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# Phylogeny of the genus *Apodemus* with a special emphasis on the subgenus *Sylvaemus* using the nuclear IRBP gene and two mitochondrial markers: cytochrome *b* and 12S rRNA

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**Abstract**

Phylogenetic relationships among 17 extant species of Murinae, with special reference to the genus *Apodemus*, were investigated using sequence data from the nuclear protein-coding gene IRBP (15 species) and the two mitochondrial genes cytochrome *b* and 12S rRNA (17 species). The analysis of the three genes does not resolve the relationships between *Mus*, *Apodemus*, and *Rattus* but separates *Micromys* from these three genera. The analysis of the two mitochondrial regions supported an association between *Apodemus* and *Tokudaia* and indicated that these two genera are more closely related to *Mus* than to *Rattus* or *Micromys*. Within *Apodemus*, the mitochondrial data sets indicated that 8 of the 9 species analyzed can be sorted into two main groups: an *Apodemus* group, with *A. agrarius*, *semotus*, and *peninsulae*, and a *Sylvaemus* group, with *uralensis*, *flavicollis*, *alpicola*, *sylvaticus*, and *hermonensis*. The position of *Apodemus mystacinus* is ambiguous and might be either included in *Sylvaemus* or considered a distinct subgenus, *Karstomys*, more closely related to *Sylvaemus* than to *Apodemus*. Estimation of the divergence time for these taxa suggests a separation between 7 and 8 My ago for the three groups (*mystacinus* and the two subgenera *Apodemus* and *Sylvaemus*). Within each subgenus, divergence times are between 5.4 and 6 My for *Apodemus* and between 2.2 and 3.5 My for *Sylvaemus* and *mystacinus*. 

## 1. Introduction

The speciose genus *Apodemus* is widespread throughout the Palearctic region. Morphologically, *Apodemus* seems distinct from both *Mus* and *Rattus*, perhaps indicative of an ancient isolation (early Vallesian) of its ancestor (Martin-Suarez and Mein, 1998). Nevertheless, DNA/DNA hybridization data (Catzeflis, 1987; Catzeflis and Denys, 1992; Catzeflis et al., 1993; P. Chevret, unpublished data) and sequence analyses (Martin et al., 2000; Suzuki et al., 2000) tend to associate *Apodemus* with *Mus*.

Since the review of Musser et al. (1996) all but one of the *Apodemus* species have been divided into two subgenera: *Sylvaemus* (including most of the western Palearctic species) and *Apodemus* (in which *A. agrarius* and the ancient eastern Palearctic *Alsomys* subgenus, excluding *A. argenteus*, are included). The remaining *A. argenteus* seems to be distinct from the others. This hypothesis has been confirmed by Serizawa et al. (2000) based on sequences from the mitochondrial cytochrome *b* and the nuclear IRBP genes. Moreover, these authors proposed a fourth monotypic group, *A. gurkha*, the Himalayan field mouse.

At present, many questions concerning the phylogenetic relationships both of the genus *Apodemus* within the Murinae and between the different species within each of the *Apodemus* subgenera still remain unanswered.

According to several authors (Filippucci et al., 1989, 1996; Musser and Carleton, 1993; Musser et al., 1996;

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Orlov et al., 1996; Vorontsov et al., 1992), 13 different species are presently recognized within the subgenus *Sylvaemus* in the western Palearctic region: *A. sylvaticus*, Linnaeus (1758); *A. flavicollis*, Melchior (1834); *A. alpicola*, Heinrich (1952); *A. uralensis*, Pallas (1881); *A. mystacinus*, Danford and Alston (1877); *A. hermonensis*, Filippucci et al. (1989); *A. fulvipectus*, Ognev (1924); *A. mosquensis*, Orlov et al. (1996); *A. ciscaucasicus*, Orlov et al. (1996); *A. ponticus*, Sviridenko (1936); *A. hyrcanicus*, Vorontsov et al., 1992; *A. arianus*, Blanford (1881); and *A. wardi*, Wroughton (1908). Species within the subgenus *Sylvaemus* are phenotypically very similar, and traditional morphometrics are often at a loss to distinguish between them (Michaux et al., 2002; Zagorodnyuk, 1996). For this reason, several authors have employed protein electrophoresis (Britton-Davidian et al., 1991; Csaikl et al., 1980; Engel et al., 1973; Filippucci, 1992; Filippucci et al., 1996, 2002; Gemmeke, 1980; Hartl et al., 1992; Mezhzherin and Zykov, 1991) and traditional cytogenetics (Bulatova et al., 1991; Nadjafova et al., 1993; Zima, 1984) in an attempt to unravel relationships. Unfortunately, similar to morphometrics, cytogenetics were not successful in delimiting species, given the uniformity in karyotypic characteristics of this subgenus. More recently, new molecular methods such as restriction fragment length polymorphism (Chelomina, 1996; Michaux et al., 1996, 1998a, 1998b), random amplified polymorphic DNA (RAPD) (Bellinvia et al., 1999), and sequencing of mitochondrial and/or nuclear genes (Chelomina, 1998; Chelomina et al., 1998; Martin et al., 2000; Serizawa et al., 2000) were used with greater success. However, most of these studies included only a limited number of *Sylvaemus* species and although they gave very interesting results concerning the intraspecific and interspecific phylogenetic relationships within this subgenus, many inconsistencies among the different studies remain.

The aims of the present study were fourfold. First, we wanted to determine the sister group to the genus *Apodemus*, adding novel 12S rRNA mitochondrial DNA sequences to the already existing data of Serizawa et al. (2000) and Suzuki et al. (2000). In addition, we followed a combined data approach (Kluge, 1989) using three genes (12S rRNA, cytochrome *b*, and IRBP, available for 15 taxa) in concert. Second, we wanted to clarify the phylogenetic relationships among six European and Near Eastern *Sylvaemus* species, *A. sylvaticus*, *A. flavicollis*, *A. uralensis*, *A. alpicola*, *A. mystacinus*, and *A. hermonensis*, using new sequences from two mitochondrial regions (cytochrome *b* and 12S rRNA). Third, we wanted to propose more robust estimations of divergence dates between the different murine genera, the *Apodemus* subgenera, and the *Sylvaemus* species. Finally, we wanted to discuss the evolutionary history of this speciose genus.

## 2. Material and methods

### 2.1. DNA sequencing of cytochrome b, 12 S rRNA, and IRBP

DNA was extracted from ethanol-preserved tissue for the species listed in Table 1 following Sambrook et al. (1989). These tissues were taken from the *Apodemus* tissue collection of J.R. Michaux and the mammal tissue collection housed at the Institut des Sciences de l'Evolution de Montpellier (Catzeflis, 1991). Whenever possible, we selected two individuals for each species (Table 1) to minimize the effect of long-branch attraction (Felsenstein, 1978).

