1993; Bibikov, 1996a; Le Berre *et al.*, 1996). The recent history of this group, its expansion during the last glaciations, and the relationships between the different species from both parts of the Bering Strait have been addressed by several studies (Vorontsov *et al.*, 1969; Vorontsov and Lyapunova, 1970, 1984; Hoffmann and Nadler, 1968, Hoffmann *et al.*, 1979; Nadler and Harris, 1967; Nadler *et al.*, 1971a,b, 1982, 1984; Rausch and Rausch, 1971).

[1] Laboratoire de Socioécologie & Conservation (J.E. 1942), UCB Lyon I, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France.
[2] Institut des Sciences de l'Evolution (UMR 5554 CNRS), Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.
[3] To whom correspondence should be addressed. e-mail: giboulet-o@cons-dev.univ-lyon1.fr

1064-7554/97/1200-0271$12.50/0

However, the origin of this group and the relationships between its constituent taxa are still unclear. It is usually assumed that the long-clawed ground squirrel (*Spermophilopsis leptodactylus*) is a particular species of spermophile and that it is very closely related to the genus *Spermophilus* and, also, that prairie dogs form a recent sister group of ground squirrels (Hollister, 1916; Bryant, 1945; Black, 1963; Hafner, 1984). Ammospermophiles are considered to present some ancestral characters and to have originated in the Miocene from *Miospermophilus* (James, 1963, cited by Hafner, 1984), but no fossil records are available before the Clarendonian (early Pliocene) at this time. The relative position of marmots in the tribe is more dubious. For a long time, paleontologists have interpreted *Protospermophilus*, an early fossil form of the Oligocene, as the ancestor of marmots, whereas ground squirrels were thought to derive from the fossil form *Miospermophilus*. Consequently, the two main extant genera (*Marmota* and *Spermophilus*) were considered to form two distinct monophyletic lineages in the tribe (Black, 1963, 1972; Mein, 1992).

Several biochemical comparative studies have proposed the hypothesis that *Miospermophilus* was the common ancestor of both *Marmota* and *Spermophilus* (Ellis and Maxson, 1980; Hight *et al.*, 1974; Hafner, 1984). More recently, Thomas and Martin (1993) compared complete mitochondrial cytochrome *b* sequences in four species of *Spermophilus* and one of *Marmota*. Their study yielded only a tentative phylogeny, due to saturation problems encountered with this fast-evolving gene. Yet Thomas and Martin (1993) could confirm a recent origin of marmots, with regard to more ancient dichotomies in the squirrel family.

To test the various biochemical and morphological hypotheses dealing with marmot and ground squirrel relationships, we used another molecular method in systematics, namely, nonrepeated DNA–DNA hybridization. DNA-DNA hybridization has proven valuable for establishing the relationships between closely related taxa of mammals, such as species and genera within a family (Kirsch *et al.*, 1993; Chevret *et al.*, 1993, 1994). Our comparative experiments involved four species of *Marmota*, two of *Spermophilus*, one *Ammospermophilus*, and one *Tamias*. The tribe Tamiini is considered to be the sister-group of Marmotini (Ellis and Maxson, 1980; Hight *et al.*, 1974; Mein, 1992). The outgroup taxon was represented by a tree squirrel (*Sciurus vulgaris*) and a sun squirrel (*Heliosciurus gambianus*).

MATERIALS AND METHODS

DNA/DNA Hybridization Experiments

Tissue samples come from the Collection of Preserved Mammalian Tissues held at the Institut des Sciences de l'Evolution, Montpellier (Catzeflis, 1991). Table I lists the 10 taxa involved in this study, their geographic origins, and collectors' names. DNAs used for single-copy nuclear DNA (scnDNA) hybridization experiments were extracted from 95% ethanol-preserved tissues, purified, and sheared into fragments with an average size of 500 base pairs (bp) (range, 200–1000 bp). The scnDNA fractions were isolated by removing on hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) columns, the highly repeated sequences that reassociated with a C_0t value of 1000 $M \cdot$ sec (C_0t is the product of DNA concentration by the time of reassociation) in 0.48 M phosphate buffer at 50°C. These nonrepeated DNA fractions were chemically labeled with ^{125}I (Werman *et al.*, 1990; Sibley and Ahlquist, 1990).

Table I. List of Taxa Used in this Study with Their Geographic Origin and Collectors' Name

Species	Geographic origin	Collector
Ammospermophilus leucurus[a]	Mexico (Baja, California)	C. Baudoin
Heliosciurus gambianus	Senegal	J. M. Duplantier
Marmota bobac	Russia (Oural)	M. Le Berre
Marmota camtschatica[a]	Russia (Yakutia)	R. Ramousse
Marmota marmota[a]	France (Savoie)	M. C. Bel
Marmota monax[a]	Canada (Yukon)	F. Catzeflis
Sciurus vulgaris[a]	Switzerland (Geneve)	P. Clerc
Spermophilus major[a]	Russia (Oural)	G. Olenev
Spermophilus parryii	Canada (Yukon)	R. S. Hoffmann
Tamias striatus	Canada (Nova Scotia)	V. Volobouev

[a] Radioactively labeled species.

