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Mitochondrial phylogeny of African wood mice, genus *Hylomyscus* (Rodentia, Muridae): Implications for their taxonomy and biogeography

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Abstract

This paper investigates the usefulness of two mitochondrial genes (16S rRNA and cytochrome *b*) to solve taxonomical difficulties within the genus *Hylomyscus* and to infer its evolutionary history. Both genes proved to be suitable molecular markers for diagnosis of *Hylomyscus* species. Nevertheless the resolving powers of these two genes differ, and with both markers (either analyzed singly or in combination), some nodes remain unresolved. This is probably related to the fact that the species emerged during a rapid diversification event that occurred 2–6 Myr ago (4–5 Myr ago for most divergence events). Our molecular data support the recognition of an "*aeta*" group, while the "*alleni*" and "*parvus*" groups are not fully supported. Based on tree topology and genetic divergence, two taxa generally recognized as subspecies should be elevated at the species level (*H. simus* and *H. cf kaimosae*). *H. stella* populations exhibit ancient haplotype segregation that may represent currently unrecognized allopatric species. The existence of cryptic species within *H. parvus* is questioned. Finally, three potentially new species may occur in West Central Africa. The Congo and Oubangui Rivers, as well as the Volta and Niger Rivers and/or the Dahomey gap could have formed effective barriers to *Hylomyscus* species dispersal, favoring their speciation in allopatry. The pronounced shifts in African climate during the late Pliocene and Miocene, which resulted in major changes in the distribution and composition of the vegetation, could have promoted speciation within the genus (refuge theory). Future reports should focus on the geographic distribution of *Hylomyscus* species in order to get a better understanding of the evolutionary history of the genus.

Keywords: 16S rRNA; Cytochrome *b*; Muridae; Phylogeny; Tropical African forests; Woodmice

1. Introduction

Woodmice of the genus *Hylomyscus* (Thomas, 1926) are small rodents belonging to the family Muridae. They are restricted to tropical Africa, where they are abundant in forests and dense vegetation. During recent decades the validity of the genus *Hylomyscus* and its relationships with the genera *Praomys*, *Mastomys*, and *Myomys* have been highly debated (reviewed by Lecompte et al., 2002a). Recent phylogenies based on morphological (Lecompte et al., 2002a) and molecular data (Lecompte, 2003; Lecompte et al., 2002b) confirmed the monophyly of the genus *Hylomyscus*. There is now a general agreement on the validity of this genus, despite the fact that phylogenetic studies have covered only a few taxa in the genus. *Hylomyscus* species are morphologically rather similar and, as a

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result, the taxonomy at the species level is debated (Robbins et al., 1980; Rosevear, 1969). Description of most species or subspecies of *Hylomyscus* relied on a small number of specimens with a brief description of their external morphology and skull measurements (e.g., Heller, 1912; Osgood, 1936; Sanderson, 1940; Thomas, 1904, 1906, 1911). To our knowledge, only three attempts were made to document morphometrical variability between populations or species: Bishop (1979) for species inhabiting East Africa, Robbins et al. (1980) for species inhabiting Southern Cameroon, and Dudu et al. (1989) for *H. parvus*. The karyotypes of several species were also published (Iskandar et al., 1988; Lecompte et al., 2005; Maddalena et al., 1989; Matthey, 1963, 1967; Robbins et al., 1980; Tranier and Dosso, 1979; Viégas-Péquignot et al., 1983). Taking into account the results of all these studies, the most recent check-list (Musser and Carleton, in press) includes eight species (Fig. 1): *H. aeta* (Thomas, 1911), *H. alleni* (Waterhouse, 1837), *H. baeri* (Heim de Balsac and Aellen, 1965), *H. carillus* (Thomas, 1904), *H. denniae* (Thomas, 1906), *H. grandis* (Eisentraut, 1969), *H. parvus* (Brosset et al., 1965) and *H. stella* (Thomas, 1911). However, previous investigations on the genus considered a higher number of species, several forms having been described and later on synonymized within *H. aeta* (*laticeps* (Osgood, 1936); *shoutedeni* (Dollman, 1914); *weileri* (Lönnberg and Gyldenstolpe, 1925)), *H. alleni* (*canus* (Sanderson, 1940); *montis* (Eisentraut, 1969); *simus* (Allen and Coolidge, 1930)), *H. denniae* (*anselli* (Bishop, 1979); *endorobae* (Heller, 1910); *vulcanorum* (Lönnberg and Gyldenstolpe, 1925)), and *H. stella* (*kaimosae* (Heller, 1912)).

In this study, we will mainly focus on the species inhabiting the lowland forests of West Central Africa (i.e., *H. aeta*, *H. alleni*, *H. stella*, and *H. parvus*), where our team members conducted extensive surveys during the last 10 years. There are several taxonomical uncertainties concerning the *Hylomyscus* species of this region.

(1) Taxonomic difficulties have resulted from incomplete definitions and inadequate diagnoses of the species, particularly *alleni* and *stella*. The first species to be described, *H. alleni*, was named by Waterhouse in 1837 from a juvenile specimen from Bioko (Fernando Pô, Fig. 1) for which the exact locality of capture is unknown. Several authors have attempted to rediagnose *H. alleni* and to determine its distribution and relationships with other species (Brosset et al., 1965; Hatt, 1940; Heim de Balsac and Aellen, 1965; Heim de Balzac and Lamotte, 1958; Robbins et al., 1980; Rosevear, 1966, 1969). A good review of these studies is available in Rosevear (1969) and Robbins et al. (1980). Briefly, the question of the validity of *H. alleni*, and its affinities with *H. simus*, described from Liberia (Fig. 1), were highly debated. At the present time, *simus* is considered as a subspecies of *H. alleni* (Musser and Carleton, in press). In several studies, another species, *H. stella* was found to be sympatric with *H. alleni* (Brosset et al., 1965 in Gabon; Eisentraut, 1969 in Bioko and adjacent Cameroon; Robbins et al., 1980 in Southern Cameroon). However, the authors agreed that the distinction between these two species is difficult because the morphological differences are slight and vary geographically. In Cameroon, the karyotypes of these two species were shown to have the same number of chromosomes (2N = 46), and only differed slightly in fundamental number (FN = 68–70; Robbins et al., 1980). However, this difference could represent intra-populational variation (Musser and Carleton, 1993, in press). Actually, a specimen of *H. stella* from Burundi had a karyotype rather distinct

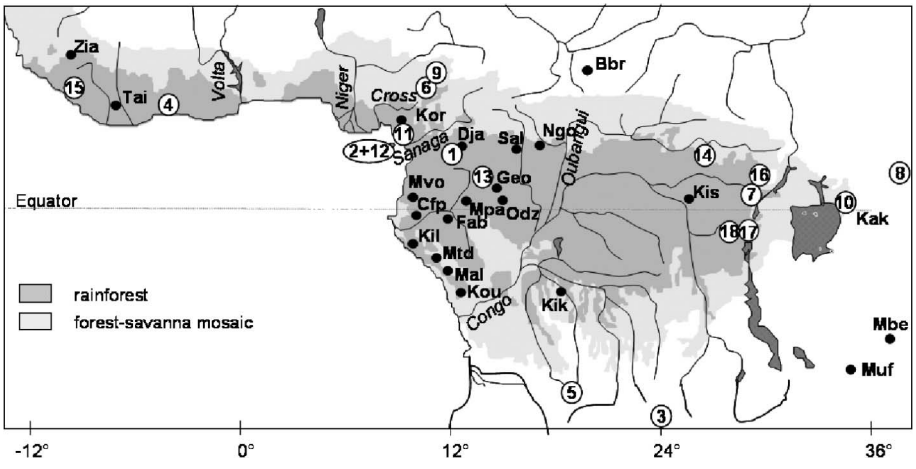

Fig. 1. Map showing the geographical position of collecting localities for the *Hylomyscys* specimens included in this study (black circles), and type localities (white circles with numbers) of the different taxa of the *Hylomyscus* genus. (1) *aeta* (Thomas, 1911), (2) *alleni* (Waterhouse, 1837), (3) *anselli* (Bishop, 1979), (4) *baeri* (Heim de Balsac and Aellen, 1965), (5) *carillus* (Thomas, 1904), (6) *canus* (Sanderson, 1940), (7) *denniae* (Thomas, 1906), (8) *endorobae* (Heller, 1910), (9) *grandis* (Eisentraut, 1969), (10) *kaimosae* (Heller, 1912), (11) *laticeps* (Osgood, 1936), (12) *montis* (Eisentraut, 1969), (13) *parvus* (Brosset et al., 1965), (14) *shoutedeni* (Dollman, 1914), (15) *simus* (Allen and Coolidge, 1930), (16) *stella* (Thomas, 1911), (17) *vulcanorum* (Lönnberg and Gyldenstolpe, 1925), and (18) *weileri* (Lönnberg and Gyldenstolpe, 1925). See Table 1 for the definition of the abbreviations of the collecting localities.