A large portion of cytochrome *b* (971 bp) was amplified using the Universal PCR primers L7 (5′-ACCAA TGACATGAAAAATCATCGTT-3′) and H16 (5′-AC ATGAATYGGAGGY-CAACCWG-3′) (Kocher et al., 1989). The complete 12S rRNA was amplified using the PCR primers R1 and S2 (Sourrouille et al., 1995). Finally, the complete IRBP gene was amplified using the PCR primers I1, J1, I2, and J2 (Stanhope et al., 1992). Amplification reactions were carried out in 2× 50-μl volumes including 25 μl of each 2 μM primer, 20 μl of 1 mM dNTP, 10 μl of 10× reaction buffer, 10 μl of purified water, and 0.2 μl of 5 U/μl Promega *taq* DNA polymerase. Approximately 200 ng of DNA extract was used per PCR amplification. Amplifications were performed in a Labover PTC 100 thermal cycler employing 33 cycles (20 s at 94 °C, 30 s at 50 °C, and 1 min 30 s at 68 °C) with a final extension cycle of 10 min at 68 °C. PCR products were purified using the Ultra-free DA Amicon kit (Millipore) and directly sequenced. Both strands were sequenced using a Bigdye terminator (Perkin–Elmer) sequencing kit and ran on an ABI 310 (Applied Biosystems) automated sequencer.

### 2.2. Sequence alignment and saturation analysis

Published sequences for cytochrome *b*, 12S rRNA, and IRBP were extracted from GenBank (see Table 1) and aligned to our new sequences using the ED editor (MUST package; Philippe, 1993). The program AFAS (MUST package; Philippe, 1993) was used to combine the aligned matrices of IRBP, 12S rRNA, and cytochrome *b*.

Following Hassanin et al. (1998) and Philippe and Douzery (1994), we examined the IRBP, cytochrome *b*, and 12S rRNA data sets for saturation. Using the matrices of patristic and inferred substitutions calculated by PAUP 4b8 (Swofford, 2000), the pairwise numbers of observed differences was plotted against the corresponding values for inferred substitutions (Philippe and Douzery, 1994). The slope of the linear regression (S) was used to evaluate the level of saturation (Hassanin et al., 1998). When no saturation is observed in the data

Table 1
References of rodent tissues used for the experiments

| Family | Subfamily | Species | Tissue sample | Geographic origin | Accession numbers (IRBP gene) | Accession numbers (cyt. *b* gene) | Accession numbers (12S rRNA) |
|---|---|---|---|---|---|---|---|
| Muridae | Spalacinae | *Spalax ehrenbergi* | T-1016 | Diyarbakir, Turquie | | AJ311138 | Chevret et al. (2001) |
| | | *Spalax zemni* | GenBank | | Stanhope et al. (1996) | | |
| | Cricetinae | *Cricetulus griseus* | GenBank | | Suzuki et al. (2000) | Suzuki et al. (2000) | |
| | | *Cricetulus migratorius* | T-325 | IZEA Lausanne élevage | | | Hänni et al. (1999) |
| | Murinae | *Diplothrix legata* | HS-1163 | | Suzuki et al. (2001) | Suzuki et al. (2001) | AJ311143 |
| | | *Tokudaia osimensis* | T-XXX | | Suzuki et al. (2002) | Suzuki et al. (2002) | AJ311133 |
| | | *Mus musculus* | GenBank | | Stanhope et al. (1992) | Bibb et al. (1981) | Sourrouille et al. (1995) |
| | | *Mus caroli* | DNA-1413 | Laboratory strain (F. Bonhomme) | Suzuki et al. (2000) | Suzuki et al. (2000) | AJ279437 |
| | | *Rattus norvegicus* | GenBank | | Suzuki et al. (2000) | Gadaleta et al. (1989) | Kobayashi et al. (1981) |
| | | *Micromys minutus* | T-1196 | Giverny (Orne, France) | Suzuki et al. (2000) | Suzuki et al. (2000) | AJ311139 |
| | | *Apodemus semotus* | T-248 | Taiwan: Mt. Ari | Serizawa et al. (2000) | Serizawa et al. (2000) | AJ311136 |
| | | *Apodemus peninsulae* | T-668 | Baikalsk; Lake teletskoje; Kracnojozsk | Serizawa et al. (2000) | Serizawa et al. (2000) | AJ311142 |
| | | *Apodemus agrarius* | JRM-265 | Czech Republic, Koprivnice | Serizawa et al. (2000) | AJ311144 | AJ311130 |
| | | | T-780 | Estonia, Tallin | | AJ311145 | AJ311140 |
| | | *Apodemus mystacinus* | JRM-261 | Syria | AJ311158 | AJ311146 | AJ311141 |
| | | | JRM-281 | Greece, Peloponnese | | AJ311147 | AJ311132 |
| | | *Apodemus sylvaticus* | JRM-168 | Italy, Latium, Rome | Serizawa et al. (2000) | AJ311148 | AJ311126 |
| | | | JRM-269 | France, Pyrénées-Orientales | | AJ311149 | AJ311131 |
| | | *Apodemus flavicollis* | JRM-199 | Italy, Abruzzo, Penne | Serizawa et al. (2000) | AJ311150 | AJ311127 |
| | | | T-666 | France, Allier | | AJ311151 | AJ311164 |
| | | *Apodemus alpicola* | JRM-202 | France, Savoie | Serizawa et al. (2000) | AJ311152 | AJ311137 |
| | | | T-1595 | Austria, Vorarlberg | | AJ311153 | AJ311135 |
| | | *Apodemus uralensis* | JRM-257 | Czech Republic, Moravia | | AJ311154 | AJ311128 |
| | | | JRM-258 | Turkey, Yalnizcam gecidi | | AJ311155 | AJ311125 |
| | | *Apodemus hermonensis* | JRM-259 | Turkey, Hakkari | | AJ311156 | AJ311134 |
| | | | JRM-260 | Turkey, Dogubayazit | | AJ311157 | AJ311129 |

*Note.* The taxonomic arrangements follow Wilson and Reeder (1993). The GenBank accession numbers are given for the new sequences obtained in this study.

set, the slope equals one, whereas the slope tends toward zero as the level of saturation increases.

### 2.3. Phylogenetic reconstructions

Phylogenetic analyses followed three approaches. First, we analyzed each gene separately. Second, we used the combined matrix for IRBP (782 bp), 12S rRNA (904 bp), and cytochrome *b* (971 bp) sequences to determine the phylogenetic position of *Apodemus* within Murinae. Third, to determine the relationships within *Apodemus* and more precisely within the subgenus *Sylvaemus*, we used a combined matrix of 12S rRNA (904 bp) and cytochrome *b* (971 bp) sequences.

Before combining these different genes into single matrices, the level of incongruence between genes was tested using PAUP4b5 (option Hompart). This approach uses the incongruence length difference (ILD) test with the parsimony criterion; 1000 randomizations were performed on variable sites only (Farris, 1985).

The aligned sequences were treated by different approaches: the GTR and the Kimura two-parameter (K2P) estimator were used for the calculation of genetic distances. The GTR analyses were performed assuming a gamma distribution for substitution rates across sites, where the parameter alpha (Yang, 1996) and the proportion of invariant sites were estimated with the maximum-likelihood (ML) method assuming the GTR phylogeny using PAUP 4.0b8. Maximum-parsimony (MP: heuristic search; TBR branch swapping option) and maximum-likelihood (GTR model of sequence evolution) analyses were also conducted using PAUP 4.0b8 (Swofford, 1998).