The procedures used here are similar to those published by Sibley and Ahlquist (1983, 1990), Catzeflis (1990), Chevret *et al.* (1993, 1994), Springer *et al.* (1990), and Veron and Catzeflis (1993). DNA/DNA hybrids, formed by one part of labeled scnDNA (tracer) and 1000 parts of nonlabeled total DNA (driver), were permitted to reassociate, after heat denaturation, to an equivalent C_0t of 16,000 at 60°C in 0.48 *M* phosphate buffer. Thermal elutions were started at 55°C, with 2.5°C increments up to 95°C, and the raw data are the radioactive counts eluted at each fractionation temperature.

Calculation of Thermal Stability Indices and Genetic Distances

Several statistics can be calculated to estimate the difference between the thermal elution curves of the homoduplex (tracer and driver of the same species) and those of the heteroduplex (tracer and driver of different species) hybrids. These distance indices are ΔT_m, ΔMode, ΔNPH, and ΔT50H, and they have been described and discussed in detail by Sheldon and Bledsoe (1989), Catzeflis (1990), Sibley and Ahlquist (1990), and Werman *et al.* (1990). In this paper Mode and T_m were used, because they are less variable than the other statistics (Kirsch *et al.*, 1990, 1993). Briefly, the Mode index is the highest point of the melting curve of radioactive counts versus temperature, and the T_m value is the temperature at which 50% of the hybrid DNA has been dissociated between 62.5 and 95°C.

Data Treatment and Phylogeny Reconstruction

The raw data (ΔMode and ΔT_m) were interpreted using three approaches. First, an almost-complete distance matrix was analyzed, which involved six radioactively labeled taxa: *Ammospermophilus leucurus, Marmota camtschatica, M. marmota, M. monax, Spermophilus major*, and *Sciurus vulgaris*, which is the outgroup taxon. Due to tissue and DNA availability, one comparison could not be done, and its corresponding cell in the complete Mode and T_m matrix was filled by the average value of the reciprocal comparisons. A bootstrap procedure was applied 1000 times to these two (Mode, T_m) distance matrices, as described by Krajewski and Dickerman (1990) and Sheldon *et al.* (1992). The bootstrap is performed on uncorrected distance values (ΔMode and ΔT_m). It samples, with replacement, the replicate measures in each cell. For each of the 1000

bootstrapping procedures, a pseudo-replicate matrix was constructed by recalculating the average distance for each cell and corrected for nonreciprocity by the symmetrization procedure described by Sarich and Cronin (1976). Each of these 1000 symmetrized pseudo-replicate matrices was treated by the FITCH program [Version 3.3; options power = 0, global rearrangements, 10 random inputs of order of species, from the PHYLIP package (Felsenstein, 1990)] to estimate its best-fit tree. The 1000 resulting topologies were recorded and treated by the CONSENSE program in PHYLIP, Version 3.4 (Felsenstein, 1990), to get a majority-rule consensus tree. Each node of this consensus tree is characterized by the frequency at which the dichotomy of interest has been found among the 1000 pseudo-replicate trees.

Second, we used the approach developed by Lapointe and Kirsch (1995) for completing a lacunose matrix to get a complete 10 × 10 matrix with the ΔMode values. The matrix was first symmetrized following the procedure of Sarich and Cronin (1976) applied to the common known row and column values. The different lacunose cells were completed by reciprocal values for hemilacunose cells and using the ultrametric procedure when the two values were missing (Lapointe and Kirsch, 1995). The FITCH program [Version 3.5c; options power = 0, global rearrangements (Felsenstein, 1993)] and the neighbor joining (NJ) program (Saitou and Nei, 1987) were then used to analyze this complete 10 × 10 matrix and derive a branching pattern. The original matrix presents 40% of lacunose cells, among which approximately one-third (37.5%) are holo-lacunary cells (as defined by Lapointe and Kirsch, 1995).

Finally, the departure from rate uniformity was estimated through the relative rate test, by measuring the $|AC - BC|/(AC + BC)$ percentage value in the various combinations of three taxa (A, B, C), where C is the outgroup for A and B. Then the symmetrized ΔMode values were transformed into percentage base pair mismatch (%bpm) estimates by the relation of 1°C ΔMode = 1% bpm (Brownell, 1983). These estimates were finally transformed into percentage nucleotide substitutions (% nucl. subst.) by the Jukes and Cantor formula (1969), which corrects for multiple substitutions. A KITSCH procedure [Version 3.5 c; options P = 0, global rearrangements (Felsenstein, 1993)] was applied to this new 10 × 10 matrix, to derive a tree with the hypothesis of rate equality. The percentage nucleotide substitution values were calibrated against the geological time provided by the fossil record. In our study the tree squirrel/ground squirrel dichotomy was estimated at 29–30 Ma (Black, 1963; Thomas and Martin, 1993).

RESULTS

6 × 6 Complete Matrix

The results (average values and standard deviations for n hybrids), expressed as ΔMode and ΔT_m, are presented in Tables II and III. The experimental variability is indicated by the comparable repeatability of the ΔMode and ΔT_m values (average SD of 0.66 and 0.62, respectively). The deviation from perfect symmetry can be estimated indirectly by the correcting factors provided by the Sarich and Cronin (1976) procedure. As shown in Tables II and III, these correcting factors range from 0.939 to 1.009 for Mode and from 0.998 to 1.001 for T_m. The percentage of nonreciprocity (following the formula of Champion *et al.*, 1974) was, after symmetrization, 2.88% for the ΔMode matrix and 3.72% for the ΔT_m matrix.

