Systematics of African lowland rainforest *Praomys* (Rodentia, Muridae) based on molecular and craniometrical data

V. NICOLAS[1,2]*, E. VERHEYEN[3], W. VERHEYEN[4], J. HULSELMANS[4], M. DILLEN[5], B. AKPATOU[2,5,6], A. DUDU[7], W. WENDELEN[8] and M. COLYN[2]

[1]*MNHN, Département de Systématique et Evolution, UMR 5202, USM 601, Laboratoire Mammifères et Oiseaux, 55 rue Buffon, 75005 Paris, France*
[2]*UMR CNRS 6552–6553, Université de Rennes 1, Station Biologique, 35380 Paimpont, France*
[3]*RBINS, Vertebrate department, Vautierstraat 29, 1000 Brussels, Belgium*
[4]*UA, Department of Biology, Groenenborgerlaan 171, 2020 Antwerp, Belgium*
[5]*Center for Tropical Research, University of California, Los Angeles, USA*
[6]*Université de Cocody, UFR Bio-Sciences, Laboratoire de zoologie et biologie animale, Université de Cocody, Abidjan, Republic of Ivory Coast*
[7]*Université de Kisangani, Kisangani, Democratic Republic of Congo*
[8]*RMCA-Tervuren, Zoology department, Leuvense steenweg 13, 3080 Tervuren, Belgium*

Representatives of the genus *Praomys* occur throughout the African intertropical zone. It is unclear how many species this genus contains, nor do we know the exact distribution ranges and phylogenetic relationships of these taxa. Using molecular (16S rRNA gene sequencing) and morphological (multivariate craniometry) analyses we clarify the taxonomy and phylogenetic relationships among the *Praomys* occurring in Africa's lowland tropical rainforests. We studied most species known from this area, based on specimens collected in seven countries (Guinea, Ivory Coast, Cameroon, Central African Republic, Gabon, Congo Republic and Democratic Republic of Congo). In our study, *Praomys* appears to be monophyletic. Our results identify two species complexes: the *jacksoni* complex includes at least two species (*P. jacksoni* and *P. mutoni*) and the *tullbergi* complex contains at least four species (*P. tullbergi*, *P. rostratus*, *P. misonnei*, *P. petteri*). Although the 16S rRNA gene appears insufficient to resolve the phylogenetic relationships among all the members of the *tullbergi* species complex, it is suitable for the identification of most of the studied species, and its use has allowed us to redefine the geographical limits of several species. © 2005 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2005, **145**, 539–553.

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INTRODUCTION

The African genus *Praomys* is widely distributed in the intertropical zone. Its species are morphologically similar, rendering species taxonomy unclear. Musser & Carleton (in press) recognized 15 species: *P. daltoni* (Thomas, 1892), *P. degraaffi* Van der Straeten & Kerbis Peterhans, 1999, *P. delectorum* (Thomas, 1911), *P. derooi* Van der Straeten, 1978, *P. hartwigi* Eisentraut, 1968, *P. jacksoni* (DeWinton, 1897), *P. lukolelae* (Hatt, 1934), *P. minor* Hatt, 1934, *P. misonnei* Van der Straeten & Dieterlen, 1987, *P. morio* (Trouessart, 1881), *P. mutoni* Van der Straeten & Dudu, 1990, *P. petteri* Van der Straeten, Lecompte & Denys, 2003, *P. rostratus* Miller, 1900, *P. tullbergi* (Thomas, 1894) and *P. verschureni* Verheyen & Van der Straeten, 1977. *P. daltoni* and *P. derooi* are usually allocated to *Myo-*

*Corresponding author. E-mail: vnicolas@mnhn.fr

mys (= *Myomyscus*; Rosevear, 1969; Van der Straeten & Verheyen, 1978; Musser & Carleton, 1993), and *P. verschureni* is usually allocated to *Malacomys* (Musser & Carleton, 1993; Verheyen & Van der Straeten, 1977). The affiliation of these three last species to the genus *Praomys* is only partly supported by molecular data (Lecompte *et al.*, 2002b; Lecompte, 2003), and they were not considered in our study.

Four of these species are restricted to mountain forests: *P. morio* and *P. hartwigi* in the Cameroon region, and *P. degraaffi* and *P. delectorum* in eastern Africa. Little is known about the taxonomy and distribution ranges of the eight species occurring in lowland forests. The taxonomical problems concerning these *Praomys* are summarized in the following paragraphs.

1. The taxonomic status of some populations of Cameroon, Congo Republic (C.R.) and Central African Republic (C.A.R.) has repeatedly changed: first called *P. morio* by Matthey (1965) and Petter (1965), they were later identified as *P. lukolelae* (Petter, 1975; Genest-Villard, 1980; Granjon, 1991; Chevret *et al.*, 1994), later named as *Praomys* sp. (Lecompte, Denys & Granjon, 2001; Weltz, 1998; Lecompte, Granjon & Denys, 2002a), until the new species *P. petteri* was described by Van der Straeten *et al*. (2003). Three specimens morphologically similar to *P. petteri* but with a distinct karyotype ($2n = 42$, autosomal fundamental number (NFa) = 40; instead of $2n = 42$, NFa = 62 in *P. petteri*) are known from Pointe Noire, C.R. (Matthey, 1963) and Doudou Mounts, Gabon (Lecompte *et al.*, 2005). In the absence of the C-banding pattern of the type and paratypes of *P. petteri* it is not possible to identify the cause of the NFa variation observed. Additional karyological data are needed to clarify the significance of the observed differences: either *P. petteri* is karyotypically polytypic or two cryptic species are present.

2. Some populations from Gabon (Duplantier, 1982) and C.A.R. (Petter, 1975; Genest-Villard, 1980) have been identified as a subspecies of *P. tullbergi* called *minor*, without supporting evidence. All these specimens were not included in the last revision by Van der Straeten *et al*. (2003), who only considered *P. tullbergi* specimens from West Africa. The terminology is quite confusing, but *P. t. minor* is distinct from *P. minor*.

3. *P. misonnei* is morphologically very similar to *P. tullbergi*. According to Musser & Carleton (1993, in press), the possibility that *P. misonnei* simply represents populations of *P. tullbergi* at the eastern margins of its geographical range needs to be considered. Although the eastern limit of the distribution range of *P. misonnei* is known to be somewhere in the north of the Democratic Republic of Congo (D.R.C.) the identity of some specimens from Uganda and Kenya remains controversial (Qumsiyeh *et al.*, 1990; Lecompte *et al.*, 2002a, b; Musser & Carleton, in press).