from that of specimens from Cameroon (2N = 48 and NF = 86; Maddalena et al., 1989). Based on electrophoretic and karyological data, Iskandar et al. (1988) suggested that *H. stella* could comprise two species in Gabon. Finally, Heller (1912) described *H. stella kaimosae* from Kakamega forest in Kenya (Fig. 1), but the taxonomical status of this form was never thoroughly investigated. Thus, the definition of both *H. alleni* and *H. stella* needs clarification.

(2) Several forms of *H. aeta* were described and then put in synonymy (Musser and Carleton, 1993, in press), but a detailed analysis of the significance of the variation in body size between sampling locations still needs to be assessed. The karyotype of *H. aeta* was described for the first time by Matthey (1967) for a male specimen from Bioko as having 2N = 52 and NFa = 78. However Robbins et al. (1980) found in Cameroon (at Bua and Yaoundé) two specimens identified as *H. aeta* and possessing 2N = 54 and NFa = 86. In the absence of chromosome banding data, it is difficult to precise the exact nature of the observed chromosomal differences, but these results suggest the existence of several forms within *H. aeta*. Finally, a specimen morphologically close to *H. aeta* but probably representing an undescribed species was reported from the Doudou Mounts (Gabon; Lecompte et al., 2005). Its mammary formula is 2 + 2 = 8 instead of 1 + 2 = 6 in *H. aeta*, and its karyotype is characterized by 2N = 56 and NFa = 86.

This brief review of the literature data highlights a clear need for taxonomic revision. Molecular approaches using mitochondrial DNA have proven useful in addressing phylogenetic relationships both within and among species in a variety of taxa (reviewed in Simon et al., 1994), and a growing literature data has emerged on the usefulness of DNA sequences as taxon "barcodes" (Hebert et al., 2003, 2004a,b; Moritz and Cicero, 2004). This method suffers a number of limitations, and more empirical studies are needed to determine its range and modalities of application (Moritz and Cicero, 2004). In order to clarify the taxonomy of the genus *Hylomyscus*, we analyzed the mitochondrial 16S rRNA gene of 92 specimens coming from nine countries and 22 localities. The Cytochrome *b* (Cytb) gene was also used for a restricted number of specimens. Our expectation was that the obtained DNA sequences, linked to voucher specimens, could be used in the future as DNA barcodes that may facilitate the identification of *Hylomyscus* species. Our study mainly focused on the species inhabiting the West Central African region. However, in order to get a more comprehensive understanding of the evolution of this genus, additional forms inhabiting outside this geographic area were also included: *kaimosae*, inhabiting East Africa and considered by Musser and Carleton (in press) as a subspecies of *H. stella*; *simus*, inhabiting West Africa, and considered as a subspecies of *H. alleni*, and *H. baeri* inhabiting West Africa. By using a carefully calibrated molecular clock, we also intended to estimate the divergence times between the different *Hylomyscus* species, which allowed us to discuss the evolutionary history of the representatives of this genus.

2. Material and methods

2.1. Specimens examined

A total of 92 individuals collected in nine countries and 22 localities were examined (Table 1 and Fig. 1). All the species described for West Central and West Africa (*H. aeta*, *H. alleni*, *H. baeri*, *H. parvus*, and *H. stella*) were included in our analysis, except one species known to be restricted to mountain forests for which we were unable to obtain any sample (*H. grandis* in Mount Oku, Cameroon). Two additional taxa considered either as species or subspecies in the literature (*kaimosae* and *simus*) were also included in our study. At least two specimens were analyzed per species, and for the most problematic species (*H. alleni*, *H. parvus*, and *H. stella*) up to 43 individuals were used. Whenever possible, individuals from different localities were sampled to increase the reliability of the phylogenetic analysis. Before the genetic analysis, the skins and the morphology of the skulls of *H. aeta*, *H. alleni*, *H. baeri*, *H. parvus*, *H. simus*, and *H. stella* were qualitatively compared to type specimens to ascertain correct identification of species. We mainly focused on the following characters for species identification: body size, coloration of the fur, number of mammae, shape of the interorbital area, presence or absence of suparorbital ridges, shape and breadth of molars, angle of incisors, length and breadth of rostrum, and size of the tympanic bullae (cf. Robbins et al., 1980; Rosevear, 1969). As the genetic analysis identified two clades within *H. stella* and *H. parvus*, a comparison of our specimens with type specimens was done again after the genetic analysis, but it confirmed our first identification. Identification of *H. kaimosae* is uncertain as no direct comparison with the type specimen was made, and the locality of capture of these specimens (Mufindi in Tanzania) is rather far away from the type locality (Kakamega forest in Kenya). Thus, these specimens will be referred to as *H. cf kaimosae*. We also included samples of uncertain taxonomic origin, which could represent three undescribed or unrecognized taxa. The first one (taxon1) is morphologically close to *H. aeta* but has a distinct mammary formula and karyotype (Lecompte et al., 2005). The second one (taxon2) was previously identified by Lecompte et al. (2002b) as *H. parvus*. However, after a carefully examination of the skull of the four available specimens, one of us (W. Verheyen) concluded that it was a new undescribed species. Finally, the third undescribed species (taxon3) is morphologically close to *H. parvus*, but it has smaller molars.

Most mitochondrial sequences used in this paper are published for the first time and were deposited in the Gen-

Table 1
Reference and locality of collect of the specimens examined in this study