Table II. The 6 × 6 Symmetrized Matrix of Average ΔMode Values (°C)[a]

	Tracer					
Driver	*Sciurus vulgaris*	*Ammo-spermophilus leucurus*	*Marmota monax*	*Marmota marmota*	*Marmota camtschatica*	*Spermophilus major*
Sciurus vulgaris		14.86 ± 1.25 (n = 3)	13.56 ± 0.80 (n = 4)	15.37 ± 4.20 (n = 3)	13.64 ± 0.40 (n = 2)	14.72 ± 0 .50 (n = 3)
Ammospermophilus leucurus	14.62 ± 0.40 (n = 4)		4.40 ± 0.11 (n = 3)	4.57 ± 0.66 (n = 4)	4.07 (n = 1)	4.70 ± 0.10 (n = 3)
Marmota monax	13.99 ± 0.80 (n = 3)	4.40 ± 0.14 (n = 3)		1.14 ± 1.00 (n = 3)	0.59 ± 0.59 (n = 2)	2.97 ± 0.3 (n = 3)
Marmota marmota	15.03 ± 0.66 (n = 4)	4.57 ± 0.66 (n = 4)	1.433 ± 0.12 (n = 4)		0.59 (n = 1)	2.90 ± 0.80 (n = 3)
Marmota camtschatica	13.62 ± 0.40 (n = 2)	4.40 (n = 1)	0.47 (n = 1)	0.59 (n = 1)		2.63 ± 0.10 (n = 2)
Spermophilus major	14.72 ± 0.50 (n = 3)	4.14 ± 0.30 (n = 3)	3.24 ± 0.29 (n = 4)	2.90 ± 0.80 (n = 3)	2.85 (n = 1)	
Correcting factor	1.004	1.009	0.992	0.999	0.939	0.990

[a] For each average value, the standard deviation and number of replicates (n) are given. The percentage of nonreciprocity after symmetrization was 2.88.

Table III. The 6 × 6 Symmetrized Matrix of Average ΔT_m Values (°C)[a]

	Tracer					
Driver	*Sciurus vulgaris*	*Ammo-spermophilus leucurus*	*Marmota monax*	*Marmota marmota*	*Marmota camtschatica*	*Spermophilus major*
Sciurus vulgaris		10.95 ± 0.28 (n = 3)	11.2 ± 0.40 (n = 3)	11.68 ± 3.00 (n = 2)	11.25 (n = 1)	11.64 ± 0.80 (n = 3)
Ammospermophilus leucurus	11.24 ± 1.30 (n = 4)		4.54 ± 0.10 (n = 3)	4.44 ± 0.90 (n = 4)	3.05 (n = 1)	4.67 ± 0.20 (n = 3)
Marmota monax	11.05 ± 1.00 (n = 2)	4.69 ± 0.30 (n = 3)		2.27 ± 1.50 (n = 3)	1.14 (n = 1)	3.01 ± 0.30 (n = 3)
Marmota marmota	11.43 ± 0.60 (n = 4)	4.44 ± 0.90 (n = 4)	2.32 ± 0.30 (n = 4)		0.95 (n = 1)	3.61 ± 0.06 (n = 2)
Marmota camtschatica	11.28 ± 0.20 (n = 2)	3.67 (n = 1)	0.87 (n = 1)	0.71 ± 0.40 (n = 2)		3.08 ± 0.20 (n = 3)
Spermophilus major	11.64 ± 0.80 (n = 3)	4.20 ± 0.40 (n = 3)	3.25 ± 0.20 (n = 4)	3.69 ± 0.50 (n = 3)	3.24 ± 0.30 (n = 2)	
Correcting factor	1.000	0.999	0.999	0.998	0.999	1.001

[a] For each average value the standard deviation and number of replicates (n) are given. The percentage of nonreciprocity after symmetrization was 3.72.

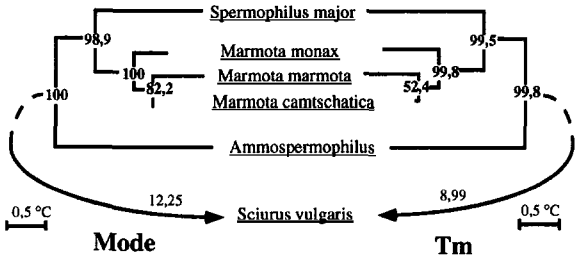

Fig. 1. Consensus trees resulting from bootstrap procedure on ΔMode and ΔT_m values. Numbers at nodes are bootstrap percentages of 1000 pseudo-replicates and values along ancestral segments are branch lengths as degrees.

The consensus trees (Fig. 1), resulting from the bootstrap procedure applied to the data in Tables II and III, present the same topology for both distance indices (Mode, T_m). The three marmot species are always more closely related to each other (with a bootstrap support of more than 99%) than to *Spermophilus major*, which is their sister-group in more than 98% of the topologies. Then come *Ammospermophilus leucurus* and *Sciurus vulgaris* (the outgroup), which is the most distant taxon (12.25°C of ΔMode). The lengths of the different segments of the trees presented in Fig. 1 are average values calculated from a random sample of 20 replicate trees.