Employing mainly craniometric data, Van der Straeten & Dieterlen (1987) and Van der Straeten & Dudu (1990) divided *Praomys* into three species complexes: (1) the *tullbergi* complex (*P. hartwigi*, *P. tullbergi*, *P. misonnei*, *P. morio*, *P. petteri*, *P. rostratus*); (2) the *jacksoni* complex (*P. degraaffi*, *P. jacksoni*, *P. minor*, *P. mutoni*); and (3) the *delectorum* complex (with only one species: *P. delectorum*). Except for the results of one morphologically based cladistic study of nine species (Lecompte *et al.*, 2002a), the phylogenetic relationships within and among *Praomys* species complexes remain largely unknown. Thus far, three molecular studies that included some species of *Praomys* and related genera were performed: one was based on DNA/DNA hybridization (Chevret *et al.*, 1994) and two were based on DNA sequences (cytochrome *b* sequences, Lecompte *et al.*, 2002b; IRBP sequences, Lecompte, 2003).

The aim of our study is to revise the taxonomy of tropical forest lowland *Praomys*, based on specimens collected in seven countries (Guinea, Ivory Coast, Cameroon, C.A.R., Gabon, C.R. and D.R.C.). The use of mitochondrial DNA has 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 body of literature propagates the usefulness of DNA sequences as taxon 'barcodes' (Hebert *et al.*, 2002, 2004a, b; Moritz & Cicero, 2004). To untangle the above-mentioned taxonomic problems, we used nucleotide sequences of the 16S rRNA gene fragment and cranio-dental measurement data from the same specimens. We performed craniometric analyses to test if monophyletic clades that can be identified by mtDNA sequences for specimens that co-occur in West Central Africa (i.e. Gabon, C.A.R., R.C.) can also be identified based on morphometrical features. This approach was also used to measure the degree of craniometrical similarity between members of the *tullbergi* species complex. Finally, by using a carefully calibrated molecular clock, we estimated divergence times between the different *Praomys* species occurring in the African lowland rainforests, which provides context for recovering the evolutionary history of species in the African lowland rainforest.

MATERIAL AND METHODS

In this study, we use the name *Praomys* 'MI' to designate *P. t. minor* specimens collected in West Central Africa.

MOLECULAR ANALYSIS

Species included in this study (Appendix 1 and Fig. 1)
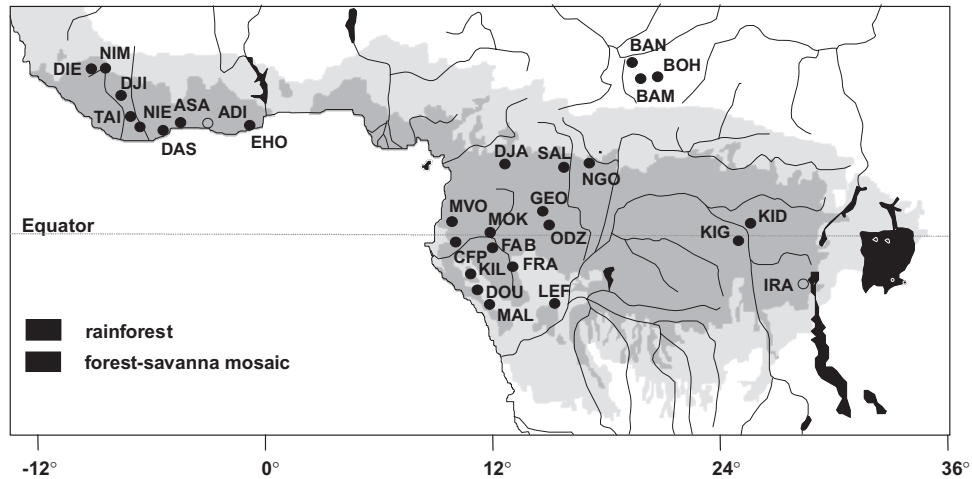A careful examination of the skulls of all specimens of *Praomys* ($N = 1219$) that were collected by team mem-

Figure 1. Location of collecting localities of the *Praomys* specimens included in our study. Black circles: collecting localities of the specimens included in our molecular analyses; white circles: collecting localities of the specimens only included in our morphometrical analyses. ADI, Adiopodoumé; ASA, Assagny; BAM, Bamingui-Brendja; BAN, Bangoran; BOH, Bohou; CFP, Carrière Four Place; DAS, Dassieko; DIE, Diécké; DJA, Dja; DJI, Djidoubaye; DOU, Doudou Mounts; EHO, Ehotile; FAB, forêt abeilles; FRA, Franceville; GEO, Grand Escarpement Odzala; IRA, Irangi; KID, Kisangani, right bank of the Congo River; KIG, Kisangani, left bank of the Congo River; KIL, Kili; LEF, Léfini; MAL, Malounga; MOK, Mokekou; MVO, Mvoum; NGO, Ngotto; NIE, Niégré; NIM, Mont Nimba; ODZ, Odzala National Park; SAL, Salo; TAI, Taï National Park.

bers in West Central Africa over the past 10 years allowed us to select 67 specimens from three species (*P. jacksoni*, *Praomys* 'MI' and *P. petteri*) for our molecular analyses (Appendix 1).

In these analyses we also included samples of *P. tullbergi* (*N* = 8) and *P. rostratus* (*N* = 19) from West Africa (Guinea and Ivory Coast) and *P. mutoni* (*N* = 4), *P. misonnei* (*N* = 4) and *P. jacksoni* (*N* = 10) from Kisangani (D.R.C.). The 112 specimens included in our molecular phylogeny represent all but one of the species that are known to occur in lowland rainforest (Appendix 1). Indeed, we were not able to obtain a sample for typical *P. minor*, which is only known from three specimens from the type locality (Lukolela, R.D.C.).

Isolation, amplification and sequencing of DNA
DNA was isolated from ethanol-preserved muscle by the Chelex method (Walsh, Metzger & Higuchi, 1991). The primers used for amplification and sequencing were the 16Sar L primer 5′-CGCCTGTTTAACA AAAACAT-3′ and the 16Sm H primer 5′-AGATCACG TAGGACTTTAAT-3′ (Palumbi *et al.*, 1991). The PCR consisted of 30 cycles: 60 s at 94 °C, 60 s at 46 °C and 90 s at 72 °C. The double-stranded PCR product (550 bp) 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 sequencing kits (Thermosequenase 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.

Data analyses
Sequences were aligned by using CLUSTAL W version 1.5 (Thompson, Higgins & Gibson, 1994) with the default settings. The alignment obtained was corrected by eye, on the basis of the secondary structure of the 16S rDNA gene fragment for *Mus* (De Rijk, 1995).