Putative species	Country	Locality	Code	References
Hylomyscus aeta	Cameroon	Dja reserve	Dja	**R14476**
	D.R.C.	Kikwit	Kik	915,2359,2727
	R.C.	Odzala N.P.	Odz	**R23181**
Hylomyscus alleni	Cameroon	Dja reserve	Dja	**R14659**, R14771
	C.A.R.	Bamingui Brendja	Bbr	NC0438
	C.A.R.	Ngotto	Ngo	R12381, R13001, R13800, R13839, R18723, R18835, R19304
	C.A.R.	Salo	Sal	R13187
	Gabon	Four-Place Quarry	Cfp	GA0093
	Gabon	Mvoum	Mvo	GA0013
	R.C.	Odzala N.P.	Odz	**R22966**
	R.C.	Great escarpment	Geo	R16844
Hylomyscus baeri	Guinea	Ziama	Zia	**P0712**, **P1540**
Hylomyscus kaimosae	Tanzania	Mbete	Mbe	4011
	Tanzania	Mufinfi	Muf	10871, **11036**
Hylomyscus parvus	Gabon	Forêt des Abeilles	Fab	**G10022**, R16137
	Gabon	Kili	Kil	**GA0256**
	Gabon	Monts Doudou	Mtd	GA1089
	R.C.	Odzala N.P.	Odz	R22243, **R22265**, **R23146**
Hylomyscus simus	Guinea	Ziama	Zia	**P0266**, P0716
	Ivory Coast	Taï N.P.	Taï	R24225, **R24278**
Hylomyscus stella	Cameroon	Dja reserve	Dja	R14014, R14028, R14220, R14477, R14510, R14703
	C.A.R.	Ngotto	Ngo	R12586, R12626, R13061
	C.A.R.	Salo	Sal	R13657, R13714
	D.R.C.	Kisangani (right bank of the Congo River)	Kis	Z2559, **Z2654**, Z2739
	Gabon	Forêt des Abeilles	Fab	G10003, G10006, R16004, **R16014**, R16038, R16132
	Gabon	Kili	Kil	GA0217, GA0225
	Gabon	Malounga	Mal	GA0131.GA0166, GA0199
	Gabon	Monts Doudou	Mtd	GA0293, GA0484, GA1158, GA1998, GA3549
	Gabon	M'Passa	Mpa	T0415
	Kenya	Kakamega	Kak	**SP5032**, SP5058
	R.C.	Great escarpment	Geo	R16841
	R.C.	Kouilou	Kou	T0799
	R.C.	Odzala N.P.	Odz	R22014, **R22018**, R22062, R22226, R22302, R22353, R22354, R23116
Taxon1	Gabon	Malounga	Mal	GA0132
	Gabon	Monts Doudou	Mtd	**GA0528**, GA0635, GA1204, **GA3646**
Taxon2	Cameroon	Korup N.P.	Kor	**SP10502**, SP10504, SP10507, **SP10514**
Taxon3	Gabon	Four-Place Quarry	Cfp	GA0087, GA0092, **GA0119**
	Cameroon	Dja reserve	Dja	**R14497**

Numbers in bold correspond to specimens selected for the reduced data set. All specimens were collected by team members of the Station Biologique de Paimpont and will be deposited at the National Museum of Natural History (Paris), except those captured in Kikwit (provided by H. Leirs), Mbete and Mufindi, (W. Verheyen), M'Passa (V. Nancé), Kouilou (L. Granjon and F. Catzeflis), Kakamega, and Korup (D. Schlitter).

Bank library (accession numbers DQ078137-DQ078228 for 16S rRNA sequences and DQ078229-DQ078245 for Cytb sequences), except Cytb sequences of the following specimens that were extracted from the GenBank database: *H. kaimosae* 11036 (GenBank Z83894); taxon2 SP10502 (GenBank AF518329), SP10514 (GenBank AF518330), and SP5032 (GenBank AF518331).

Two taxa belonging to the subfamily murinae were chosen as outgroups: *Rattus norvegicus* (GenBank X14848 for both 16S and Cytb) and *Mus musculus* (GenBank J01420 for both 16S and Cytb).

2.2. Mitochondrial DNA sequencing

DNA was extracted from ethanol-preserved muscle by the Chelex method (Walsh et al., 1991). For all specimens, the second half of the 16S rRNA gene was amplified using the primers 16Sar L (5′-CGCCTGTTTAACAAAAACAT-3′; Palumbi et al., 1991) and 16S-Hm (5′-AGATCACGTA GGACTTTAAT-3′; Quérouil et al., 2001). A large proportion of the Cytb was also amplified, for a restricted number of specimens, using Universal PCR primers L7 (5′-ACCA ATGACATGAAAAATCATCGTT-3′) and H15915

(5′-TCTCCATTTCTGGTTTACAAGAC-3′; Kocher et al., 1989). The PCR consisted of 30 cycles: 60 s at 94 °C, 60 s at 46 °C for the 16S rRNA gene and at 52 °C for the Cytb gene, and 90 s at 72 °C. The double-stranded PCR product was purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The samples were sequenced with both primers using an automated ALF Express DNA sequencer (Amersham Biosciences) and two different sequencing kits (Thermo Sequenase Cy5 Dye Terminator and Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kits) following the manufacturer's protocol (Amersham Biosciences). The cycle-sequencing reaction consisted of 30 cycles: 36 s at 94 °C, 36 s at 50 °C, and 80 s at 72 °C.

2.3. Data analyses

2.3.1. Patterns of sequence variation and saturation analyses

Sequences were aligned by CLUSTAL W version 1.5 (Thomson et al., 1994) with the default settings. For the 16S rRNA gene, the obtained alignment was optimized visually on the basis of the secondary structure of the 16S rRNA gene fragment for *Mus* (Burk et al., 2002).

Base composition was estimated for all taxa and homogeneity among taxa was tested using χ^2 tests of contingency tables of nucleotide counts, as implemented in PAUP. Base composition was estimated separately for stems and loops for the 16S rRNA gene, and for the three codon positions for the Cytb gene. Bias in base composition for each of the five resulting data sets was analyzed using χ^2 tests.

The amount of homoplasy was measured through the consistency index CI (Kluge and Farris, 1969) for each substitution type (i.e., C–T, A–G, A–C, A–T, and G–T) at each of the three codon positions separately (cf. Hassanin et al., 1998) for the Cytb gene, and separately for stem and loop regions for the 16S rRNA gene.

Following Philippe and Douzery (1994) and Hassanin et al. (1998), we examined the 16S rRNA and Cytb data sets for saturation for each substitution type. Using the matrices of patristic and inferred substitutions calculated by PAUP, the pairwise numbers of observed differences was plotted against the corresponding values for inferred substitutions (Philippe and Douzery, 1994) using the program TransPAUPMatrix (http://lis.snv.jussieu.fr/apps/contrib/TPM/TransPaupMatrix.htm). The slope of the linear regression (S) was used to evaluate the level of saturation (Hassanin et al., 1998). The slope equals one when no saturation is observed in the data set and tends toward zero as the level of saturation increases. Because of differential rates at codon positions, saturation plots for Cytb were analyzed separately at first, second, and third codon position. Likewise, stems and loops were assessed independently for the 16S rRNA gene.

2.3.2. Phylogenetic analyses

Phylogenetic inferences involved two steps:

(1) A neighbor-joining (NJ) phylogenetic analysis was conducted on all the 16S rRNA sequences ($n = 94$). Gaps were treated as missing data. Bootstrap replications (1000 replicates) were done in order to evaluate support for the main branches and to select taxa for further analyses. Pairwise uncorrected distances were calculated to assess within and among species differences. This approach allowed us to select a restricted data set containing 25 sequences (23 ingroup and 2 outgroup sequences) and representing all the species and most of the genetic diversity included in the entire data set. For each putative taxon, we selected the longest and best quality sequences. The first half of the Cytb gene was sequenced or extracted from GenBank for these 25 specimens.

(2) Several phylogenetic analyses were performed on the restricted data set. Phylogenetic trees were constructed using maximum parsimony (MP) and maximum likelihood (ML) approaches. Data of the two genes were first analyzed separately and then in combination. Before combination, the congruence between the two data sets was evaluated with a partition homogeneity test (Farris et al., 1995), implemented in PAUP 4.0b10 (Swofford, 2002).

All MP analyses were performed using the tree-bisection-reconnection (TBR) branch swapping option with 10 random addition replicates. We estimated the robustness of internal nodes by 1000 bootstrapping replicates (each with a single replication of random addition of taxa). MP analyses were conducted with either equal weighting or differential weighting of the character–state transformations using the consistency index (CI), the slope of saturation (S), and the product $CI * S$. The relevance of these weighting schemes was discussed by Hassanin and Douzery (1999) and Hassanin et al. (1998). These estimations of homoplasy were used to weigh each substitution type at each of the three codon positions for the Cytb gene and at stems and loops for the 16S rRNA gene. Unconstrained and constrained trees (constraining monophyly of species) were constructed and statistical comparisons of tree length were performed using the non-parametric KH test (Kishino and Hasegawa, 1993).