10 × 10 Complete Matrix

We completed the ΔMode matrix to get a complete 10 × 10 matrix following the procedure of Lapointe and Kirsch (1995), the result of which is presented in Table IV. No resampling was done on this matrix because about two-fifths of the cells were represented by just one measurement. The general topology of the tree obtained with the FITCH procedure (Fig. 2) and the NJ procedure is the same as that derived from the previous bootstrap procedure on six taxa and, again, indicates that marmots and ground squirrels are recent monophyletic sister taxa (separated by only 3°C ΔMode), whereas *Ammospermophilus* seems to have diverged earlier. These three genera appear to constitute a monophyletic group. The sister-group of the Marmotini tribe appears to be a Tamiini rather than a Sciurini.

All branch lengths between the four species of *Marmota* are smaller than 0.5°C ΔMode, that is, at most 1% nucleotide substitutions. These values are very small compared to the available standard deviation of measures derived from DNA/DNA hybridization experiments for the same hybrids (see Tables II and III), and the branching order of the four species of marmots obtained differs with regard to the different procedures used. Thus, it might well appear that the radiation of these species of *Marmota* is such a recent event that the scnDNA–DNA hybridization approach is not adapted to decipher the relationships between these almost genetically identical taxa.

Temporal Calibration

The FITCH and NJ procedures do not make the assumption of rate equality between the taxa, and Figs. 1 and 2 illustrate that there are differences in rates of DNA change

Table IV. The 10 × 10 Matrix of Symmetrized Average ΔMode Values (°C), Completed by the Procedure of Lapointe and Kirsch (1995)[a]

	Tracer									
Driver	*Sciurus vulgaris*	*Ammo-spermophilus leucurus*	*Marmota monax*	*Marmota marmota*	*Marmota camtschatica*	*Spermophilus major*	*Marmota bobac*	*Spermophilus parryii*	*Tamias striatus*	*Heliosciurus gambianus*
Sciurus vulgaris		14.86	13.56	15.37	13.64	14.72	**13.95**	**15.76**	**13.6**	**14.44**
Ammospermophilus leucurus	14.62		4.40	4.57	4.07	4.70	**3.93**	**4.93**	**9.13**	**12.16**
Marmota monax	13.99	4.40		1.14	0.59	2.97	**0.402**	**4.15**	**9.44**	**12.00**
Marmota marmota	15.03	4.57	1.433		0.593	2.90	**0.33**	**2.88**	**9.42**	**11.06**
Marmota camtschatica	13.62	4.40	0.471	0.594		2.63	**0.413**	**2.70**	**9.28**	**14.80**
Spermophilus major	14.72	4.14	3.24	2.90	2.85		**2.67**	**2.70**	**8.93**	**12.17**
Marmota bobac	13.95	3.93	0.402	0.33	0.413	2.67		**2.70**	**9.13**	**11.06**
Spermophilus parryii	15.76	4.93	4.15	2.88	2.70	2.70	**2.70**		**9.13**	**11.06**
Tamias striatus	13.60	9.13	9.44	9.42	9.28	8.95	**9.13**	**9.13**		**11.06**
Heliosciurus gambianus	14.44	12.16	12.00	11.06	11.80	12.17	**11.06**	**11.06**	**11.06**	

[a]Values corresponding to hemilacunary cells are in boldface, and those to hololacunary cells are in boldface and underlined. The percentage of nonreciprocity after symmetrization was 2.02.

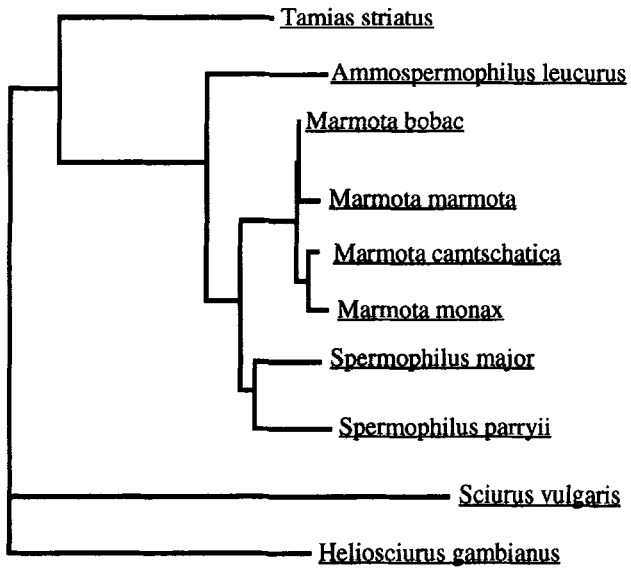

Fig. 2. Tree derived from the 10 × 10 complete matrix of ΔMode values, reconstructed with the Fitch procedure (no hypothesis of rate constancy).

between the different branches of the trees. That these differences are small is suggested by the results of the relative rate test: the mean departure from rate uniformity is 3.99%, with a standard error of ±4.33. Thus, we believe it acceptable to apply a KITSCH procedure (that is, with the hypothesis of a molecular clock) to build a tree on which we could estimate the geological age of the divergences between the different taxa. To calibrate the molecular time scale, 30 My was used for the age of the tree sciurid/ground sciurid divergence (Black, 1963; Thomas and Martin, 1993).

Figure 3 illustrates the result of this procedure and Table V indicates the age of several nodes. No discrepancy in the branching pattern was observed between the KITSCH tree and the previous trees. The Tamiini split is estimated at about 18 My. Within Marmotini, *Ammospermophilus* diverged first at about 8.5 My, while the true ground squirrels and the marmots separated later, at about 6 My. It appears that there was an explosive period of diversification around 1 My in *Marmota* s.s., which gave rise to the different species of marmots. The relationships within this group still cannot be discriminated.