The entire data set was analysed with the neighbour-joining method (NJ) after correction of the distances by using the Tamura–Nei formula (Tamura & Nei, 1993). Bootstrap analyses (1000 replicates) were performed to define support for the main branches and to select taxa for further analyses. This approach allowed us to construct a 'reduced data' set that contains 27 sequences that represent all the species and most of the genetic diversity included in the entire data set. For each putative taxon we selected the longest sequences. As a result, sequences retained for phylogenetic analyses were longer in the restricted data set than those in the entire data set. Using this restricted data set, phylogenetic inferences were made using the NJ, maximum parsimony (MP) and maxi-

mum likelihood (ML) analyses with PAUP version 4b10 (Swofford, 2000). The genetic distance was corrected by using the Tamura & Nei (1993) formula, which allows differential substitution rates for transitions (TS) and transversions (TV) and substitution rate heterogeneity along the sequences. For MP and ML analyses a heuristic algorithm was used with the TBR option and ten replicate searches (stepwise addition, randomly ordered addition sequence). For MP analysis, a uniform weighting scheme was employed. We estimated the robustness of internal nodes by bootstrapping: 10 000 NJ and 1000 MP replicates (each with a single replication of random addition of taxa). Prior to ML analyses, we used Modeltest 3.04 to select the substitution model which best fitted the data according to a hierarchical ratio test (Posada & Crandall, 1998). We computed likelihood-ratio tests to evaluate branch length significances.

Because using different methods of treating gaps may influence the resulting phylogenetic hypotheses (Simmons & Ochoterena, 2000), we first treated gaps as missing data. In a second analysis, gaps were treated as characters (in NJ and MP analyses) by using the simple indel coding method proposed by Simmons & Ochoterena (2000), which is implemented in the program GapCode (Healy & Young, 2001).

We also inferred phylogenetic trees using Weighbor (Bruno, Socci & Halpern, 2000). Its weighted neighbour-joining (WNJ) algorithm reconstructs trees in such a way that the variance and covariance of the distances are taken into account for the choice of the sequences to be linked and for the calculation of the new distance matrix. Simulations show that it performs nearly as well as ML even in instances where long branches are present (Bruno *et al.*, 2000). Two options were defined: the number of variable sites (L) was set to 97 and the number of bases corrected for the nucleotide bias (b) was set to 3.85.

To examine both a priori- and a posteriori-selected relationships, we used the SH test (Shimodaira & Hasegawa, 1999) as implemented in PAUP. The appropriate use of this test is discussed in Goldman, Anderson & Rodrigo (2000).

Levels of genetic variation were evaluated by calculating the proportion of nucleotide transitions and transversions between all pairs of specimens and by estimating the genetic distance using the Kimura two-parameter model (Kimura, 1980). To assess the level of saturation of base substitutions (i.e. the occurrence of multiple substitutions at the same nucleotide site), we plotted the total number of transitions (TS) and transversions (TV) against the Kimura two-parameter distances.

The choice of outgroups to be used is important because 'all details of ingroup topology are sensitive to the selection of outgroup taxa' (Milinkovitch & Lyons-Weiler, 1998). Rooting with more than one outgroup presents several advantages, one of which is that this allows us to test the monophyly of the ingroup (Barriel & Tassy, 1998). To designate an appropriate outgroup we used several outgroup taxa as proposed by Smith (1994) (data not shown), such as *Gerbillus nigeriae* (GenBank accession no. AF141257), *Rattus norvegicus* (GenBank X14848), *Mus musculus* (GenBank J01420), *Hylomyscus stella* (GenBank AY647968) and *Heimyscus fumosus* (GenBank AY647969). For the final analysis (data presented in this paper) we retained only the two outgroups that were found to be the closest relatives to the ingroup (i.e. *H. stella* and *He. fumosus*).

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 & Huchon, 2000). We used two palaeontological records to calibrate our molecular clock: first, the *Mus*/*Rattus* dichotomy at 12 Mya (Jaeger, Tong & Denys, 1986; Jacobs & Downs, 1994) and second, the divergence time between *Mus* and *Gerbillus* estimated at 16 Mya (Tong & Jaeger, 1993). The distance matrix derived from the NJ tree was used to estimate times of divergence between clades.

CRANIOMETRICAL ANALYSIS

We took 20 craniometrical measurements on 211 skulls of adult specimens: M1 = condylobasal length, M2 = henselion–basion length, M3 = henselion–palation length, M4 = length of palatal foramen, M5 = length of disatema, M6 = distance between alveolus M^1 and cutting edge of upper incisor, M7 = smallest interorbital breadth, M8 = smallest palatal breadth, M9 = length of upper cheekteeth, M10 = breadth of upper dental arch, M11 = greatest breadth of first upper molar, M12 = smallest breadth of zygomatic plate, M13 = greatest breadth of nasals, M14 = greatest length of nasals, M15 = length of mandibular teeth, M16 = length of auditory bulla, M17 = greatest breadth of braincase, M18 = depth of upper incisor, M19 = mediosagittal projection of rostrum height, M20 = greatest rostrum breadth.

To provide reliable species identifications for the populations investigated, we compared in a series of multivariate analyses these populations with the metric data of the holotypes of all relevant species. Stepwise forward canonical analyses were performed with the statistical package STATISTICA 6.0. Only adult specimens of both sexes were included in our analyses.

We performed two canonical analyses. (1) In the first (Appendix 2), we only included measurements of specimens that were previously identified by their 16S rDNA gene sequences as belonging to three OTUs (operational taxonomic units): *P. jacksoni* ($N = 16$), *Praomys* 'MI' ($N = 30$) and *P. petteri* ($N = 17$). This allowed us to test if the three monophyletic groups, determined on the basis of their mtDNA sequences, and occurring in sympatry at least in West Central Africa, could also be identified using morphometric data. In this analysis, variables M1 and M15 were not included because they were missing for the holotype of *P. jacksoni*. Holotypes of *P. petteri* and *P. jacksoni* were plotted on the graph. (2) The aim of the second analysis (Appendix 3) was to determine the morphometric similarity between *Praomys* 'MI' from Gabon (Doudou Mounts, $N = 37$ including eight specimens identified based on their DNA sequences) and specimens of *P. tullbergi* ($N = 26$) and *P. rostratus* ($N = 24$) from Ivory Coast (Adiopodoumé) and of two populations of *P. misonnei* from the D.R.C., one from Irangi ($N = 14$), which is the type locality, and one from Kisangani ($N = 28$), from which we had sequenced four specimens. Variables M6 and M14 were not included in this analysis because they were missing for several specimens. Holotypes of *P. tullbergi* and *P. rostratus* could not be plotted in the root 1/root 2 scatterplot because several measurements were missing (skulls in poor condition).

RESULTS

Molecular analysis

Sequence composition and alignments (Fig. 2)
For all the nucleotide sequences for the ingroup taxa, 402 out of the 494 molecular characters are constant, 92 are variable and 74 are parsimony informative. For the reduced data set (see Data analysis above), 437 out of the 515 characters are constant, 78 are variable and 62 are parsimony informative when indels are treated as missing data. Treating indels as characters leads to the addition of 18 characters, all variables, 11 of them being parsimony informative.

The hierarchical ratio test reveals that the model which best fits the data is the GTR model (Rodriguez *et al.*, 1990) with a gamma parameter of 0.55 and a proportion of invariable sites of 0.67. Estimated substitution rates are 4.59 for A–C, 9.09 for A–T, 1.02 for C–G and 1.00 for G–T transversions, and 12.92 for A–G and 25.22 for C–T transitions. Base frequencies are equal to 0.35, 0.19, 0.19 and 0.27, respectively, for A, C, G and T.