The robustness of inferences was assessed by bootstrap resampling (1000 random repetitions for MP and distance analyses and 100 for ML). Bremer's support index (BSI) (Bremer, 1988) was also calculated on the most parsimonious tree with enforcement of topological constraints.

Likelihoods of alternative topologies were compared with MOLPHY 2.3b3 (Adachi and Hasegawa, 1996) and PUZZLE (Strimmer and von Haeseler, 1996). Following Kishino and Hasegawa (1989), an alternative hypothesis was rejected when $\delta \ln L > 1.96$ SE, where $\delta \ln L$ is the difference between the log-likelihoods of the best and the evaluated trees, and SE is the standard error of this difference.

### 2.4. Divergence time

To determine divergence times within *Apodemus* and more particularly within *Sylvaemus*, we used cytochrome *b* and 12S rRNA. These two genes were chosen since they comprised full data for the majority of the species. Two calibration points derived from paleontological data were used: first, the *Mus*/*Rattus* dichotomy at 12 millions years (My) ago (Jacobs and Downs, 1994; Jacobs et al., 1989, 1990; Jaeger et al., 1986), and second, the divergence time between *A. mystacinus* and all the "small" *Sylvaemus* species estimated at approximately 7 My (Aguilar and Michaux, 1996; Michaux et al., 1997).

To determine whether there are differences in rates of cytochrome *b* and 12S rRNA changes between the different murine genera included here, the *Apodemus* subgenera, and the *Sylvaemus* species, relative-rate tests were conducted with each of them against the remaining lineages. The relative-rate tests were done with RRTree, version 1.0 (Robinson et al., 1998), which improves the test of Wu and Li (1985) by taking into account taxonomic sampling and phylogenetic relationships. Relative-rate tests were performed at the subfamilial (*Mus*, *Rattus*, and *Apodemus* genera), subgeneric (among *Apodemus* subgenera), and intrasubgeneric (*Sylvaemus* species) levels. The two mitochondrial DNA regions (12S rRNA and cytochrome *b*) were analyzed separately. The ML tree for each gene was chosen as the reference topology. *Micromys* was used as outgroup. For coding sequences (cytochrome *b*), relative-rate tests were performed on the proportions of synonymous ($K_s$) and nonsynonymous ($K_a$) substitutions. For noncoding regions (12S rRNA), relative-rate tests were performed on the proportion of all the substitutions types (K).

To apply a molecular clock and to estimate times of divergence, we estimated the ML (search constrained to clock-like evolution) tree based on the cytochrome *b* data set with *Micromys* as outgroup. The inferred maximum-likelihood distances were used to estimate separation times.

## 3. Results

### 3.1. New sequences

All sequences generated in the present study were deposited in GenBank under Accession Nos. AJ311127 to AJ311143 and AJ311164 (12S rRNA), AJ311144–AJ311157 (cytochrome *b*), and AJ311158 (IRBP) (Table 1).

The alignment of the IRBP sequences of 15 taxa comprises 782 nucleotides of which 234 (30%) are variable and 120 (15%) parsimony informative. The average ratio of transition/transversion (TS/TV) is 2.39, ranging from 1.2 to 5.1. The alignment of the cytochrome *b* gene consists of 971 nucleotides for 24 taxa, 298 (37%) of which are variable and 215 (26%) parsimony informative. The average ratio of TS/TV is 1.79, ranging from 0.80 (*A. flavicollis* (JRM-199)/*Micromys minutus*) to 5.34 (*A. flavicollis* (JRM-199)/*A. hermonenis* (*JRM-260*)). The complete alignment of the 12S rRNA mitochondrial gene comprises 987 sites for 24 individuals. When the

indels are removed from the matrix, 904 sites are included in the analyses, 455 in loops and 499 in stems. Of these 258 (29%) are variable and 139 (15%) parsimony informative. The average ratio of TS/TV is 1.56, ranging from 0.8 to 6.7. The concatenated data matrix for the 15 species of Muridae comprises 2657 nucleotide sites, 776 (29%) variable and 451 (26%) parsimony informative.

### 3.2. Saturation Analysis

Saturation analysis of the IRBP data set (15 taxa, 782 nucleotides) indicates that there is no saturation for transition and transversion at 1st and 2nd codon positions, with slopes (S) ranging from 0.91 to 0.97, and only light saturation for the 3rd codon position (TS: S = 0.81; TV: S = 0.76). Therefore we included all events at the three codon positions for all phylogenetic analyses.

Saturation analyses of the 12S rRNA data sets were performed on four partitions: transitions and transversions were analyzed separately in loop and stem regions. There is no saturation for TV in stems (S = 0.99) and only moderate saturation for TS in stems (S = 0.80) and for both substitution types in loops (TS: S = 0.76; TV: S = 0.73).

Saturation analysis of the cytochrome *b* data showed that TS and TV at positions 1 and 2 and TV at position 3 are moderately affected by homoplasy (TS 1: S = 0.59; TV 1: S = 0.69; TS 2: S = 0.54; TV 2: S = 0.98; TV 3: S = 0.52). In contrast, transitions at 3rd position are highly saturated (S = 0.26). Therefore, all further analyses were conducted excluding TS at 3rd codon position.

### 3.3. Phylogenic relationships of Apodemus within the Murinae

Notwithstanding minor discrepancies between the branching patterns obtained from the 12S rRNA and those from the cytochrome *b* data sets, the ILD test showed no significant incongruence between the two mitochondrial markers ($P = 0.15$). Despite significant incongruences (see below for some examples of incongruence between the different topologies) found when all three genes are combined ($P = 0.001$), we chose to concatenate these three genes for the 15 species common to these markers. This allowed us more sites for analysis and a combination of one nuclear and two mitochondrial markers.