DISCUSSION

The existence of the separate genus *Tamias* [proposed by Black (1963) and based on fossil material] is validated by our molecular data. A closer relationship between *Tamias* and Marmotini than between *Tamias* and Sciurini has already been suggested by biochemical approaches (Ellis and Maxson, 1980; Hight *et al.*, 1974; Hafner, 1984);

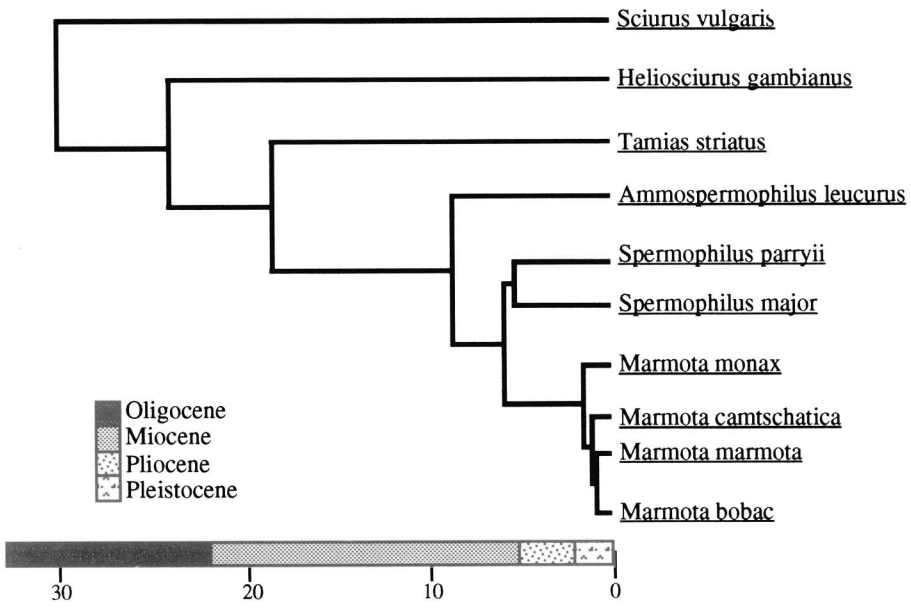

Fig. 3. Tree reconstructed with the hypothesis of rate equality (KITSCH procedure) from the modal values of the 10 × 10 complete matrix. ΔMode values were transformed into percentage nucleotide substitutions (see text for procedure). The time scale was calibrated by taking 30 My for the tree squirrel/ground squirrel divergence.

Table V. Percentages of Nucleotide Substitutions and Estimated Divergence Times Between Several Taxa Represented in Fig. 3[a]

Dichotomy	% nucl. subst.	Age (My)
Sciurini/Tamiini & Marmotini	16.02	30
Tamiini/Marmotini	9.83	18.4
Ammospermophilus/ Spermophilus & Marmota	4.55	8.5
Spermophilus/Marmota	3.04	5.7
Within *Marmota*	0.74	1.4

[a] The molecular clock concept was calibrated by the tree squirrel/ground squirrel split at approximately 30 million years (My).

this result is retrieved also through DNA hybridization experiments. With regard to the dating of the Tamiini speciation, biochemical and paleontological approaches suggest 22 My (early Miocene) (Ellis and Maxson, 1980), whereas our molecular approach yields a date of 18 My (middle Miocene), which is a little younger. As distance values in our 10 × 10 matrix were not complete for *Tamias striatus*, we should interpret this preliminary result cautiously.

As for other lines of evidence, such as fossil material (Black, 1963), dental characters (Bryant, 1945), chromosomal morphology (Nadler and Sutton, 1962), and bio-

chemical analysis (Hight *et al.*, 1974; Ellis and Maxson, 1980; Hafner, 1984), the DNA/DNA hybridization data also indicate that *Ammospermophilus* might have an older origin than the other Nearctic ground squirrels. The absence of fossil records before the Clarendonian for this genus is more and more questionable. We hope that a fossil Miocene ancestor for *Ammospermophilus* will soon be discovered, as was recently the case for *Cynomys* (Korth, 1996). The age of 8.5 My for its speciation, proposed by our molecular approach, is somewhat younger than what has been calculated in other molecular studies [10–15 My/middle Miocene period (Ellis and Maxson, 1980; Hight *et al.*, 1974)], but the discrepancy is still acceptable. Thus, *Ammospermophilus* seems to have originated precociously, which could explain why this genus appears to have shared a long history of association with North American deserts (Hafner, 1981). It is probable that this taxon specialized to become an arid-adapted genus as the climate of North America became drier during the Miocene period. For example, the uplift of the Rocky Mountains and Sierra Nevada was accompanied by the transformation of the Mojave-Sonora and Great Basin areas into incipient deserts (Axelrod, 1950, 1979).

Our study indicates that *Marmota* and *Spermophilus* are recent sister-groups. This is in agreement with the hypothesis of a *Miospermophilus* origin for the genus *Marmota*, proposed by Hafner (1984) on the basis of biochemical data, and by Thomas and Martin (1993) from gene sequence data. Thus the alleged *Protospermophilus* origin of marmots (Black, 1963, 1972; Mein, 1992) seems definitively less plausible. In fact Black (1963) himself remarked that the first fossils of true marmots appear only in the Clarendonian (9–11.5 My). This means that all the marmot-like fossil forms (probably derived from *Protospermophilus*) observed before this period would have been only some large ground squirrels appearing in the early Miocene. Consequently, these now-extinct taxa could have disappeared as the result of direct competition with the more specialized true ground squirrels. *Protospermophilus* presents some granivorous dental characters (Black, 1963), and during the Miocene its preferred woodland habitat became reduced considerably, giving way to the open land, inhabited by herbivorous specialists such as *Spermophilus*.