A transition bias is apparent but there is no evidence of saturation for transitions or for transversions (Fig. 2). The average TS/TV ratio is 1.55 for ingroup taxa; it is 2.07 between species of the *jacksoni* complex, 2.33 between species of the *tullbergi* complex and 1.20 between species of the *jacksoni* and *tullbergi* complex.

Phylogenetic relationships within the genus Praomys (Figs 3, 4)
When the entire data set is considered, putative species are always resolved as monophyletic (Fig. 3) with a bootstrap support above 66% (range 66–100%). Within-species divergence ranged from 0.00% in *P. misonnei* or *P. mutoni* to 2.94% in *P. jacksoni*. Between-species divergence ranged from 2.24% between *P. misonnei* and *Praomys* 'MI' to 6.59% between *P. mutoni* and *Praomys* 'MI'.

For the restricted data set, the trees obtained by different reconstruction methods do not differ significantly (log-likelihood values, $P > 0.05$). The MP analysis recovered 28 equally parsimonious trees of
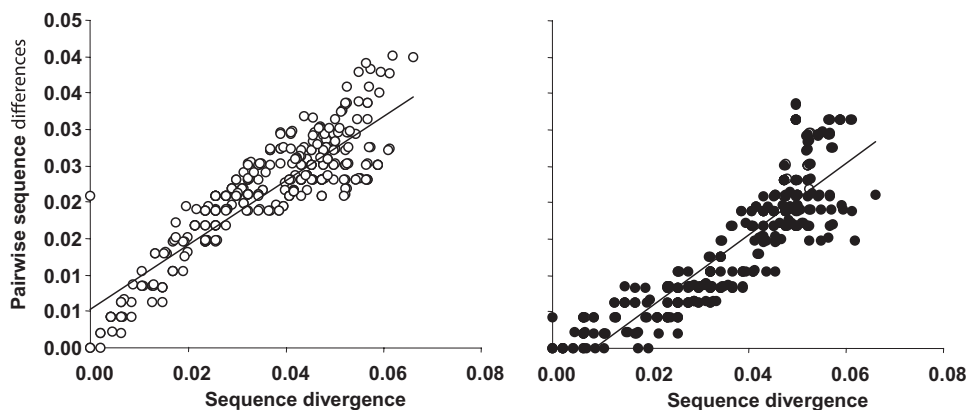

Figure 2. Plot of pairwise sequence differences in transitions (right) and transversions (left) against sequence divergences given the Kimura two-parameter distances. Only the ingroups were included in this analysis.
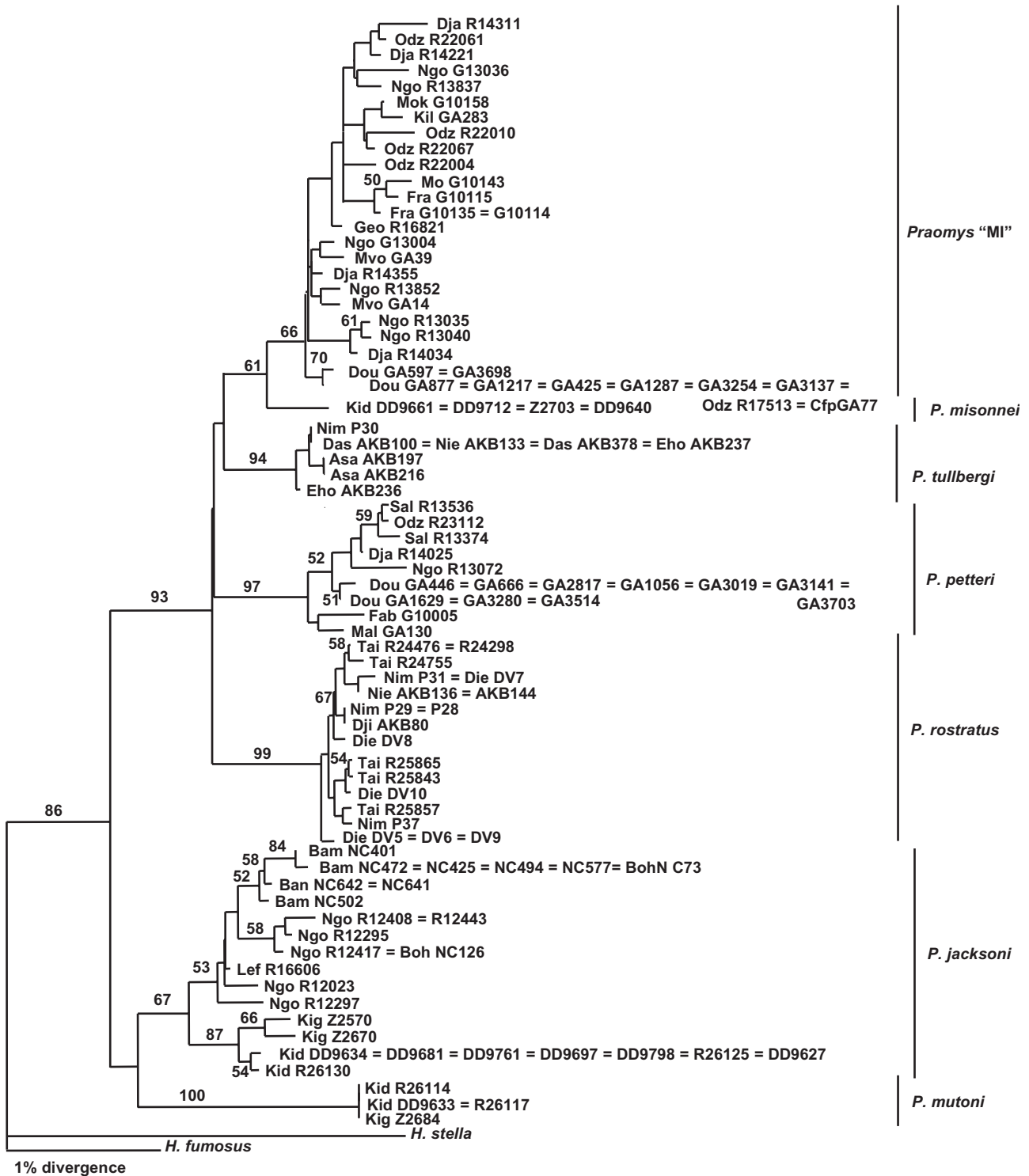
Figure 3. Phylogenetic relationships among nucleotide sequence of the 16S rRNA gene of 112 specimens of *Praomys* using the neighbour-joining algorithm with the Tamura–Nei distance correction. The murid rodents *Hylomyscus stella* and *Heimyscus fumosus* were used as outgroups. The numbers above branches indicate bootstrap scores (1000 replicates). For locality name abbreviations see Fig. 1.