ML analyses were conducted on both separate and combined data sets. Prior to ML analyses, we used Modeltest 3.04 (Posada and Crandall, 1998) to select the substitution model which best fitted the data according to a hierarchical likelihood ratio test. Heuristic searches were performed using the selected model and the TBR branch swapping option with 10 random addition replicates. We computed likelihood ratio tests to evaluate branch length significance.

2.3.3. Divergence time estimates

We tested whether the data were consistent with the molecular clock hypothesis. Substitution rates among sequences were compared using the relative rate test as

implemented in the program RRTree (Robinson-Rechavi and Huchon, 2000). Relative rate tests were conducted at the species level. The two mitochondrial DNA regions (16S rRNA and Cytb) were analyzed separately. For non-coding regions (16S rRNA), relative rate tests were performed on the proportion of all substitutions types (K). For coding sequences (Cytb), relative rate tests were performed on the proportions of synonymous (K_s) and non-synonymous (K_a) substitutions. We used the *Mus*/*Rattus* dichotomy estimated at 12 million years (Myr) (Jacobs and Downs, 1994) to calibrate our molecular clock. We used distances produced by the ML analyses to estimate divergence times.

3. Results

3.1. Entire data set (94 specimens, 16S rRNA gene)

The alignment of the 94 sequences (92 ingroup + 2 outgroup sequences) comprises 512 nucleotides, of which 163 are variable and 95 are parsimony informative. The alignment of the 92 ingroup sequences comprises 101 variable characters and 85 being parsimony informative.

Grouping of haplotypes (NJ, Fig. 2) agrees with our morphological determinations for *H. aeta*, *H. alleni*, *H. baeri*, *H. cf kaimosae*, *H. simus*, taxon1, taxon2, and taxon3. These monophyletic groups are supported by high bootstrap values, ranging from 89 to 100%. Within species divergence (uncorrected pairwise distances) is small, ranging from 0.21% in *H. simus* to 0.58% in *H. cf kaimosae*. Between species divergence ranges from 2.52% between *H. alleni* and taxon2, to 6.90% between *H. cf kaimosae* and taxon1. Two specimens per species were retained for subsequent analyses, except for *H. cf kaimosae* for which only one Cytb sequence was available in GenBank.

Specimens morphologically identified as *H. stella* split in two clades; each clade being supported by high bootstrap values (93 and 100%, respectively): the first one (called later on *H. stella1*) includes all Central and East African specimens, and the second one (called later on *H. stella2*) includes all West Central African specimens. Within clade divergence is 0.53% in the first one and 0.43% in the second one. Between clade divergence is 2.48%. These two clades cluster with a low bootstrap value (51%). These results suggest that two cryptic forms may be present in the specimens morphologically identified as *H. stella*. Thus, two specimens of each of these two clades were retained in subsequent analyses.

Specimens morphologically identified as *H. parvus* also split in two clades; each clade being supported by high bootstrap values (93 and 92%, respectively). The first one (called later on *H. parvus1*) includes specimens from the Congo Republic (CR) and Gabon, while the second one (called later on *H. parvus2*) only includes specimens from Gabon. Within clade divergence is 0.76% in the first one and 1.16% in the second one. Between clade divergence is 3.44%. These two clades cluster with a low bootstrap value (52%). These results suggest that at least two cryptic forms could be present in the specimens morphologically identified as *H. parvus*. Thus, two specimens of each of these two clades were retained in subsequent analyses.

3.2. Restricted data set (25 specimens, 16S rRNA, and Cytb genes)

3.2.1. 16S rRNA

The alignment of the 25 sequences of 16S rRNA (23 ingroup + 2 outgroup sequences) comprises 512 nucleotides of which 152 are variable and 80 are parsimony informative. The alignment of the 23 ingroup sequences comprises 85 variable characters and 67 being parsimony informative.

Loops exhibit a bias in base composition (χ^2 test, $P < 0.05$), with an under-representation of guanine (13.4%) and cytosine (17.6%) and a higher representation of adenine (44.4%; Table 2). Stems show no significant bias in base composition ($P > 0.05$). The pattern of base composition in stems and loops is not significantly different among taxa (χ^2 test, $P = 1.000$).

The comparison of the raw CI and S values indicates that the amounts of homoplasy and the levels of saturation for a given type of substitution are greater for loops than for stems (Table 3). There is one exception to this pattern: the rarest type of substitution, A–T transversions, is more saturated in stems than in loops. Transitions are not more homoplastic and saturated than transversions, and high variation in the level of homoplasy and saturation exists between the different types of transitions and transversions. Among transitions, C–T substitutions are more homoplastic and saturated than A–G substitutions in both stems and loops. Among transversions, A–T substitutions are more homoplastic than other substitution types in both stems and loops.

The MP analysis with equal weighting of all types of substitutions leads to two most parsimonious trees of 313 steps. All weighted MP analyses lead to a single most parsimonious tree. Its length varies from 114,752 steps when CI $*$ S weights are used to 185,419 steps when CI weights are used. The hierarchical ratio test reveals that the model which best fits the data is the TrN model with an α parameter of 0.36 and a proportion of invariable sites of 0.49. All trees obtained tend to have similar ingroup topologies (Fig. 3A). The monophyly of the different species is well supported (87–100%), except for *H. stella* and *H. parvus*. West Central African specimens of *H. stella* do not form a monophyletic group with those from Central and East Africa. However, imposing a *H. stella1*/*H. stella2* clade in the MP analysis with an equal weighting scheme does not require additional steps. *H. parvus1* and *H. parvus2* do not cluster, or cluster with a low bootstrap value (<52%). *H. aeta* and taxon1 are sister species (76–88%), and always cluster with *H. baeri* (62–93%). *H. alleni* clusters with taxon2 (59–86%). *H. parvus1*, *H. parvus2*, *H. cf kaimosae*, and taxon3 form a monophyletic group (52–75%). In all analyses, *H. cf kaimosae* clusters with taxon3 (55–67%).