The combined analysis was performed with two nonmurine species of Muridae (*Cricetulus* (Cricetinae) and *Spalax* (Spalacinae)) as outgroups. A consensus tree, constructed from the topologies retrieved by MP, ML, and neighbor-joining with bootstrap support (NJB), is presented in Fig. 1. For the NJ analysis, branching patterns and bootstrap values were similar using either MUST or PAUP. The monophyly of the Murinae is strongly supported (MP = 100/+36; NJ = 100; ML = 76). The most basal murine genus is *Micromys* (MP = 60/+7; NJ = 75). Three main groups can be distinguished for the remaining Murinae taxa: a *Rattus–Diplosthrix* group, *Mus*, and *Apodemus–Tokudaia*. The monophyly of both *Rattus–Diplothrix* (MP = 100/+50; NJB = 100; ML = 100) and *Mus* (MP = 100/+39; NJB = 100; ML = 100) is well supported. The support for *Apodemus–Tokudaia* is moderate with the three methods (MP = 47/+4; NJB = 73; ML = 55). Based on the combined data set, it is impossible to determine sister group relationships among *Rattus–Mus–Apodemus*. One reason for this lack of resolution is that mitochondrial and nuclear data sets provide conflicting results. The mitochondrial data support *Mus* and *Apodemus* as sister taxa, whereas the IRBP data associate *Mus* and *Rattus* to the exclusion of *Apodemus*, hence an unresolved multitomy in the combined analysis. Interestingly, *Apodemus* and *Rattus* are never grouped together. At a lower taxonomic level, the genus *Apodemus* appears monophyletic (MP = 75/+7; NJB = 88; ML = 68) and is divided into three groups: *A. mystacinus*; a *Sylvaemus* group containing *flavicollis*, *alpicola* and *sylvaticus*; and an *Apodemus* group including *agrarius*, *semotus*, and *peninsulae*. Within the subgenus *Apodemus*, *semotus*, and *peninsulae* are more closely related to each other than to *agrarius*, whereas within *Sylvaemus*, *alpicola*, and *flavicollis* are more closely related than either of them are to *sylvaticus*. *A. mystacinus* is related to *Sylvaemus* although with a low support (MP = 40/0; NJB = 61; ML = 41).

### 3.4. Phylogenetic analysis within apodemus

To determine relationships within the genus *Apodemus* and the subgenus *Sylvaemus*, we focused our analyses on the murine taxa, with a larger mitochondrial DNA sampling of *Apodemus* species. We included two individuals from each of the different species of *Sylvaemus*, *alpicola*, *uralensis*, *flavicollis*, *sylvaticus*, and *hermonensis*, two *mystacinus* and *agrarius* specimens, and one sample each from *semotus* and *peninsulae*. *Tokudaia*, *Micromys*, *Rattus*, *Diplothrix*, and *Mus* were also included in the analysis. We used *Micromys* to root the topologies (see Fig. 1).

The results from the different analyses are presented in Fig. 2. Again, for the NJ analysis, branching patterns and bootstrap values were similar using either MUST or PAUP. *Mus* appeared more closely related to *Apodemus* (MP = 76/+4; NJB = 74; ML = 88) than to *Rattus*. Although not statistically supported (MP = 34/0; NJB = 48; ML = 76), all three methods grouped *Tokudaia* as sister taxon to *Apodemus*, the latter representing a monophyletic group (MP = 54/+2; NJB = 68; ML = 73).

Within *Apodemus*, the monophyly of the different species, when represented by two individuals, are well

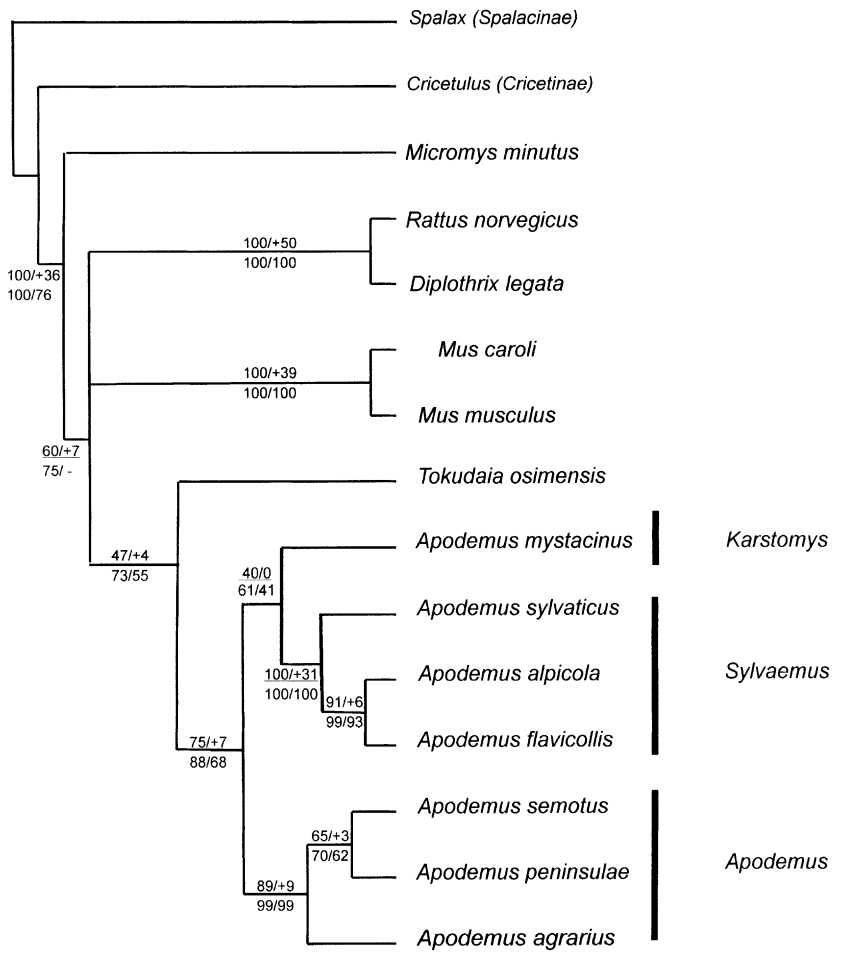


Fig. 1. Consensus tree obtained from the analysis of the concatenation of the three genes for 15 taxa and 2657 positions, with the exclusion of transitions at 3rd codon positions for the cytochrome *b*. *Spalax* and *Cricetulus* are used to root the tree. *Apodemus* subgenera names are indicated on the right side. For each node the different robustness indices are indicated as

$$\frac{\text{Maximum--parsimony bootstrap support/BSI}}{\text{NJBoot support/ML Bootstrap support}}.$$

When conflicting relationships are obtained wuth the different analysis a trichotomy is indicated.

supported (=98–100%). The *Apodemus* subgenus comprises *agrarius*, *semotus*, and *peninsulae* (MP = 60/+3; NJB = 82; ML = 67) with *semotus* and *peninsulae* more closely related to each other (MP = 63/+3; NJB = 52; ML = 63) than they are to *agrarius*. *A. mystacinus* is more closely related to *Sylvaemus* than to the *Apodemus* subgenus (MP = 74/+7; NJB = 75; ML = 71).

Within *Sylvaemus*, *alpicola*, and *uralensis* are sister species (MP = 74/+3; NJB = 67; ML = 72). These two species cluster with *flavicollis* (MP = 91/+5; NJB = 91; ML = 81). *A. sylvaticus* is associated either with the *flavicollis* group (MP = 56/+2; ML = 45) or with *A. hermonensis* (NJB = 71).

A summary of the Kimura two-parameter sequence divergence values is presented in Table 2 for the three regions, both within and between the studied genera. Both nuclear (1RBP) and mitochondrial (12S rRNA) sequences indicate a close relationship between *mystacinus* and *Sylvaemus* (3.6 and 4.8%, respectively, versus 4.5 and 5.2% between *mystacinus* and *Apodemus*), whereas cytochrome *b* shows the three groups to be equidistant (8.8%). Genetic divergence values within the *Apodemus* subgenus appear higher (average of 3.8%, 5%, and 6.8% for IRBP, 12S rRNA, and cytochrome *b*, respectively) than those within *Sylvaemus* (average of 1.3%, 2%, and 4%). Finally, the divergence between the two *mystacinus* specimens coming from different regions (Greece and Syria) is in the same order (2.5% for 12S rRNA and 4.4% for cytochrome *b*) as those observed within the *Sylvaemus* subgenus.