212 steps when indels were treated as missing data [consistency index (CI) = 0.637, retention index (RI) = 0.842, rescaled consistency index (RCI) = 0.536], and it recovered 20 equally parsimonious trees of 256 steps when indels were treated as characters (CI = 0.648, RI = 0.850, RCI = 0.551). The phylogenies inferred using different methods reveal that the genus *Praomys* contains two monophyletic clades supported by bootstrap values above 72% (88–95% for the first, 72–90% for the second; Fig. 4). The first clade groups specimens that belong to the *tullbergi* complex (*P. tullbergi*, *P. rostratus*, *P. misonnei*, *Praomys* 'MI', *P. petteri*). The second clade groups the species belonging to the *jacksoni* complex (*P. jacksoni*, *P. mutoni*). In addition, in all analyses *P. misonnei* and *Praomys* 'MI' cluster with low to high bootstrap support (69–93%). The phylogenetic relationship between other species of the *tullbergi* complex are not resolved (bootstrap values < 61%) and vary according to the used tree-building method: in MP (gaps treated either as miss-

ing data or characters), as well as in ML and NJ (gaps treated as characters) we observed a dichotomy between *P. rostratus* and other species. By contrast, in WNJ and NJ (gaps treated as missing data) we observed a trichotomy for *P. petteri*, *P. rostratus* and *P. tullbergi-misonnei-*'MI'.

Finally, all our analyses identified three *P. jacksoni* clades. The clustering of *P. jacksoni* from West Central Africa and from Kisangani, either on the left or on the right bank of the Congo River, is supported by low to high bootstrap values (59–98%), but the phylogenetic relationships between these taxa differ when other tree-building methods are employed. Whereas we observe a trichotomy between the three clades with MP and ML analyses, a well-supported dichotomy between *P. jacksoni* from Kisangani and those from West Central Africa (bootstrap values > 84%), and a dichotomy between specimens from Kisangani coming from the left bank and those coming from the right bank of the Congo River (boot-
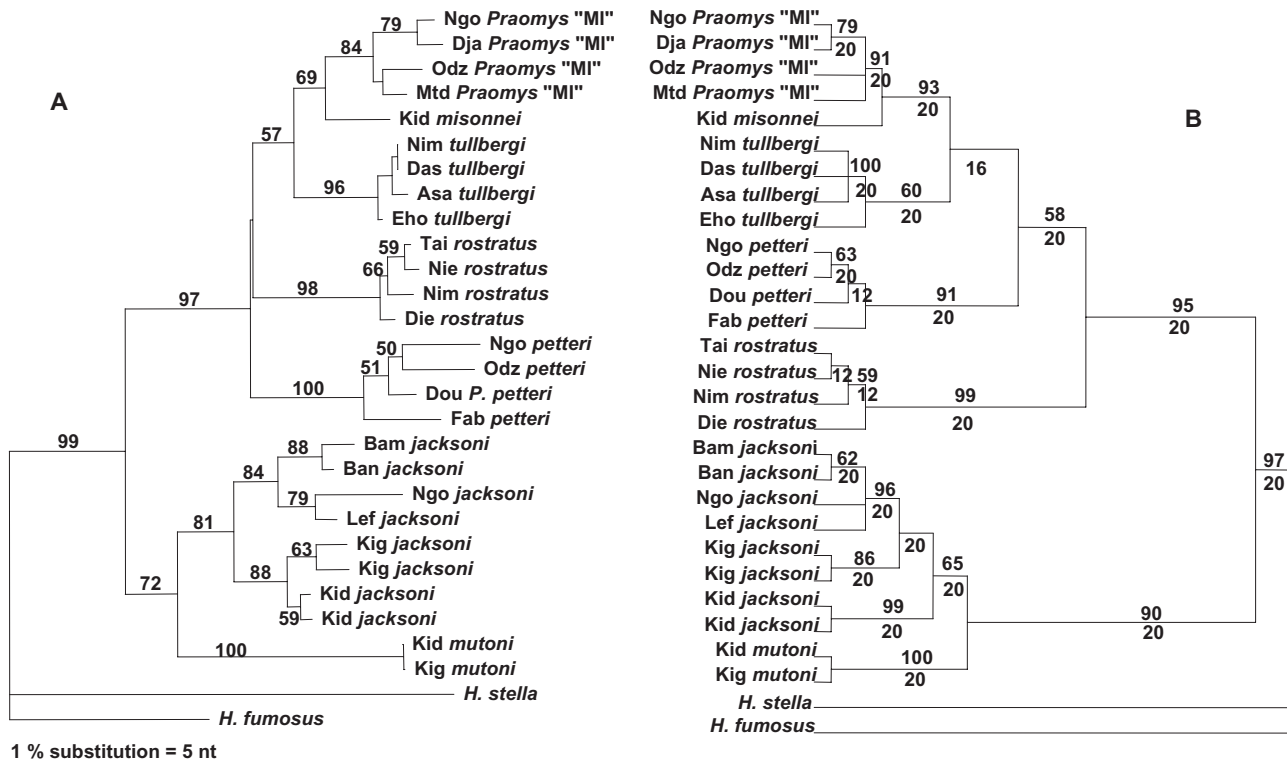

Figure 4. Phylogenetic relationships among sequences of the 16S rRNA gene of 27 specimens of *Praomys* using the neighbour-joining (NJ) (A) and maximum parsimony (MP) (B) algorithms. Numbers above branches indicate bootstrap scores (10 000 and 1000 replicates, respectively) and numbers below branches (MP) represent the number of trees in which each branch was found. In the NJ analysis, gaps were treated as missing and in the MP analysis gaps were treated as characters following the simple coding of Simmons & Ochoterena (2000). For NJ analysis, genetic distance was corrected by the Tamura & Nei (1993) correction. For MP analysis an heuristic algorithm was used with the TBR option and ten replicate searches (stepwise addition, randomly ordered addition sequence). For locality name abbreviations see Fig. 1.

Table 1. Estimates of divergence times (per Myr) using two calibration dates

Taxa 1	Taxa 2	Calibration *Mus–Rattus* times ± σ	Calibration *Mus–Gerbillus* times ± σ	Mean times ± σ
Mus	*Rattus*	−12	14.4	13
Mus	*Gerbillus*	13.4	−16	14.8
Heimyscus fumosus – Hylomyscus stella	*Praomys* species	4.3 ± 0.9	5.1 ± 1.1	4.7 ± 1.0
jacksoni complex	*tullbergi* complex	3.2 ± 0.4	3.9 ± 0.4	3.5 ± 0.4
Praomys mutoni	*P. jacksoni*	2.5 ± 0.4	3.0 ± 0.5	2.8 ± 0.4
P. jacksoni West Central	*P. jacksoni* Kisangani	1.4 ± 0.3	1.6 ± 0.4	1.5 ± 0.4
P. jacksoni Kisangani right bank	*P. jacksoni* Kisangani left bank	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
P. petteri	*P. rostratus*	2.4 ± 0.3	2.8 ± 0.4	2.6 ± 0.4
P. petteri	*P. tullbergi*	2.0 ± 0.2	2.4 ± 0.2	2.2 ± 0.2
P. petteri	*P. misonnei – Praomys* 'MI'	2.3 ± 0.3	2.8 ± 0.3	2.5 ± 0.3
P. tullbergi	*P. rostratus*	1.6 ± 0.1	1.9 ± 0.2	1.8 ± 0.1
P. tullbergi	*P. misonnei – Praomys* 'MI'	1.4 ± 0.3	1.7 ± 0.4	1.5 ± 0.3

strap values > 59%) are observed in the NJ and WNJ trees.