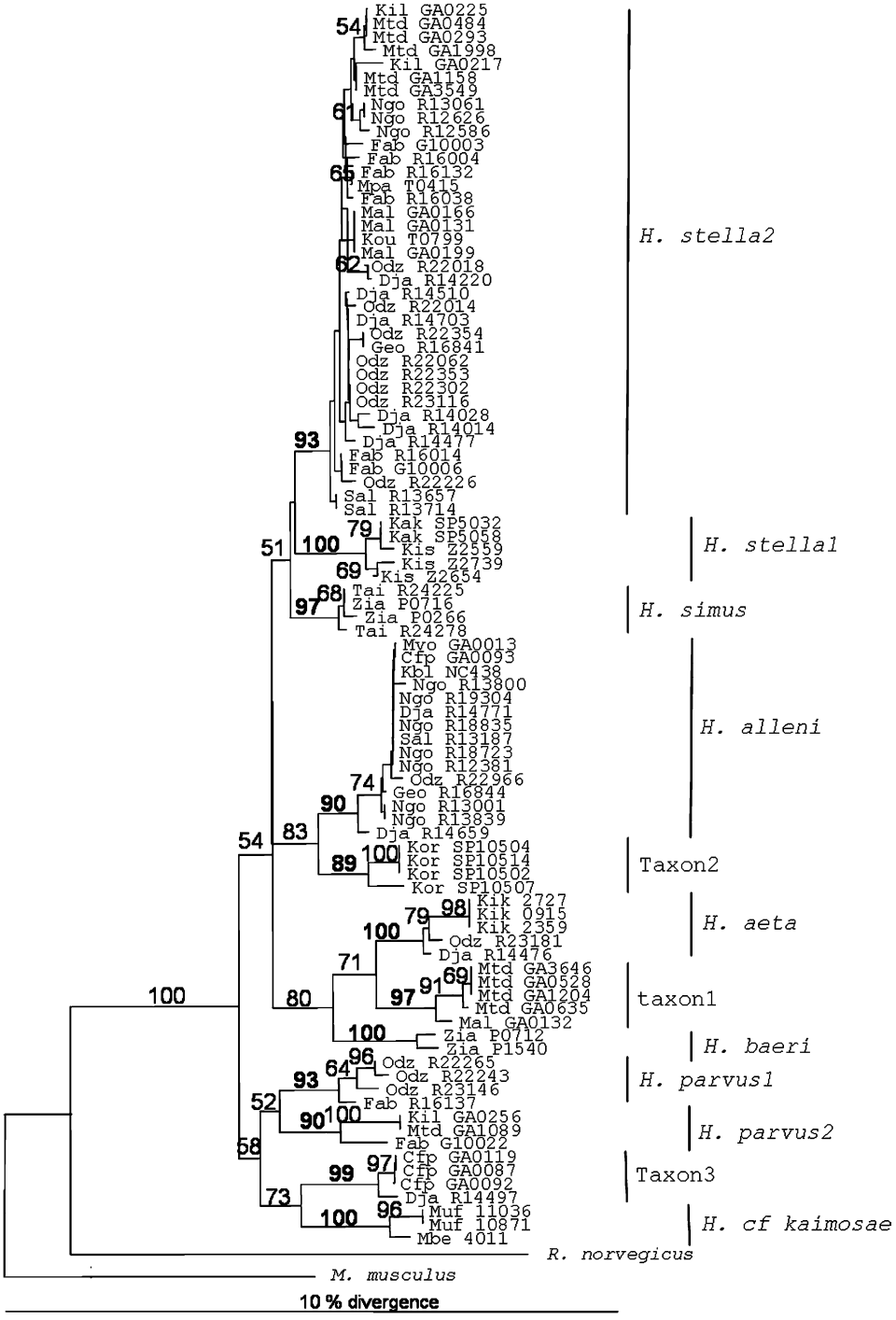

Fig. 2. Phylogenetic relationships among 16S rRNA sequences of 92 specimens of *Hylomyscus* inferred by NJ. The murid rodents *Mus musculus* and *Rattus norvegicus* were used as outgroups. The numbers above branches indicate bootstrap scores (1000 replicates). For locality name abbreviations see Table 1.

3.2.2. Cytochrome b

The alignment of the 25 Cytb sequences (23 ingroup + 2 outgroup sequences) comprises 421 nucleotides of which 144 are variable and 107 are parsimony informative. The alignment of the 23 ingroup sequences comprises 124 variable characters and 104 being parsimony informative.

At the three codon positions, all taxa are similar in base composition (χ^2 test, $P = 1.000$, 1.000, and 0.562, respectively, for the first, second, and third positions), having the same nucleotide frequency biases at each of the three codon positions. At the second codon position there is an under-representation of A (18.5%) and G (17.3%) and a higher representation of T (39.8%; Table 2). At the third codon position there is a marked under-representation of G (1.7%) and a higher representation of A (50.8%). At the first codon position there is no significant bias in nucleotide frequency ($P > 0.05$).

Table 2
Percentage (and mean standard deviation) base composition for the 16S rRNA (partitioned into stems and loops) and Cytb (according to codon position) genes

Nucleotide	16S rDNA		Cytb codon position		
	Stems	Loops	First	Second	Third
A	23.0 (0.5)	44.4 (1.2)	28.2 (0.8)	18.5 (0.3)	50.8 (3.5)
C	22.2 (0.9)	17.6 (0.7)	17.5 (1.5)	24.4 (0.4)	26.9 (3.9)
G	28.0 (0.3)	13.4 (0.6)	23.5 (1.0)	17.3 (0.5)	1.7 (1.3)
T	26.8 (0.8)	24.6 (1.1)	30.8 (1.5)	39.8 (0.7)	20.6 (2.6)

The comparison of the raw CI and *S* values indicates that the amounts of homoplasy and the levels of saturation for a given type of substitution are the greatest at the third position and the lowest at the second position of each triplet (Table 3). There is hardly any variable character at the second codon position. Contrary to expectations, transitions are not more saturated and homoplastic than transversions. Among transitions, C–T substitutions are more homoplastic and saturated than A–G substitution at every codon position. Among transversions, the substitution types which involve G rather than A are rarer and more strongly saturated.

The MP analysis with equal weighting of all types of substitutions leads to two most parsimonious trees of 383 steps. The CI and weighted MP analyses lead to a single most parsimonious tree, and the CI $*$ *S* weighted analysis leads to two most parsimonious trees. Length of tree varies from 83,980 steps when CI $*$ *S* weights are used to 213,390 steps when CI weights are used. The hierarchical ratio test reveals that the model which best fits the data is the TrN model with an α parameter of 1.15 and a proportion of invariable sites of 0.57. As for the 16S rRNA data set, the monophyly of the different species

Table 3
Number of variable and informative sites, amount of homoplasy measured through the consistency index (CI), intensity of saturation evaluated by the slope of the linear regression (*S*), and products CI $*$ *S* for each substitution type for the 16S rRNA (partitioned into stems and loops) and Cytb (according to codon position) genes, used for weighting MP trees

	Nb of variable sites	Nb of informative sites	Amount of homoplasy (CI)	Level of saturation (slope *S*)	Product CI $*$ *S*
16SrDNA					
Loops					
A–C	60	36	0.725	0.520	0.377
A–G	48	25	0.788	0.622	0.490
A–T	73	47	0.702	0.635	0.446
C–G	26	10	0.906	0.510	0.462
C–T	65	34	0.643	0.544	0.350
G–T	36	14	0.927	0.762	0.706
Stems					
A–C	7	2	1.000	0.789	0.789
A–G	9	4	1.000	0.995	0.995
A–T	8	4	0.800	0.558	0.446
C–G	2	0	1.000	0.543	0.543
C–T	15	6	0.789	0.721	0.569
G–T	4	0	1.000	1.000	1.000
Cytb					
First positions					
A–C	7	1	1.000	0.876	0.876
A–G	7	4	0.778	0.802	0.624
A–T	2	1	1.000	0.881	0.881
C–G	2	0	1.000	0.185	0.185
C–T	13	9	0.619	0.569	0.352
G–T	1	0	1.000	0.041	0.041
Second positions					
A–C	0	0			
A–G	2	0	1.000	1.000	1.000
A–T	1	0	1.000	0.917	0.917
C–G	0	0			
C–T	3	2	0.750	0.866	0.650
G–T	0	0			
Third positions					
A–C	43	22	0.710	0.546	0.388
A–G	27	15	0.771	0.521	0.402
A–T	40	23	0.769	0.401	0.308
C–G	15	7	0.938	0.377	0.354
C–T	76	65	0.385	0.194	0.075
G–T	11	6	1.000	0.199	0.199