### 3.5. Likelihoods of alternative topologies

Given that the partition homogeneity test (Farris, 1985) indicated some conflict between the data sets, it was not surprising to find different topologies in the

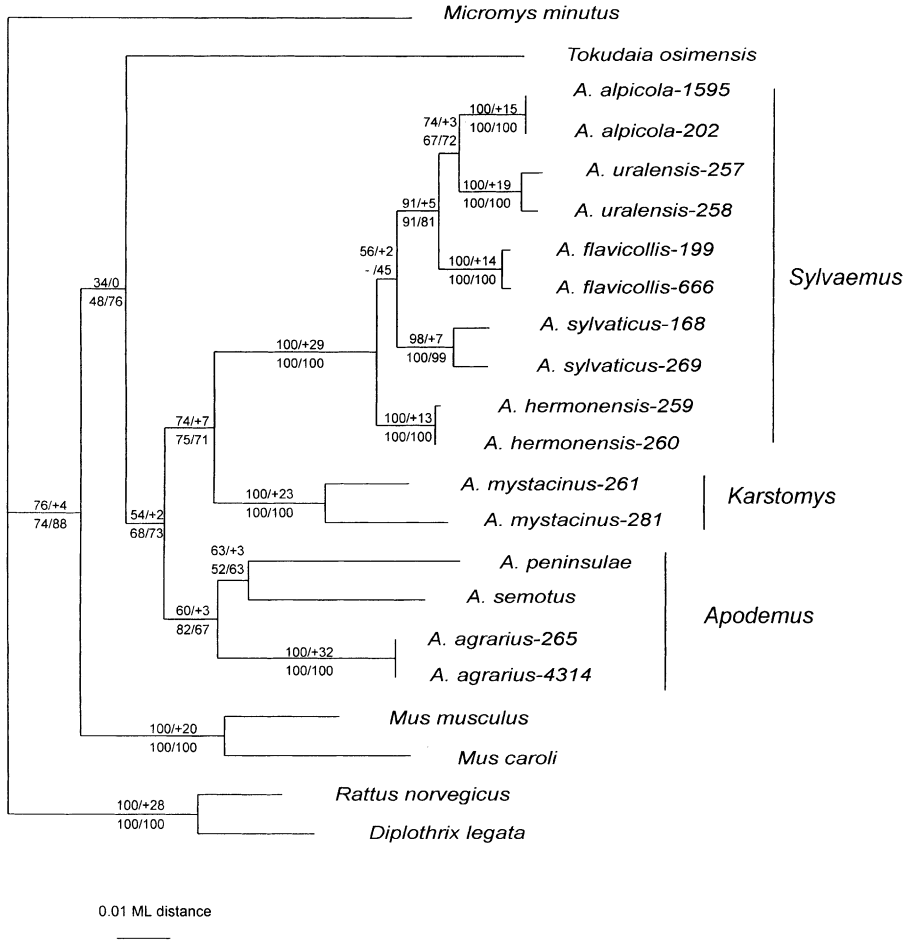


Fig. 2. Highest-likelihood tree derived from the analysis of the concatenation of the two mitochondrial genes for 22 taxa and 1875 positions, with the exclusion of transition at 3rd codon position for the cytochrome *b*. *Micromys* is used to root the tree. Each *Apodemus* sample is identified by its name and DNA number. Subgenera names are indicated on the right side. For each node the different robustness indices are indicated as

$$\frac{\text{Maximum--parsimony bootstrap support/BSI}}{\text{NJBoot support/ML Bootstrap support}}.$$

Table 2
Degree of within- and between-genus sequence divergence with Kimura two-parameter distance for the IRBP, 12S, and cytochrome *b* genes

| Taxa compared | IRBP d (all events) | 12S d (all events) | Cytochrome *b* d (12 TVTS + 3 TV) |
|---|---|---|---|
| Among the Murinae genera | 7.2% (5.7–8.2) | 7.7% (7.1–8.3) | 10.6% (9.8–12.1) |
| *Diplothrix*/*Rattus* | 1.80% | 3.8% | 3.4% |
| *Apodemus* subgenus/*Sylvaemus* | 4.8% (3.9–5.8) | 6.2% (4.8–6.9) | 8.5% (7.3–10) |
| *Apodemus* subgenus/*Karstomys* | 4.5% (3.8–5.1) | 5.2% (4.8–5.5) | 8.8% (8.3–10) |
| *Karstomys*/*Sylvaemus* | 3.6% (3.5–3.7) | 4.8% (4–5.4) | 8.8% (7.9–9.9) |
| Within the *Apodemus* subgenus | 3.8% (3.7–3.8) | 5.0% (4.8–5.3) | 6.8% (6.8–6.9) |
| Within the *Sylvaemus* subgenus | 1.3% (1.1–1.4) | 2.0% (1.3–2.8) | 4.0% (3.2–4.5) |
| *mystacinus* Europe/Near East | – | 2.5% | 4.4% |

IRBP and mitochondrial DNA gene trees. We wanted to determine whether either of these conflicting topologies were significantly better. To this end, we constructed the highest–likelihood tree for each of the IRBP and combined mtDNA data sets. The tree obtained from the IRBP data ($\mathrm{Ln}L = -3067.88$) included 15 murid species, whereas the one based on the cytochrome *b* + 12S rRNA sequences ($\mathrm{Ln}L = -9381.73$) included 24 murine sequences. They were identified with PUZZLE (Strimmer and von Haeseler, 1996) among 945 alternative trees constructed using MOLPHY 2.3b3 (Adachi and Hasegawa, 1996).

The best tree obtained from the two mitochondrial genes was identical to the one presented in Fig. 2. The IRBP topology was similar with the exception that it relates *Mus* to *Rattus* rather than to the *Tokudaia–Apodemus* group.

These two trees with the highest likelihoods were used as reference topologies to apply the test of Kishino and Hasegawa (1989) (KH) on each DNA data set for assessing the following phylogenetic relationships: (1) *Mus*, *Apodemus*, and *Rattus*; (2) *Tokudaia* and *Apodemus*; (3) *Apodemus mystacinus* and *Sylvaemus*; and (4) *Sylvaemus species* (mitochondrial gene matrices only). To do so, we tested different alternative hypotheses derived from traditional morphological, paleontological, and molecular studies (Table 3).

For the tests based on the IRBP tree, none of the alternative topologies exhibited a significantly worse log-likelihood score than the highest-likelihood IRBP tree (Table 3). In sharp contrast, for the combined cytochrome *b* and 12S rRNA, the alternative hypotheses 1, 3, 7, 8, 10, 11, and 12 exhibited significantly worse log-likelihood scores (Table 3). It appears that the most probable relationships are similar to those observed in Fig. 2 with the exception of the distinct placement of *A. hermonensis* with regard to the other *Sylvaemus* species.