Age estimates (Table 1)

Because the rates of evolution are not significantly different across the lineages examined (for all comparisons between lineages $P > 0.213$), the estimation of the timing of divergence events based on a molecular clock is justified. However, because age estimates are known to yield large confidence limits, the values presented here should be interpreted cautiously (Hillis, Moritz & Mable, 1996) and viewed as preliminary hypotheses of the timing of important events in the history of the *Praomys* genus (Table 1). The deepest split within the genus occurred approximately 3.5 Mya (*P. tullbergi* complex/*P. jacksoni* complex), whereas *P. jacksoni* and *P. mutoni* diverged approximately 2.8 Mya. The *jacksoni* clade from West Central Africa and the *jacksoni* clade from Kisangani diverged approximately 1.5 Mya, and the divergence between the clades from the Left and Right bank of the Congo River occurred about 0.6 Mya. Finally, *Praomys* 'MI' and *P. misonnei* separated about 1.2 Mya and the other species of the *tullbergi* complex (*P. tullbergi*, *P. rostratus* and *P. petteri*) branched off between 1.5 and 2.6 Mya.

CRANIOMETRICAL ANALYSIS

Comparison of Praomys 'MI', P. petteri and P. jacksoni (Fig. 5)

After a stepwise forward canonical analysis, 15 variables were retained. Seventy-one per cent of the total variation is expressed in root 1 and 29% is expressed in root 2. Wilk's Lambda (= 0.04) is significantly lower

than one ($P < 0.000$), indicating a good discrimination between OTUs. The scatter diagram of root 1 vs. root 2 (Fig. 5) shows that the three OTUs are clearly differentiated (probability of correct classification: 98%). The OTUs are well differentiated on root 1, and *Praomys* 'MI' is separated from other OTUs on root 2. The variables 'smallest breadth of zygomatic plate' (M12), 'henselion–palation length' (M3), 'length of diastema' (M5), 'distance between alveolus M^1 and cutting edge of upper incisor' (M6) are the most positively correlated with root 1, and 'length of the auditory bulla' (M16) is the most negatively correlated with root 1. Variables 'length of palatal foramen' (M4) and 'greatest breadth of first upper molar' (M11) are the most positively correlated with root 2. To conclude, the three OTUs are well differentiated morphometrically, a finding that – combined with mtDNA sequence data – favours the recognition of at least three species in West Central Africa. At least two of these belong to the *tullbergi* complex.

Comparison of Praomys 'MI', P. tullbergi, P. rostratus and P. misonnei (Fig. 6)

To allocate *Praomys* 'MI' taxonomically, we compared it to other species of the *tullbergi* complex from the African lowland rainforest. After a stepwise forward canonical analysis, 16 variables were retained in the discriminant function. Sixty-eight per cent of the total variation is expressed in root 1 and 17% is expressed in root 2. Wilk's Lambda (= 0.02) is significantly lower than one ($P < 0.000$), indicating a good discrimination between OTUs. Figure 6 shows that *Praomys* 'MI' is clearly differentiated from *P. tullbergi* and *P. rostratus* along the first axis. The variables that are the most
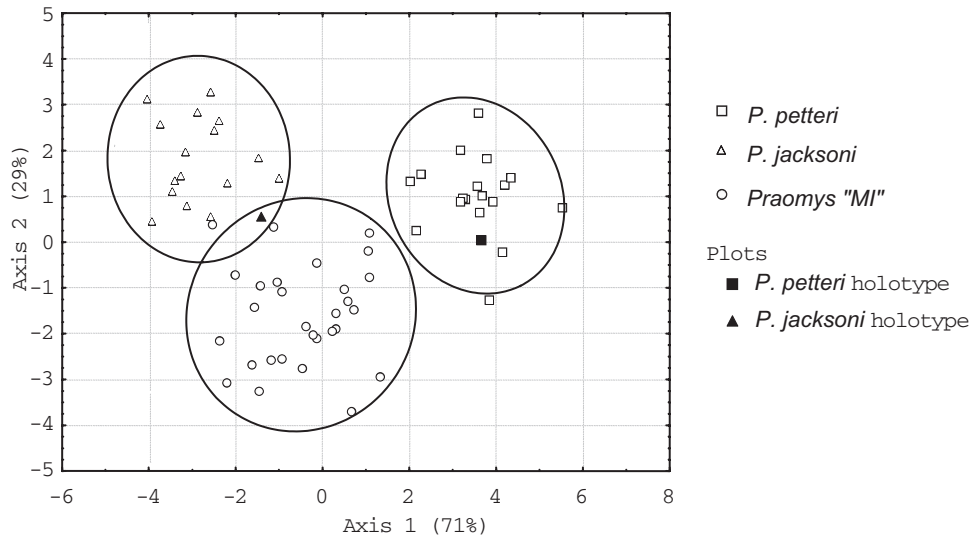
Figure 5. Canonical analysis on a selected craniometrical data-set of *Praomys jacksoni*, *P. petteri* and *Praomys* 'MI' (sequenced specimens only).
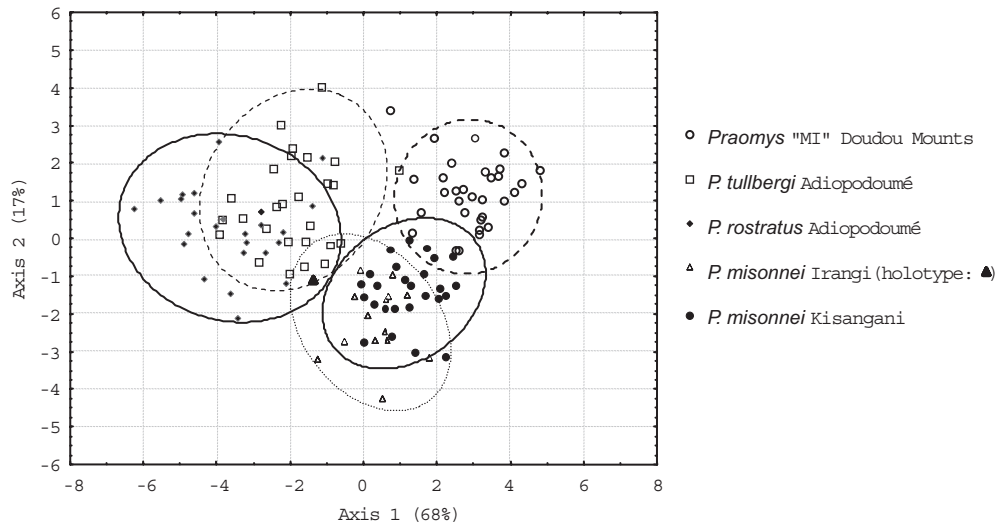