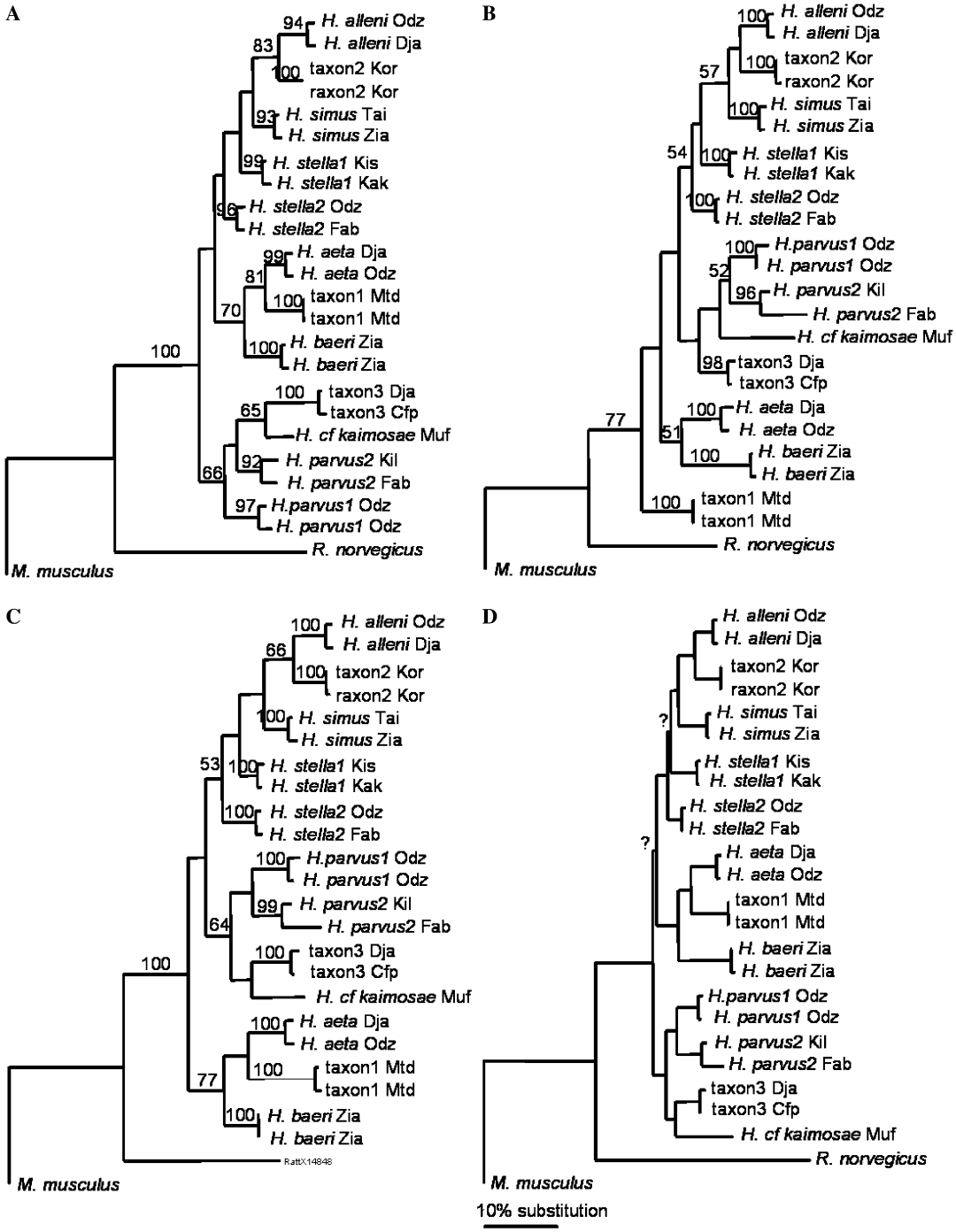

Fig. 3. Results of MP and ML analyses based on 16S (A), Cytb (B) and combined 16S + Cytb (C and D) sequences. (A) MP with *S* weighting; (B) MP with CI ∗ *S* weighting, (C) MP with CI weighting, and (D) ML with TrN + I + G model. Numbers above branches indicate bootstrap scores (1000 replicates). Question marks indicate branch length not significantly different from zero, according to a likelihood ratio test. For locality name abbreviations see Table 1.

is well supported in all trees (Fig. 3B; 86–100%), except for *H. stella* and *H. parvus*. West Central African specimens of *H. stella* do not cluster with those from Central and East Africa. Constraining the *H. stella1*/*H. stella2* clade in the MP analysis with an equal weighting scheme did not require additional step. Depending on the analyses, *H. parvus1* does not cluster with *H. parvus2* or clusters with a low bootstrap value (<55%). In all analyses, there is little resolution above the species level, bootstrap values being mainly under 60%.

3.2.3. Combined data set

The partition homogeneity test suggests that the data partitions (16S rRNA and Cytb) did not undergo significantly different processes or patterns ($P = 0.61$). The

combined data set is 932 bp long, with 295 variable characters, 186 being parsimony informative.

All the MP analyses lead to a single most parsimonious tree. Its length varies from 698 steps when all substitutions are equally weighted to 496,602 steps when CI weights are used. The hierarchical ratio test reveals that the model which best fits the data is the TrN model with an α parameter of 0.58 and a proportion of invariable sites of 0.52. In all analyses, the monophyly of most species is highly supported (Fig. 3C and D; 99–100%). *H. parvus1* always clusters with *H. parvus2*, but the support of this node varies from one tree to another (53–72%). *H. stella1* and *H. stella2* do not cluster, or cluster with a bootstrap value lower than 50%. There is little resolution above the species level. Only two clades are supported in all analyses: (1) *H. aeta*, taxon1 and *H. baeri* form a monophyletic group (70–89%), and (2) *H. alleni* and taxon2 always cluster with a high bootstrap support (66–82%). In the ML analysis and in the MP analyses with equal or CI weights, *H. parvus1*, *H. parvus2*, taxon3, and *H. cf kaimosae* cluster (64–84%). Phylogenetic relationships between other taxa vary from one tree to another, and the support of these nodes is generally low (<60%).

3.3. Divergence times

The relative rate test indicates no significant rate heterogeneity for 16S rRNA and Cytb between species. Thus, the estimation of divergence times based on a molecular clock is justified. The ML distance between *Mus* and *Rattus*, which diverged 12 Myr ago, is 0.440. This value gives a rate of 0.037 ML distance per million years. Most divergence events between *Hylomyscus* species are estimated to have occurred about 4–5 Myr ago (Fig. 4). The most ancient divergence event (between *H. baeri* and *H. cf kaimosae*) occurred 5.9 (±0.1) Myr ago, and the most recent one (between *H. alleni* and taxon3) occurred 1.8 (±0.1) Myr ago. The divergence between *H. baeri* and its sister species (taxon1 and *H. aeta*) occurred 2.7 (±0.0) to 3.6 (±0.1) Myr ago. The divergence between *H. simus* and its sister species (*H. stella1*, *H. stella2*, taxon2, and *H. alleni*) occurred 2.2 (±0.1) to 2.7 (±0.1) Myr ago.

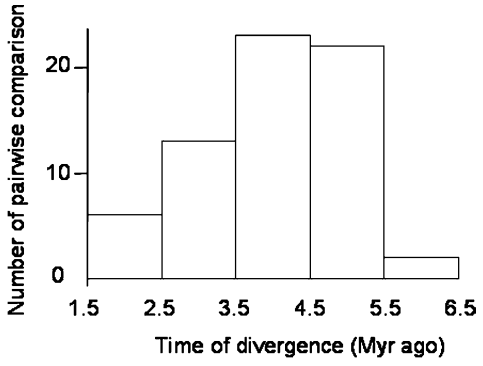

Fig. 4. Histogram of the estimated time of divergence (in Myr) between pairs of species.

4. Discussion

4.1. Properties and resolving power of the analyzed gene fragments

Most of our taxonomic knowledge of the systematic of the genus *Hylomyscus* is based on morphological and cytological data. Recently, two molecular Cytb-based phylogenies including three to five species of the genus *Hylomyscus* were published (Lecompte, 2003; Lecompte et al., 2002b). Our study includes a higher number of species. Both the 16S rRNA and the Cytb genes proved to be suitable molecular markers for diagnosis of *Hylomyscus* species. Nevertheless the resolving powers of these two genes differ, and with both markers (either analyzed singly or in combination), some nodes remain unresolved. This could be due to the relatively low number of substitutions which could either be related to the length of the gene fragments used or to the fact that they evolved too slowly. According to our results there is little saturation of the 16S rRNA gene. For the Cytb gene, the level of saturation is high at the third codon position. However, a differential weighting of the different types of substitutions at each of the three codon position did not increased tree resolution. Thus, it is improbable that the lack of resolution is due to the fact that these two genes evolved too fast. Alternatively, it can reflect either very short basal branches due to a polytomic radiation or a series of rapid consecutive cladogenetic events that cannot be resolved by the information embedded in the two gene fragments. Phylogenetic relationships between species are better resolved with the 16S rRNA than with the Cytb gene. The Cytb gene is extensively used in the literature data to infer phylogenetic relationships within murid genera. In some studies it shows a high resolving power (e.g., Barome et al., 1998; Ducroz et al., 1998), whereas in others it does not (e.g., Myers et al., 1995).