### 3.6. Divergence time

The relative-rate test (Robinson et al., 1998) indicated no significant rate heterogeneity (both $K_s$ and $K_a$) for cytochrome *b* in the different murine genera, *Apodemus* subgenera, and *Sylvaemus* species. On the contrary, K comparisons for the 12S rRNA showed marked differences in evolutionary rates with both *Mus* and *A. mystacinus* exhibiting an elevated rate compared to the other taxa. This excluded the use of the 12S rRNA data for divergence estimates since both *Mus* and *A. mystacinus* are calibration points.

The ML distance between *Mus* and *Rattus* that diverged 12 My ago is 0.101. The one between *A. mystacinus* and all remaining *Sylvaemus* that diverged 7 My ago is 0.059. These values give a rate of 0.0084 (*Mus*/*Rattus* and *mystacinus*/other *Sylvaemus*) ML distance per million years. When this rate is applied to the different dichotomies within the Murinae, the following molecular datings are obtained: 9.7 My for the separation between *Mus* and *Tokudaia–Apodemus* and 7.9 My for the separation between the *Apodemus* and the *Sylvaemus* subgenera (see Table 4).

## 4. Discussion

### 4.1. Relationships of Apodemus to other Murinae

According to our data, the closest murine relative of *Apodemus* is *Tokudaia*, an endemic genus from Ryukyu Island, Japan. Notwithstanding moderate support, this finding confirms the phylogenetic hypothesis of Kawamura (1989) who proposed that *Tokudaia* is included in a group that contains *Apodemus*, Pliocene *Rhagapodemus*, and Quaternary *Rhaghamys*. In the same way, Misonne (1969) included *Tokudaia* in his *Lenothrix–Parapodemus* division but closer to *Lenothrix* than to *Apodemus*, with a possible origin in the Philippine Islands.

From a molecular point of view, Suzuki et al. (2000) proposed two conflicting topologies (*Tokudaia–Mus* (cytochrome *b*) and *Tokudaia–Micromys* (IRBP). The former association was, however, not supported (BP = 33%) and appears as an unresolved trichotomy between *Mus*, *Apodemus*, and *Tokudaia*. The same authors proposed two possible events for the colonization of the Islands by *Tokudaia*, either an ancient (12–14 My) or a recent (3–4 My) colonization event. We estimated an age of 9.6 My (Table 4) for the divergence between *Apodemus* and *Tokudaia*, a date which is more consistent with the second hypothesis.

In our analysis, the *Tokudaia–Apodemus* group either falls in a trichotomy with *Mus* and *Rattus* (combined

Table 3
Alternative hypotheses derived from traditional morphological, paleontological, and molecular studies

| Hypotheses | IRBP tree | Cytochrome *b*–12S RNA tree |
|---|---|---|
| *Mus*, *Rattus*, and *Apodemus* as a trichotomy (our combined analysis) | No | Yes |
| *Mus* sister group to *Rattus* (our IRBP data) | No | Yes |
| *Tokudaia* basal to *Apodemus* (Suzuki et al., 2000) | No | Yes |
| *Apodemus mystacinus* basal to *Sylvaemus* (Storch, 1975) | No | Yes |
| *Apodemus hermonensis* sister group of *A. flavicollis* (Filippucci et al., 1996) | No | Yes |
| *A. hermonensis* basal to the other *Sylvaemus* | No | No |
| *A. sylvaticus* sister group to *A. uralensis* (Martin et al., 2000) | No | Yes |
| *A. sylvaticus* sister group to *A. alpicola* (Bellinvia et al., 1999) | No | Yes |
| *A. uralensis* basal to the other *Sylvaemus* (Martin et al., 2000) | No | Yes |

*Note.* They were tested using the test of Kishino and Hasegawa (1989). The highest-likelihood trees obtained from the two mitochondrial genes or the nuclear IRBP gene were used as the reference topologies. "Yes" indicates that the alternative topology exhibited a significantly worse log-likelihood score than the highest likelihood tree, whereas "No" signifies the contrary.

Table 4
Estimates of the separation times of different events within muridae, on the basis of the molecular data

| Separation events | Separation | | Separation | |
|---|---|---|---|---|
| | *A. mystacinus*/other *Sylvaemus* | SE | *Mus*/*Rattus* | SE |
| *Mus*/*Rattus* | 11.9 | 0.9 | **12** | – |
| *A. mystacinus*/other *sylvaemus* | **7** | – | 7 | 0.4 |
| *Diplothrix*/*Rattus* | 2.6 | 0.5 | 2.7 | 0.5 |
| *Mus*/*Tokudaia*, *Apodemus* | 9.7 | 0.3 | 9.7 | 0.3 |
| *Tokudaia*/*Apodemus* | 9.6 | 0.3 | 9.7 | 0.3 |
| *Apodemus* subg./*Sylvaemus* subg. | 7.9 | 0.3 | 7.9 | 0.3 |
| *agrarius*/other *Apodemus* | 6 | 0.44 | 6 | 0.4 |
| *peninsulae*/*semotus* | 5.4 | 0.49 | 5.4 | 0.4 |
| *mystacinus* Europe/Near East | 3 | 0.4 | 3.1 | 0.4 |
| Radiation of *Sylvaemus* | 3.5 | 0.27 | 3.5 | 0.2 |
| *flavicollis*/other *Sylvaemus* | 2.6 | 0.2 | 2.6 | 0.2 |
| *uralensis*/*alpicola* | 2.2 | 0.2 | 2.2 | 0.2 |

*Note.* The numbers in boldface correspond to the two calibration points used for this analysis: 12 Myr for the separation between *Mus* and *Rattus* (Jaeger et al., 1986; Jacobs and Downs, 1994) and 7 Myr for the *mystacinus*/other *Sylvaemus* dichotomy (Aguilar and Michaux, 1996; Michaux et al., 1997). SE, standard error values provided by the maximum-likelihood analysis of Puzzle 4.0.

analysis of three genes) or is associated with *Mus* (mitochondrial data sets). The first possibility is certainly the result of a conflict between the nuclear (which rather associates *Mus* to *Rattus*) and the mitochondrial genes. However, the KH test shows that for IRBP, the association between *Mus* and *Tokudaia–Apodemus* is not significantly worse than that relating *Mus* to *Rattus*. Moreover, the second hypothesis is corroborated by dental morphology, which associates *Apodemus* closer to the *Progonomys–Mus* lineage than to the *Karnimata–Rattus* one (Jacobs and Downs, 1994; Jacobs et al., 1990), and previous molecular analyses (Catzeflis, 1987; Catzeflis et al., 1992, 1993; Suzuki et al., 2000; P. Chevret, unpublished).