Figure 6. Canonical analysis on a selected craniometrical data set of *Praomys* 'MI' (Doudou Mounts, Gabon), *Praomys tullbergi* and *P. rostratus* (Adiopodoumé, Ivory Coast), and *P. misonnei* (Irangi and Kisangani, D.R.C.).

negatively correlated with root 1 are 'henselion–palation length' (M3) and 'length of palatal foramen' (M4), and also 'length of diastema' (M5), 'mediosagittal projection of rostrum height' (M19), 'length of upper cheekteeth' (M9) and 'depth of upper incisor' (M18). In the root 1/root 2 scatterplot, *P. misonnei* from Kisangani overlaps with *Praomys* 'MI' (4% of *P. misonnei* individuals are misclassified), and *P. misonnei* from Irangi overlaps with *P. tullbergi* (7% of *P. misonnei* individuals are misclassified).

DISCUSSION

PHYLOGENY AND TAXONOMY

Current taxonomic knowledge of the genus *Praomys* is largely based on morphological studies (Van der Straeten & Verheyen, 1981; Van der Straeten & Dieterlen, 1987; Van der Straeten & Dudu, 1990; Weltz, 1998; Van der Straeten & Kerbis Peterhans, 1999; Lecompte *et al.*, 2002a; Van der Straeten *et al.*, 2003). Recently, two molecular studies investigated the phylogenetic

relationships among a number of *Praomys* species (Lecompte *et al*., 2002b; Lecompte, 2003). Nevertheless, we are not aware of a molecular phylogeny including all lowland rainforest *Praomys* species, nor has there been a serious attempt to determine the distribution ranges of these murines.

Our study suggests that the 16S rRNA gene is a suitable molecular marker to identify the investigated *Praomys* species. This mtDNA gene allowed us to solve some of the current taxonomic problems in this genus and to establish the relationships among several taxa. However, the resolving power of the gene fragment employed appears to be limited. This could be due to the low number of substitutions, possibly related to the length of the gene used or to the fact that it evolved too slowly. According to our results there is no evidence of saturation for transitions and transversions, and therefore it is improbable that the lack of informative characters is due to the fact that the gene evolves too fast. Alternatively, these species may be the result of a rapid succession of cladogenetic events that are always difficult to detect reliably with molecular phylogenies. This last hypothesis appears to be in concordance with the results obtained by Lecompte *et al*. (2002b) based on cytochrome *b* sequences.

In agreement with previous morphometrical and morphological studies (Van der Straeten & Dieterlen, 1987; Van der Straeten & Dudu, 1990; Lecompte *et al*., 2002a), the molecular phylogeny presented here indicates that the species inhabiting the Guinean–Congolese lowland rainforest should be divided in two species complexes:

1. The *jacksoni* complex contains three clades that represent at least two species, i.e. *P. mutoni* and *P. jacksoni*; a third species (*P. minor*) is probably present in this region but could not be included in our analysis because it is known from only three specimens from the type locality (Lukolela, on the left bank of the Congo River). Until now, *P. mutoni* was only known from the right bank of the Congo River, but our results show that it also occurs on its left bank. That our data identify three *P. jacksoni* clades may be explained by the important geographical distances between the localities where these samples were collected (between West Central Africa and Kisangani, in East Central Africa), and the presence of the Congo River, which separates the two sampling localities in Kisangani (right and left bank of the Congo River). When dealing with allopatric taxa, the genetic distances separating them are not enough to determine whether or not they belong to the same species (Fergusson, 2002). Therefore, additional data on the relationship between geographical distance and genetic divergence, as well as on eventual cytological, ecological or behavioural differences, will be required to determine how many species are contained within the three *P. jacksoni* clades.

2. Within the *tullbergi* complex, our molecular results indicate that *P. misonnei* and *P. tullbergi* are clearly differentiated. Three lines of evidence support this hypothesis: (1) genetic divergence is low within each taxon (< 0.43%); (2) the genetic divergence between these taxa (3.41%) is similar to those found between other species of the genus; and (3) the two taxa do not cluster together in the phylogenetic trees. Even if these two species are morphologically quite similar (Fig. 6), our molecular finding agrees with Lecompte *et al*. (2002a, b), who concluded that they are two valid species. In addition, based on sequence divergence and tree topology, our genetic analyses suggest that *Praomys* 'MI' and *P. misonnei* are close relatives and both clearly distinct from typical *P. tullbergi*. Moreover, based on craniometrical data, *Praomys* 'MI' more closely resembles *P. misonnei* than *P. tullbergi*. Therefore, based on molecular and craniometrical data, we suggest that West Central African *Praomys* 'MI' should be referred to as *P.* cf. *misonnei*, and not as *P. tullbergi minor* (Petter, 1975; Genest-Villard, 1980; Duplantier, 1982). It is clear that additional studies will be required to determine the taxonomic position of *P.* cf. *misonnei* compared with typical *P. misonnei*. With regard to *P. jacksoni*, the geographical distance between the collecting localities of *P. misonnei* (Kisangani, D.R.C.) and *Praomys* 'MI' (West Central Africa) may partly explain the observed amount of sequence divergence between both clades. Variation in the fundamental number of autosomes was found within *P. petteri*, but the biological significance of this variation remains unknown (see Introduction). We included in our molecular analyses specimens from the whole geographical range of *P. petteri*, and from populations with distinct karyotypes (Doudou Mounts and south-east Cameroon). Based on sequence divergence ($1.01 \pm 0.54\%$ within *P. petteri*) and tree topology there is no evidence that several cryptic species exist within *P. petteri*. However, there are examples in several rodent genera of species that have become reproductively isolated by means of chromosomal changes without other measures of genetic differentiation(Volobouev *et al*., 2002). Thus, additional cytological data are needed to clarify the significance of the observed karyological differences within *P. petteri*.

In summary, our results suggest that at least four species of the *tullbergi* complex inhabit the Guinean–Congolese lowland rainforests. *P. tullbergi* and *P. rostratus* appear to be endemic to West Africa. *P. petteri* seems to be restricted to West Central Africa, where it occurs in sympatry with *P.* cf. *misonnei*. The phylogenetic relationships among the species of the *tullbergi* complex remain unclear. Depending on

the tree-building method used, we found either a dichotomy between *P. rostratus* and other species, or a trichotomy between *P. petteri*, *P. rostratus* and *P. tullbergi-misonnei*-'MI'. Both trees differ from the morphology-based phylogenetic tree of Lecompte *et al*. (2002a), who observed a trichotomy between *P. petteri* (called in their study *Praomys* sp.), *P. misonnei* and *P. rostratus*, and a dichotomy between these three species and *P. tullbergi*. The different species groupings based on morphological and molecular data sets may be the result of the fact that these two studies included a different set of taxa. On the other hand, it should be noted that the characters used in the morpho-anatomical data set are very homoplastic, which may have affected the species groupings obtained by Lecompte *et al*. (2002a).