Stems and loops of the 16S rRNA gene exhibit differences in base composition, substitution patterns and rates of evolution, which are typical of the mammalian 16S rRNA gene (Burk et al., 2002). The three codon positions of the Cytb gene exhibit differences in base composition and substitution patterns. The Cytb is a protein-coding gene and its evolution is constrained so that the gene product remains functional. As previously observed (e.g., Hassanin and Douzery, 1999; Irwin et al., 1991), the highest levels of homoplasy and saturation are observed at third codon positions, for which almost all T_i and most T_v are synonymous.

4.2. Taxonomic implications

Our molecular data allowed us to confirm some previous hypotheses based on morphological or cytological data, to reassess the taxonomy of several species, and to discover five potentially new species.

For all but two species, the molecular phylogeny was congruent with our species assignment based on external morphology and cranio-dental features of the specimens.

The bootstrap support for each of these species was high (87–100%) and genetic divergence (16S rRNA gene) was considerably lower within (0.21–0.58%) than between species (2.52–6.90%). Pairs of species were separated by 13 to 35 diagnostic substitutions in the 512 bp of the 16S rRNA gene that we examined. The 16S rRNA gene was rarely used to investigate intra-generic phylogeny of murid rodents. Our results on among species sequence divergence are similar to those found within the genus *Praomys* (2.24–6.59%, Nicolas et al., 2005).

It has recently been proposed that DNA-based systems could soon provide a general solution to the problem of species identification in many groups (Hebert et al., 2003). Our results suggest that the 16S rRNA gene could be used to identify species of the genus *Hylomyscus* based on the percentage of sequence divergence and tree topology. Three observations support this hypothesis: (1) these sequences are easy to obtain, the same primers being used for a wide variety of taxa; (2) the high level of 16S rRNA divergence between species of the genus *Hylomyscus* contrasts with the low intra-specific values that we observed; and (3) bootstrap support for each species was high. For most species, our sampling involved specimens coming from multiple allopatric populations. Thus we are confident with our estimations of intra- and inter-specific sequence divergence. However, one inconvenience of the 16S rRNA gene for barcoding is that alignment of sequences is not straightforward due to the presence of indels. The Cytb does not suffer this limitation. The limited data available suggests that this gene might also be used for species identification purpose within the genus *Hylomyscus* (Fig. 3B), but further analyses are required to evaluate the validity of this statement. Moreover, additional studies including mountain species and all East African forms have to be performed.

It seems plausible that *H. stella* represents two cryptic species: one inhabiting Central and East Africa (DRC and Kenya), and the other one inhabiting West Central Africa (Cameroon, CAR, Gabon, CR). Four observations support this hypothesis: (1) Maddalena et al. (1989) showed a specimen from Burundi to have a distinct karyotype from specimens from Cameroon; (2) the two taxa do not cluster together in the phylogenetic trees; (3) the genetic divergence is low within each taxon (<0.53%); and (4) the genetic differentiation between these taxa (2.48%) is similar to that found between other species of the genus. Considering that *H. stella* was described from Ituri forest in DRC (Fig. 1), the taxon from Central and East Africa (i.e., *H. stella1*) probably refers to the true *H. stella*, while that from West Central Africa (i.e., *H. stella2*) would be a new species. However, studies of intra- and inter-populational morphometric variability, as well as a careful comparison with the holotype, are needed before a final decision can be taken.

Our results also highlight the possibility that *H. parvus* could include two cryptic species with a parapatric distribution in the Forêt des Abeilles (Gabon). Based on the localities of capture, *H. parvus1* probably refers to the true *H. parvus* (described from Belinga in Gabon, on the right bank of the Ogooue River; Fig. 1). Estimates of genetic divergence within (<1.16%) and between (3.44%) these clades is similar to those found within and between other species of the genus, respectively. These two clades do not cluster, or cluster with a low bootstrap support (<55%), in phylogenetic analyses of the 16S and Cytb data sets; while they cluster with a higher bootstrap support when 16S and Cytb data are combined (53–72%). Nonetheless, in the absence of any morphological or cranio-dental difference between these two taxa, the two clusters might well represent population level differences within the same species. Sequencing specimens from more localities is required to get a better understanding of the geographical distribution of each group. A particular emphasis should be put on the Forêt des Abeilles, where the two clusters are present. Additional morphological and karyological analyses will be necessary to determine whether the two clusters correspond to distinct species.

In the most recent rodent check-list (Musser and Carleton, in press), *simus* and *kaimosae* are considered as subspecies of *H. alleni* and *H. stella*, respectively. However, based on our molecular data, there is little doubt that they are distinct species: (1) *H. cf kaimosae* and *H. stella*, or *H. simus* and *H. alleni* are never sister taxa (each other's closest relative); and (2) the genetic distances between *H. alleni* and *H. simus* (3.50%) or between *H. cf kaimosae* and *H. stella* (4.94%) are similar to those found between other species of the genus. *H. simus* and *H. alleni* seem to be allopatric, the former only occurs in West Africa, while the latter is only found in West Central Africa. In contrast, *H. stella* and *H. cf kaimosae* probably have overlapping distribution ranges, at least in the Eastern part of their range (e.g., Kakamega). However, additional morphological analyses are necessary to compare *H. stella* and *H. kaimosae* from Kakamega forest, as well as the holotype of *H. kaimosae* and *H. cf kaimosae*.

For three potentially new species identified based on morphological and/or cytological data (taxon1, taxon2, and taxon3), our molecular data confirm that they form three distinct groups of haplotypes. Moreover, levels of genetic divergence within and between these taxa are congruent with those observed for other species within the genus. Before describing these three potential species, a detailed comparison with the type specimens of all described subspecies will have to be done.

4.3. Phylogenetic relationships between species

Based on cranio-dental morphology, *Hylomyscus* species were divided into three species groups (Robbins et al., 1980). The "*aeta*" group includes four species (*H. aeta*, *H. baeri*, *H. carillus*, and *H. denniae*) and is characterized by the presence of: (1) a broad, wedge-shaped interorbital area, (2) supraorbital ridges, (3) relatively large molars, and (4) a less fragile skull. The "*alleni*" group includes *H. alleni*, *H. stella*, *H. simus*, and *H. kaimosae*, and is characterized by the presence of a biconcave interorbital area and narrow incisors and molars. The last group only includes *H. parvus*;

this species being characterized by its small size, inflated braincase, pro-odont incisors, and the shape of its lower molars. In agreement with this classification, the three taxa presenting the skull characteristics of the "*aeta*" group included in our study (i.e., *H. aeta*, *H. baeri*, and taxon1) do cluster with high bootstrap values (bootstrap value ranges: 62–93% for the 16S rRNA gene, and 70–89% for the 16S and Cytb genes combined), except for the Cytb analyses. The other two usually recognized groups are not fully supported by our molecular data: while *H. alleni*, *H. simus*, *H. stella1*, and *H. stella2* often cluster with low bootstrap support, *H. cf kaimosae* clusters with representatives of the "*parvus*" group (*H. parvus1*, *H. parvus2*, taxon3; 16S rRNA gene and both genes combined).