### 4.2. Basal position of Micromys

It has been suggested that *Micromys*, the other Palearctic rodent included in our analysis, represents an early offshoot within the Murinae (Catzeflis et al., 1992; Furano et al., 1994). This association is based on the analysis of Line-1 (L1) repeated elements, more precisely a specific murine L1 subfamily named Lx (Furano et al., 1994), and scnDNA hybridization data (Catzeflis et al., 1992). Given that this genus has been known in Europe for only 5 My (Aguilar et al., 1989) it may have originated earlier in Asia where the oldest *Micromys* was found in the Miocene of China (Storch, 1987). Our results based on the combined nuclear and mitochondrial data sets confirm with rather good support (distances and MP) its basal position within the Murinae.

The basal placement of *Micromys* contradicts with the results of Suzuki et al. (2000), who placed *Micromys* as sister group to either *Rattus* (cytochrome *b*) or *Tokudaia* (IRBP), and with the results of Martin et al. (2000), who clustered *Micromys* with *Apodemus* (ML) or *Rattus* (MP). Neither of these contradicting placements received bootstrap support. A possible reason for these contradictions is the choice of *Glirulus* (Gliridae) as outgroup (Suzuki et al., 2000). This genus may be too distantly related to the Murinae, particularly when using cytochrome *b*. The tree that we constructed with *Spalax* (Spalacinae, Muridae) as outgroup indicates that *Micromys* either might be an early offshoot (distance, MP) or might be associated with *Rattus* (ML). In none of our analysis, whether we used the genes singly or in combination, did we find an association between *Mus* and *Micromys* or between *Apodemus* and *Micromys*. In Martin et al. (2000) the nodes that defined the basal divergences in the Murinae are very short and not robustly supported (bootstrap percentages below 50% for most of the nodes that separate *Mus*, *Rattus*, *Apodemus*, and *Micromys*). Most of these nodes should therefore be represented as multitomies. Given the short internal branch lenghts, it is possible that the early murines underwent a rapid radiation between 14 and 12 My ago, in which case the cytochrome *b* gene might not be the most appropriate molecular marker to determine deeper relationships.

### 4.3. Taxonomy and position of A. mystacinus

*A. mystacinus* is distributed throughout the Balkan peninsula, Asia Minor, and the Middle-East. It is clearly distinguishable from the other *Sylvaemus* species based on morphology (Rietschel and Storch, 1973; Storch, 1975) and was therefore included in a separate subgenus: *Karstomys* Martino, 1939. However, the validity of this subgenus has been questioned with several authors recognizing *mystacinus* within *Sylvaemus* (Corbet, 1978; Musser et al., 1996; Niethammer and Krapp, 1978). Enzymatic polymorphism studies confirmed a differen-

tiation of *A. mystacinus* with regard to the other *Sylvaemus* species (Britton-Davidian et al., 1991; Filippucci, 1992). However, the level of genetic distance between them falls within the range generally observed for species rather than subgenera. A recent RAPD study (Bellinvia et al., 1999) confirmed this result, although *A. mystacinus* seemed genetically more distant. On the contrary, cytochrome *b* sequences (Martin et al., 2000) suggested that the genetic distance between four *Sylvaemus* species and *A. mystacinus* was too high to include *mystacinus* within *Sylvaemus*, therefore, maintaining *Karstomys*.

Our phylogenetic analyses clearly distinguish *Karstomys* from the *Apodemus* and *Sylvaemus* groups (Figs. 1 and 2). However, a close relationship between *A. mystacinus* and *Sylvaemus* is found and is confirmed by the Kishino and Hasegawa (1989) test. Taxonomically, our results are ambiguous: the cytochrome *b* distance values separating *A. mystacinus* from the two other subgenera are of the same order as those observed between *Apodemus* and *Sylvaemus* (Table 2), suggesting a subgeneric status for *A. mystacinus*. On the contrary, the IRBP and 12S rRNA distance values observed between this species and the *Sylvaemus* group are similar to those observed within the *Apodemus* subgenus and would suggest a specific status for *A. mystacinus* within *Sylvaemus*.

According to Felten et al. (1973), two subspecies should be recognized within the species *mystacinus*: *epimelas* occurring on the Balkan peninsula and *mystacinus* inhabiting Asia Minor and the Middle-East. Storch (1975), on the basis of variation in the first upper molar, proposed these as distinct species. Our results confirm an important level of genetic divergence between the animals from these two regions, similar to those observed between different *Sylvaemus* species (Table 2). The inclusion of a larger number of individuals covering the distribution area of this species is needed to resolve the taxonomic rank of the species.

### 4.4. Phylogenetic relationships within the subgenus Sylvaemus

The five recognized *Sylvaemus* species are closely related and form a well-supported monophyletic group. This result was already observed by Britton-Davidian et al. (1991), Filippucci (1992), Filippucci et al. (1996), Chelomina (1998), Martin et al. (2000), and Serizawa et al. (2000).

Within this subgenus, three groups are evident: a *flavicollis* group, *A. hermonensis*, and *A. sylvaticus*. Although we used a relatively large matrix (1875 bp), distance, parsimony, and maximum-likelihood analyses were not congruent with regard to the phylogenetic relationships between the three groups. This can be interpreted as the result of a bush-like radiation leading to the simultaneous emergence of many of Western Palearctic *Sylvaemus* species.

Our phylogenetic placement of *A. hermonensis* is at odds with the enzymatic analyses (Filippucci et al.1992, 1996) which suggest that *A. hermonensis* is closely related to *A. flavicollis*. The Kishino and Hasegawa (1989) test rejects this, but instead supports the distinctness of *A. hermonensis* with regard to the other *Sylvaemus* species. This result was already observed in Bellinvia et al. (1999) based on RAPD data.

The taxonomic relationships of *A. hermonensis* to the other oriental species are similarly problematic. In a recent morphological comparison between different populations of *A. hermonensis* from Turkey and Israel and specimens of *A. fulvipectus* from Ukraine and *A. ponticus* from Caucasus, Filippucci et al. (1996) concluded that *A. hermonensis* is probably a junior synonym of *A. fulvipectus*. The comparison of five *A. hermonensis* cytochrome *b* sequences (data not shown) from different regions (Eastern Turkey, Israel, Iran) with partial sequences (275 bp) of five *fulvipectus* specimens and three *A. ponticus* specimens (GenBank Accession Nos. AF249761–AF249768) from the Caucasus indicates that *A. hermonensis* is clearly distinct from *A. fulvipectus* (K2P values with all substitutions: 8.9%) and from *A. ponticus* (K2P: 9.2%), whereas the last two taxa seem more closely related to each other (K2P: 2.7%). Therefore, although our results have to be confirmed by the inclusion of additional sequences and genes, we propose that the specific status of *A. hermonensis* be retained for the time being.