Our molecular results estimate the separation between *Spermophilus* and *Marmota* at about 6 My (late Miocene). This is consistent with the idea of a *Marmota/Spermophilus* divergence at the same time that the major lineages of ground squirrels diverged from each other, in the late Miocene (Hafner, 1984; Smith and Coss, 1984). This hypothesis is further supported by other comparative molecular approaches, such as mitochondrial cytochrome *b* sequences (Thomas and Martin, 1993), hemoglobin amino acid sequences (Soskic *et al.*, 1986; Sgouros *et al.*, 1986), and immunological distances (Maxson *et al.*, 1981), and recently by paleontological data, with the discovery of a new prairie dog fossil genus (Korth, 1996). The explosive radiation of the *Spermophilus* lineages during this period gave rise to numerous specialized terrestrial forms: *Cynomys* and *Xerospermophilus* (warm desert), *Poliocitellus* (tall grass), *Ictydomys* (short grass), *Otospermophilus* (xerophyllous scrub communities), *Callospermophilus* (pine forests), and *Spermophilus* (cold grassland). Marmots would be one of these numerous specialized forms, adapted to the cold and periglacial environment (Bibikov and Rumiantsev, 1993; Bibikov, 1996b). The timing of their diversification is estimated by our analysis at 1 My, during the Pleistocene, which corresponds to the glaciation periods at the beginning of the Quaternary cited by Chaline and Mein (1979) and Mein (1992). Zimina and Gerasimov (1973) considered that the expansion of the genus *Marmota* on the Eurasiatic

continent was the result of their adaptation to the periglacial areas. As the ice fields spread, ancestral marmots would have passed from North America to East Siberia, by the Bering Strait, and then on to central Asia and western Europe.

The branching order derived from this molecular approach confirms a recent appearance of the genus *Marmota* and suggests its origin from *Miospermophilus*, and it establishes the relationships among the genera *Marmota*, *Spermophilus*, and *Ammospermophilus*. Nevertheless, the scnDNA–DNA hybridization approach does not appear to be sensitive enough to decipher the relationships between the species within the genus *Marmota*, and further experiments on fast-evolving genes are needed to investigate that question.

ACKNOWLEDGMENTS

We thank M. Le Berre for the conception of this study. This work would not have been possible without the generous help of the people who collected, identified, and preserved tissue samples of sciurids in the field: C. Baudoin, M. C. Bel, M. Le Berre, P. Clerc, J. M. Duplantier, R. S. Hoffmann, G. Olenev, and V. Volobouev. This work was supported by grants from the Service Commun de Biosystématique de Montpellier and from the Centre National de la Recherche Scientifique. This is publication No. 97–135 of the Institut des Sciences de l'Evolution (UMR 5554, CNRS).

LITERATURE CITED

Axelrod, D. I. (1950). The evolution of desert vegetation in western North America. *Carnegie Inst. Wash. Publ.* **590:** 215–306.

Axelrod, D. I. (1979). Age and origin of Sonoran desert vegetation. *Occas. Papers Calif. Acad. Sci.* **132:** 1–74.

Bibikov, D. I. (1996a). *Marmots of the World*, New Brehm Book, Heidelberg.

Bibikov, D. I. (1996b). On the size of the marmots (*Marmota*), and their annual cycle and adaptation to "short summer." In: *Biodiversity in Marmots*, M. Le Berre, R. Ramousse, and L. Le Guelte, eds., pp. 141–148, International Marmot Network, Moscow–Lyon.

Bibikov, D. I., and Rumiantsev, V. Yu. (1993). Some adaptations of marmots as the result of historical modifications of their habitat conditions. In: *Abst. V Int. Conf. Marmots CIS-states*, D. I. Bibikov, A. A. Nikol'skii, V. Yu. Rumiantsev, and V. V. Suntsov, eds., pp. 44–45, Theriological Society (Russian Academy of Science), Commission on Marmots Investigation, Moscow.

Black, C. C. (1963). A review of the North American Tertiary Sciuridae. *Bull. Mus. Comp. Zool.* **130:** 109–248.

Black, C. C. (1972). Holarctic evolution and dispersal of squirrels (Rodentia: Sciuridae). In: *Evolutionary Biology*, Vol. 6, T. Dobzhansky, M. K. Hecht, and W. C. Steere, eds., pp. 305–322, Plenum Press, New York.

Brownell, E. (1983). DNA/DNA hybridization studies of muroid rodents: Symmetry and rate of molecular evolution. *Evolution* **37:** 1034–1051.

Bryant, M. D. (1945). Phylogeny of Nearctic Sciuridae. *Am. Midl. Nat.* **33:** 257–390.

Carey, C., Florant, G., Wunder, B. A., and Horwitz, B. (1993). *Life in the Cold*, Westview Press, Boulder, San Francisco, Oxford.

Catzeflis, F. M. (1990). DNA hybridization as a guide to phylogenies: Raw data in muroid rodents. In: *Evolution of Subterranean Mammals at the Organismal and Molecular Levels*, E. Nevo and O. A. Reig, eds., pp. 317–345, Wiley-Liss, New York.