4.4. Temporal and spatial insight into Hylomyscus evolution

This study allowed us to specify the geographical distribution of several species and to date divergence events within the genus. However, because age estimates are known to yield large confidence limits, the values obtained should be interpreted cautiously (Hillis et al., 1996) and viewed as preliminary hypotheses of the timing of important events in the history of the genus. Our molecular data suggested that *Hylomyscus* species diverged during a rapid diversification event that occurred 2–6 Myr ago, with most divergence events occurring 4–5 Myr ago. This result is congruent with the estimate of Lecompte (2003) based on the Cytb gene alone. It adds evidence to the recent observation that most speciation events in tropical rainforests vertebrate taxa predate the Pleistocene (Moritz et al., 2000).

Hypothesis concerning factors that promote speciation in tropical faunas are numerous (for reviews see Bush, 1994; Haffer, 1997). By and large, these hypotheses have been preoccupied with the geographic context of speciation and, for allopatric models, the physical causes of isolation (rivers and forest/savanna). Three main models were proposed. The refugia model has been the most widely discussed and rests on the premise that climatic changes caused rainforests to contract to refugia separated by dry forests and savanna and that this isolation promoted speciation through the accumulation of genetic differences over time. Initial discussions focused on Pleistocene events (Diamond and Hamilton, 1980; Haffer, 1969), but was later on extended to tertiary events (Haffer, 1997). The second major class of allopatric models invokes substantial river systems as barriers to gene flow, such that populations on either side gradually diverge to form separate species. Finally, the gradient model suggests that strong environmental gradients resulted in adaptive divergence and speciation. Different predictions can be made with these three models (Moritz et al., 2000). For example, the gradient model predicts that sister taxa should occupy distinct but adjacent habitat. All *Hylomsyscus* species inhabit forest or dense vegetation and, up to now, no study shows the existence of *Hylomyscus* species adapted to adjacent but distinct environments (e.g., rainforest–dry forest). Thus, the gradient model fails to explain the diversification within this genus. Testing of the riverine barrier hypothesis requires that speciation events are recent (end of the Quaternary), while most divergence events between *Hylomyscus* species were estimated to date back to the Pleistocene (Fig. 4). The refuge hypothesis might well explain the diversification of the genus. The study area is presently covered by rainforest, but this has not always been the case. The period of diversification of *Hylomyscus* species correspond to a period of pronounced shifts in African climate, which resulted in major changes in the distribution and composition of the vegetation (Morley, 2000). The Late Miocene (10–5 Ma) was characterized by a period of expansion of savanna, while the Early Pliocene (5–3.5 Ma) was characterized by moist climates, expansion and diversification of rain forests, and retraction of savanna. Finally, the Late Pliocene (3.5–1.6 Ma) corresponded to pronounced climatic changes with several drying and cooling phases, resulting in an extension of savannas and open environments in tropical Africa and concomitant contraction of humid forests. These climatic and environmental changes during the late Pliocene and Miocene, could have promoted speciation within the genus *Hylomyscus*. To correctly evaluate the putative role of refugia and rivers in the diversification of *Hylomyscus* species, one would need a fully resolved phylogeny, a precise knowledge of the geographical distribution of the species and data on intra-specific genetic variability. Clearly, our study is only the first step toward this knowledge. Given the presently available data, we can only hypothesize on the putative role of rivers and forest/savanna as barriers to *Hylomyscus* dispersal.

Only two species of *Hylomyscus* are recorded in West Africa (*H. simus* and *H. baeri*) and both are endemic to this region. *H. baeri* was previously known only from Ivory Coast and Ghana, but we showed that it is also present in Guinea. Similarly, several forest mammal species have a distribution range restricted to West Africa (Happold, 1996; Renaud, 1999). West African rainforests are separated from the Central African rainforests by the Dahomey gap, where Sudanian savannahs extend all the way to the sea and interrupt the forest cover for approximately 200 km, in Togo and Benin (Maley, 1996). The Dahomey gap may be as old as 3 Myr, since sedimentological evidence shows an increase in aridity and dust supply in West Africa at that time (Robert and Chamley, 1987). During the more arid and cold periods, this savannah corridor was probably wider than it presently is. It may have acted as an effective barrier preventing the dispersal of rainforest species, and has potentially led to allopatric speciation. In agreement with this hypothesis, the split between either *H. baeri* or *H. simus* and their sister species occurred 2.2–3.6 Myr ago. The Dahomey gap did not persist throughout the last 3 Myr: forest expansion completely filled the gap at least between 115,000 and 129,000 and between 4500 and 8400 years ago (Dupont et al., 2000; Salzmann and Hoelzmann, 2005). Although it seems logical to assume that during these periods *Hylomyscus* species were able to move

between these two rainforest blocks, this is not what appears to have happened. The observation that *H. baeri* and *H. simus* are restricted to the western forest block suggests that another barrier has prevented their dispersal. According to several authors, the main barrier to dispersal for many mammalian species would not be the Dahomey gap itself, but the Niger and the Volta Rivers which are bordering the gap (e.g., Booth, 1958; Robbins, 1978).

The paucity of *Hylomyscus* species in West Africa contrasts with the high diversity found in Central Africa. Four species were previously known to co-occur in West Central Africa (*H. aeta*, *H. alleni*, *H. stella*, and *H. parvus*). Our results suggest that up to eight species may actually co-occur in this region (*H. aeta*, *H. alleni*, *H. stella2*, *H. parvus1*, *H. parvus2*, taxon1, taxon2, and taxon3). In some forest localities, up to four species were captured in sympatry (e.g., Odzala, Dja). In the central African forests, the complex river system is presumed to be a major ecological factor that determines the geographic distribution of terrestrial organisms (Colyn, 1991; Colyn and Deleporte, 2002; Colyn et al., 1991; Deleporte and Colyn, 1999; Grubb, 1990; Happold, 1996). In agreement with this hypothesis, it seems that the Oubangui and Congo Rivers limit the eastward distribution of *H. alleni* and *H. stella2*. This is not the case for all species, as we found *H. aeta* both on the right (Cameroon, CR) and the left (Kikwit, in DRC) bank of the Congo River. Dudu (1989) found significant morphometrical differences between populations of *H. parvus* captured in Cameroon and in DRC. Unfortunately, we were not able to include specimens from DRC in the present study. Thus, it is not possible to discuss the taxonomic implications of these differences. *H. stella2* could be limited westward by the Dahomey gap, as its western distributional limit seems to be the Gambari forest in Nigeria (Happold, 1977). Two rivers (the Cross and Sanaga Rivers) often act as geographical barrier to West Central African mammal dispersal (Happold, 1996). As the exact western limit of the geographical distribution of other species of *Hylomyscus* inhabiting West Central Africa remains unknown, it is impossible to conclude on the role of these two potential barriers on the distribution of *Hylomyscus* species.

5. Conclusion

The number of species within the genus *Hylomyscus* is higher than suggested by the latest revision of the genus (Musser and Carleton, in press). Two taxa previously considered as subspecies are monophyletic and appear not to be the sister taxa to the species with which they were synonymized. They should be elevated at the species level (*H. simus*, and possibly *H. kaimosae*). Moreover, five potential new species were identified, which need to be further investigated or described. Although the present study covers an important number of species, it provides a still incomplete taxonomic revision of the genus, as mountain species and several East African forms were not included in the analysis. In addition, some species identifications will remain ambiguous as long as the cranial features of these specimens are not directly compared to those of the relevant types. To ascertain species identification it would also be interesting to sequence specimens from type localities or to sequence the type specimens themselves. The phylogenetic relationships between the analyzed species are still largely unresolved, which is probably explained by the fact that these species emerged as the result of a series of rapid diversification events that occurred 2–6 Myr ago, with most divergence events occurring 4–5 Myr ago.

Our results illustrate the usefulness of DNA barcoding, especially when coupled with traditional taxonomic tools, in disclosing hidden diversity (Hebert et al., 2004a,b). Moreover, it adds to the evidence that cryptic species are common in tropical regions, a critical issue in efforts to document global species richness (Berkov, 2002; Hebert et al., 2004a; Wilcox et al., 1997).

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