Within the clade uniting *A. flavicollis*, *A. uralensis*, and *A. alpicola* (Fig. 2), the latter two species are sister taxa. This clade was described by Filippucci (1992) based on allozyme electrophoresis. Moreover, the alternative topologies tested with the Kishino and Hasegawa (1989) method were always significantly worse. However, other molecular studies (Britton-Davidian et al., 1991; Mezhzherin and Zykov, 1991; Chelomina, 1998; Bellinvia et al., 1999; Martin et al., 2000; Serizawa et al., 2000) provided conflicting results, supporting an association between *A. sylvaticus* and *A. alpicola* (Martin et al., 2000) or with *A. uralensis* (Bellinvia et al., 1999) opposed to *A. flavicollis*. However, since these studies had a much smaller species representation (between three and five) of European *Sylvaemus* species and since the sequence studies (Bellinvia et al., 1999; Chelomina, 1998; Martin et al., 2000; Serizawa et al., 2000) were based on one specimen per species, it will be difficult to compare our results with theirs. Moreover, after comparisons with several other sequences performed in our laboratory, it appears that the *A. sylvaticus* cytochrome *b* sequence used in Martin et al. (2000) might be a nuclear mitochondrial pseudogene. This interpretation should now be tested with additional nuclear genes, encompassing at least a similar species

diversity to ensure adequate representation of this speciose genus.

### 4.5. Divergence time within Apodemus

The molecular clock, based on two calibration points derived from paleontological data, gives us the opportunity to estimate the dates of divergence between and within studied genera (Table 4). Thus, it would appear that the three *Apodemus* groups considered in our study (*Apodemus*, *mystacinus*, and *Sylvaemus*) diverged roughly 7–8 My ago. According to isotopic studies (Cerling et al., 1997), this period was characterized by important climatic variations leading to drastic changes of vegetation all over the world. Indeed, in many regions and notably in the Holarctic area, the forest was greatly replaced by open herbaceous habitats. These changes in vegetation would have led to drastic faunal replacements, many woodland-adapted animals being replaced by more open-adapted species. If we postulate that the ancestors of *Apodemus* had the same ecological preference as all the recent *Apodemus* species, they would have occurred in broadleaf forests of the temperate zone. We could assume that some populations would have been isolated in refuges (forested regions) during this period, leading to isolation by distance and, in some instances, allopatric speciation of the different *Apodemus* subgenera. According to Cerling et al. (1997), the European regions were relatively protected from the climatic changes and were covered by forests. Therefore, Europe might have played an important role in the speciation of *Apodemus*. This hypothesis is corroborated by paleontological data which suggested the appearance of the true *Apodemus* species in Europe during the Turolian period (Michaux and Pasquier, 1974; Michaux et al., 1997; Martin-Suarez and Mein, 1998).

Within each subgenus, the estimated divergence times between the different species are 5.4–6 My for the *Apodemus* group and 2.2–3.5 My for the *Sylvaemus* and *mystacinus* groups. Thus it appears that the European species diverged more recently than the Asiatic species Serizawa et al. (2000) proposed that these divergences could be associated with floral and geological changes during the late Cenozoic. According to isotopic (Cerling et al., 1997) and palaeoclimatic (Fauquette et al., 1998, 1999; Fluteau et al., 1999) studies, the end of the Miocene and the Pliocene continued to be characterized by numerous climatic changes: from 7 to 4.5 My ago, the climate, fauna, and vegetation of North America greatly varied. In the Palearctic region, from 5.3 to 3.1 My ago, the climate fluctuated between subtropical with warm and humid weather to cool and dry periods. From 3.1 My ago, rapid alterations of the climate which marked the first glacial periods of the Quaternary were observed (Fauquette et al., 1998). The vegetation tracked these climatic changes, being more forested during the warm and humid periods and more open landscape with steppe and forest–steppe during the cool and dry periods (Borisova, 1993; Fauquette et al., 1999, 1998).

A process similar to that described for the Miocene probably occurred during the Pliocene and led to different speciations within the *Apodemus* subgenera. The older divergence dates within the *Apodemus* subgenus could be explained by the fact that the climatic and vegetation variations appeared more dramatic in the eastern (Russia, Asia) than in the western regions (Borisova, 1993).

*Apodemus primaevus*, the ancestor of *A. mystacinus*, has been present in early Europe since the end of the Miocene (Michaux and Pasquier, 1974; Michaux et al., 1997). However, the true *A. mystacinus* only appeared during the Middle Pliocene (Martin-Suarez and Mein, 1998). During this period, the species was widespread throughout Europe, although with lower densities due to competition with many other rodents present at that time (Gliridae, Eomyidae, Sciuridae) (Michaux and Pasquier, 1974). We therefore hypothesize that one of the late Pliocene or early Pleistocene cooling periods associated with low population densities led to the disappearance of many animals and the isolation of the two main groups: one in the Balkanic region (where it is still confined at present) and the other in the Near East (Turkey, Israel). These led to the species A *mystacinus* and *A. epimelas*.

The ancestor (*A. dominans*) of *A. flavicollis* and *A. sylvaticus* appeared more recently in Europe (toward the end of the Pliocene), probably coming from the eastern regions (Martin-Suarez and Mein, 1998; Michaux et al., 1997). It rapidly diverged and gave rise to the present species, probably as a result of an allopatric speciation. Indeed, at the beginning of the Pleistocene, *A. sylvaticus* was present only in Spain and southern France, whereas the distribution area of *A. flavicollis* was central Europe (Michaux and Pasquier, 1974).

The common ancestor of *A. uralensis* and *A. alpicola* could have been isolated in two populations during the first Pleistocene glacial events, respectively, in the eastern regions (*A. uralensis* is very rare in Western Europe during the Pleistocene, suggesting a late eastern colonization (Michaux and Pasquier, 1974)) and in the Alps, giving rise to these species.

## 5. Conclusion

Analyses of the nuclear protein-coding IRBP gene (15 species) and the two mitochondrial regions cytochrome *b* and 12S rRNA (17 species) support an association between *Apodemus* and *Tokudaia* and indicate that these two genera are more closely related to *Mus* than to *Rattus* or *Micromys*. Within *Apodemus*, the mitochon-

drial data set indicates that 8 of the 9 species can be included in two main groups: an *Apodemus* group, with *A. agrarius*, *semotus*, and *peninsulae*, and a *Sylvaemus* group, with *A. uralensis*, *A. flavicollis*, *A. alpicola*, *A. sylvaticus*, and *A. hermonensis*. The 9th species, *A. mystacinus*, might be either included in *Sylvaemus* or considered a distinct subgenus, *Karstomys*, more closely related to *Sylvaemus* than to *Apodemus*. Within *mystacinus*, we showed an important level of genetic divergence between the animals from Europe and those from the Near East. This result could confirm specific status for each of these populations. Within *Sylvaemus*, we determined three groups: a *flavicollis* group, *A. hermonensis*, and *A. sylvaticus*. Unfortunately, our phylogenetic analyses do not resolve the relationships between these three groups. This could be interpreted as the result of a rapid bush-like radiation leading to the near simultaneous emergence of many of the Western Palearctic *Sylvaemus* species. Estimation of the divergence time of these taxa provide an age of 7–8 My for the divergence of the three *Apodemus* groups (*mystacinus* and the two subgenera, *Apodemus*, and *Sylvaemus*). Within each subgenus, divergence times are 5.4–6 My for *Apodemus* and 2.2–3.5 My for *Sylvaemus* and *mystacinus*. These events could be linked to important climatic and vegetation variations during the end of the Miocene and Pliocene.

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