Catzeflis, F. M. (1991). Animal tissue collections for molecular genetics and systematics. *Trends. Ecol. Evol.* **6:** 168.

Chaline, J., and Mein, P. (1979). *Les Rongeurs et l'Evolution*, Doin, Paris.

Champion, A. B., Prager, E. M., Wachter, D., and Wilson, A. C. (1974). Microcomplement fixation. In: *Biochemical and Immunological Taxonomy of Animals*, C. A. Wright, ed., pp. 397–416, Academic Press, London.

Chevret, P., Denys, C., Jaeger, J. J., Michaux, J., and Catzeflis, F. (1993). Molecular and fossil aspects of the tempo and mode of evolution in *Otomys* (Otomyinae: Muridae: Mammalia). *Biochem. Syst. Ecol.* **21:** 123–131.

Chevret, P., Granjon, L., Duplantier, J.-M., Denys, C., and Catzeflis, F. M. (1994). Molecular phylogeny of the *Praomys* complex (Rodentia: Muridae). *Zool. J. Linn. Soc. London* **112:** 425–442.

Ellis, S. L., and Maxson, L. R. (1980). Albumin evolution within New World squirrels. *Am. Mid. Nat.* **104:** 57–62.

Felsenstein, J. (1990). *PHYLIP, Phylogeny Inference Package, Version 3.3*, University of Washington, Seattle.

Felsenstein, J. (1993). *PHYLIP, Phylogeny Inference Package, Version 3.5 c*, University of Washington, Seattle.

Hafner, D. J. (1981). *Evolution and Historical Zoogeography of Antelope Ground Squirrels, Genus Ammospermophilus (Rodentia: Sciuridae)*, Ph.D. dissertation, University of New Mexico, Albuquerque.

Hafner, D. J. (1984). Evolutionary relationships of the Neartic Sciuridae. In: *The Biology of Ground-Dwelling Squirrels*, J. O. Murie and G. R. Michener, eds., pp. 3–23, University of Nebraska Press, Lincoln and London.

Hight, M. E., Goodman, M., and Prychodko, W. (1974). Immunological studies of the Sciuridae. *Syst. Zool.* **12:** 12–25.

Hoffmann, R. S., and Nadler, C. F. (1968). Chromosomes and systematics of North American species of the genus *Marmota* (Rodentia: Sciuridae). *Experientia* **24:** 740–742.

Hoffmann, R. S., Koeppl, J. W., and Nadler, C. F. (1979). The relationships of the amphiberingian marmots (Mammalia, Sciuridae). *Occas. Pap. Mus. Nat. Hist. Kans.* **83:** 1–56.

Hollister, N. (1916). A systematic account of the prairie-dogs. *North Am. Fauna* **40:** 1–37.

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. In: *Mammalian Protein Metabolism*, H. N. Munro, ed., pp. 21–123, Academic Press, Orlando, FL.

Kirsch, J. A. W., Krajewski, C., Springer, M. S., and Archer, M. (1990). DNA-DNA hybridization studies of carnivorous marsupials II. Relationships among dasyurids (Marsupialia: Dasyuridae). *Aust. J. Zool.* **38:** 673–696.

Kirsch, J. A. W., Bleiweiss, R. E., Dickerman, A. W., and Reig, O. A. (1993). DNA/DNA hybridization studies of carnivorous Marsupials III. Relationships among species of *Didelphis* (Didelphidae). *J. Mammal. Evol.* **1:** 75–79.

Korth, W. W. (1996). A new genus of prairie dog (Sciuridae, Rodentia) from the Miocene (Barstovian of Montana and Clarendonian of Nebraska) and the classification of Nearctic ground squirrels (Marmotini). *Trans. Nebr. Acad. Sci.* **23:** 109–113.

Krajewski, C., and Dickerman, A. W. (1990). Bootstrap analysis of phylogenetic trees derived from DNA hybridization distances. *Syst. Zool.* **39:** 383–390.

Lapointe, F. J., and Kirsch, J. A. W. (1995). Estimating phylogenies from lacunose distance matrices, with special references to DNA hybridization data. *Mol. Biol. Evol.* **12:** 266–284.

Le Berre, M., Ramousse, R., and Le Guelte, L. (1996). *Biodiversity in Marmots*, International Marmot Network. Moscow–Lyon.

Mein, P. (1992). Taxonomy. In: *Proc. 1st Int. Symp. Alpine Marmot Gen. Marmota*, B. Bassano, P. Durio, U. Gallo Orsi, and E. Macchi, eds., pp. 6–12, Dipartimento di Produzioni Animali, Epidemiologia e d'Ecologia, Torino.

Moore, J. C. (1959). Relationships among living squirrels of the Sciurinae. *Bull. Am. Mus. Nat. Hist.* **118:** 157–206.

Maxson, L. R., Ellis, L. S., and Song, A. R. (1981). Quantitative immunological studies of the albumins of North American squirrels, family Sciuridae. *Comp. Biochem. Physiol.* **68:** 397–400.

Murie, J. O., and Michener, G. R. (1984). *The Biology of Ground-Dwelling Squirrels*, University of Nebraska Press, Lincoln and London.

Nadler, C. F., and Harris, K. E. (1967). Chromosomes of the North American prairie dog *Cynomys ludovicianus*. *Experientia* **23:** 41–42.

Nadler, C. F., and Sutton, D. A. (1962). Mitotic chromosomes of some North American Sciuridae. *Proc. Soc. Exp. Biol. Med.* **110:** 36–38.

Nadler, C. F., Hoffmann, R. S., and Pizzimenti, J. J. (1971a). Chromosomes and serum proteins of prairie dogs and a model of *Cynomys* evolution. *J. Mammal.* **52:** 545–555.

Nadler, C. F., Hoffmann, R. S., and Greer, K. R. (1971b). Chromosomal divergence during evolution of ground squirrel populations (Rodentia: *Spermophilus*). *Syst. Zool.* **20:** 298–305.

Nadler, C. F., Hoffmann, R. S., Vorontsov, N. N., Koeppl, J. W., Deutsch, L., and Sukernik, R. I. (1982). Evolution in ground squirrels II. Biochemical comparisons in Holarctic populations of *Spermophilus*. *Z. Saügetierkunde* **47:** 198–215.

Nadler, C. F., Hoffmann, R. S., Vorontsov, N. N., and Sukernik, R. I. (1984). Evolutionary relationships of some Beringian mammals. In: *Beringia in the Cenozoic Era*, V. L. Kontrimavichus, ed., pp. 424–439, Amerind, New Delhi.

Rausch, R. L., and Rausch, V. R. (1971). The somatic chromosomes of the North American marmots (Sciuridae), with remarks on the relationships of *Marmota broweri*. *Mammalia* **35:** 85–101.

Saitou, N., and Nei, M. (1987). The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.

Sarich, V. M., and Cronin, J. E. (1976). Molecular systematics of the Primates. In: *Molecular Anthropology, Genes and Proteins in the Evolutionary Ascent of the Primates*, M. Goodman, R. E. Tashian, and J. H. Tashian, eds., pp. 141–170, Plenum Press, New York and London.

Sgouros, J. G., Kleinschmidt, T., Arnold, W., and Braunitzer, G. (1986). The primary structure of the hemoglobin of European marmot (*Marmota marmota marmota*, Rodentia). *Biol. Chem. Hoppe-Seyler* **367:** 223–228.

Sheldon, F. H., and Bledsoe, A. H. (1989). Indexes to the reassociation and stability of solution DNA hybrids. *J. Mol. Evol.* **29:** 328–343.

Sheldon, F. H., Slikas, B., Kinnarney, M., Gill, F. B., Zhao, E., and Silverin, B. (1992). DNA-DNA hybridization evidence of phylogenetic relationships among major lineages of *Parus*. *Auk* **109:** 173–185.

Sibley, C. G., and Ahlquist, J. E. (1983). Phylogeny and classification of birds based on the data of DNA/DNA hybridization. In: *Current Ornithology*, R. F. Johnston, ed., pp. 245–292, Plenum, New York.

Sibley, C. G., and Ahlquist, J. E. (1990). *Phylogeny and Classification of Birds: A Study in Molecular Evolution*, Yale University Press, New Haven, CT, and London.

Smith, D. G., and Coss, R. G. (1984). Calibrating the molecular clock: Estimates of ground squirrel divergence made using fossil and geological time markers. *Mol. Biol. Evol.* **1:** 249–259.

Soskic, V., Grujic-Injac, B., and Braunitzer, G. (1986). The primary structure of the hemoglobin of the European souslik (*Citellus citellus*, Rodentia). *Biol. Chem. Hoppe-Seyler* **367:** 1159–1166.

Springer, M. S., Kirsch, J. A. W., Aplin, K., and Flannery, T. (1990). DNA hybridization, cladistics and the phylogeny of phalangerid marsupials. *J. Mol. Evol.* **30:** 298–311.

Thomas, W. K., and Martin, S. L. (1993). A recent origin of marmots. *Mol. Phylogenet. Evol.* **2:** 330–336.

Veron, G., and Catzeflis, F. M. (1993). Phylogenetic relationships of the endemic Malagasy carnivore *Cryptoprocta ferox* (Aeluroidea): DNA/DNA hybridization experiments. *J. Mammal. Evol.* **1:** 169–176.

Vorontsov, N. N., and Lyapunova, E. A. (1970). Chromosome numbers and speciation in the Sciuridae: Xerinae and Marmotinae of Holarctica. *Bull. Mus. Soc. Nat. Ser. Biol.* **5:** 122–136.

Vorontsov, N. N., and Lyapunova, E. A. (1984). Genetics and problems of the trans-Beringian connections of Holarctic mammals. In: *Beringia in the Cenozoic Era*, V. L. Kontrimavichus, ed., pp. 441–463, Amerind, New Delhi.

Vorontsov, N. N., Lyapunova, E. A., and Zagorujko, N. G. (1969). The comparative karyology and evolution of isolating mechanism in the genus *Marmota* (Rodentia: Mammalia). *Zool. Zurn.* **48:** 317–334.

Werman, S. D., Springer, M. S., and Britten, R. J. (1990). Nucleic acids. I. DNA/DNA hybridization. In: *Molecular Systematics*, D. M. Hillis and G. Moritz, ed., pp. 204–249, Sinauer Associates, Sunderland, MA.

Zimina, R. P., and Gerasimov, I. P. (1973). The periglacial expansion of marmots (*Marmota*) in middle Europe during late Pleistocene. *J. Mammal.* **54:** 327–340.