AGE ESTIMATES AND EVOLUTIONARY HISTORY OF TROPICAL LOWLAND *PRAOMYS* SPECIES

The estimated divergence time separating the two *Praomys* species complexes is about 3.5 Myr. This estimate is similar to that obtained by Chevret *et al*. (1994) based on DNA/DNA hybridization, but is lower than the figure given by Lecompte *et al*. (2002b) using cytochrome *b* sequences (5.2 Myr). We estimated the divergence between species within each complex to range between 1.5 and 2.6 Myr for the *tullbergi* complex, and approximately 2.8 Myr for the *jacksoni* species complex. These estimations are similar to those obtained by Lecompte *et al*. (2002b) (respectively 2.2 Myr and 3.5 Myr). However, Chevret *et al*. (1994) determined a divergence time of 0.4 Myr between *Praomys* 'MI' (called in their study *P. tullbergi*) and *P. petteri* (called in their study *P. lukolelae*), an estimate lower than the figure determined by us (1.2 Myr).

Lecompte *et al*. (2002a) calibrated their molecular clock using the same fossil record as used here (*Mus–Rattus*, 12 Myr), whereas Chevret *et al*. (1994) used a divergence time estimate between *Mus* and *Rattus* of 10 Myr. Thus, the divergence time estimations given by Chevret *et al*. may be expected to be inconsistently lower than ours, which is not the case. The observed differences between these three studies illustrate how care is needed when using a single gene for age estimations, as each gene exhibits a unique pattern of evolution.

Taxa used to calibrate the molecular clock diverged much earlier than *Praomys* species. It is therefore possible that, because of a certain amount of saturation of the substitutions, we underestimated the substitution rate, which would result in an overestimation of divergence times. In agreement with this hypothesis, divergence time estimates based on the split between *Mus* and *Gerbillus* (16 Myr) were always slightly higher than those based on the split between *Mus* and *Rattus* (12 Myr).

Relationships within each species complex are not fully resolved. This could indicate that the 16S rRNA gene is inappropriate for the study of the evolutionary history of the genus *Praomys*. Alternatively, it may also indicate that the emergence of *Praomys* species occurred during a rapid adaptive radiation that took place during the Pliocene. This appears to be congruent with the finding that the cytochrome *b* phylogeny of Lecompte *et al*. (2002b) revealed a similar weak resolution among *Praomys* species. Most of the estimated divergence times between species of a complex range between 1.5 and 2.8 Mya, which corresponds with a period of pronounced shifts in African climate with an alternation of periodically wet and dry climates (DeMendocal, 1995).

Species distribution ranges are usually determined by ecological and geological factors. In Central African forests, the complex river system is presumed to be the major ecological factor that determines the geographical distribution of terrestrial organisms (Grubb, 1990; Colyn, 1991; Colyn, Gautier-Hion & Verheyen, 1991; Happold, 1996; Deleporte & Colyn, 1999; Colyn & Deleporte, 2002). In agreement with this hypothesis, we found the distribution range of some species to be restricted to one side of the Congo River (e.g. *P. misonnei* only occurs on the right bank) and those of other species to be restricted to one side of the Oubangui–Congo River (e.g. *P. petteri* occurs only on the right bank). However, this observation does not seem to be valid for other species (e.g. *P. jacksoni* and even *P. mutoni* appear to be present on both sides of the Congo River). In addition, patterns of genetic divergence between populations present on each side of the Congo River differ between species: for *P. jacksoni* we found a mean sequence divergence of 0.87% per Myr between populations, while exactly the same haplotype was found in the *P. mutoni* specimens ($N = 3$) from the left bank and the single specimen from the right bank. If future larger samples support this finding, this would suggest that there has been recent gene flow between *P. mutoni* populations occurring on both sides on the river, whereas no trace of gene flow is observed between *P. jacksoni* populations. Unfortunately, our knowledge of the ecology and the dispersal capacity of these *Praomys* species is insufficient to explain the observed haplotype distribution pattern.

Similar genetic distances were found between *P. jacksoni* populations from Kisangani, on the right bank of the Congo River, and from West Central Africa ($1.91 \pm 0.30\%$ sequence divergence) and between *P.* cf. *misonnei* populations from the same localities ($1.59 \pm 0.39\%$). This may indicate that the evolutionary history of these two species was similar. Finally,

while the Congo River is usually thought to be one of the main barriers to Central African species distribution, it appears that for *P. jacksoni* the genetic distance was lower between populations on the two sides of this river than between populations from Kisangani and West Central Africa. However, several other rivers (e.g. the Ubangui River) are also likely to provide barriers. Future investigations will look at the separating effects of the Congo and other rivers based on a more appropriate data set.

CONCLUSIONS

By combining craniometric data and mtDNA sequence analysis, our study allowed us to characterize most *Praomys* species of the Guinean–Congolese lowland rainforest. Each taxon can be linked to a particular 16S rRNA gene sequence, which can be useful as an additional identification tool. The phylogenetic relationships among all the taxa could be resolved fully because of what appears to have been a rapid diversification event in this genus. In order to gain a complete picture of the relationships among all the taxa within the genus *Praomys*, future studies must include data on montane species. This will allow a better understanding of the speciation processes that have led to the origin of the observed diversity in this genus. Moreover, more cytological analyses are required to elucidate the significance of the karyotype differences observed within *P. petteri*. Finally, additional sampling in the western part of the D.R.C. will be required to establish whether West Central African *P. jacksoni* and *P.* cf. *misonnei* are conspecific to those that are reported in the D.R.C.

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APPENDIX 1

List of specimens included in molecular analysis with geographical origin and collection number. Specimens in bold type are those which were used in the analysis of the restricted data set. All specimens will be deposited at the MNHN.

Praomys 'MI' (*N* = 33): Cameroon, Dja Reserve: **R14034**, R14311, R14221, R14355. R.C., Odzala National Park: R17513, **R22004**, R22010, R22061, R22067; Grand Escarpement Odzala: R16821. C.A.R., Ngotto: R13004, R13035, R13036, R13040, R13837, R13852. Gabon, Mokekou: G10143, G10158; Kili: GA283; Franceville: G10114, G10115, G10135; Mvoum: GA14, GA39; Carrière Four Place: GA77; Doudou Mounts: GA425, GA597, GA877, GA1217, GA1287, GA3137, GA3254, GA3698.

P. misonnei (*N* = 4): D.R.C., Kisangani, right bank of the Congo River: DD9640, **DD9661**, DD9712, **Z2703**.

P. tullbergi (*N* = 8): Guinea, Mont Nimba: P30. Ivory Coast, Dassieko: **AKB100**, AKB378; Niégré: AKB133; Assagny: **AKB197**, AKB216; Ehotile: **AKB236**, AKB 237.

P. petteri (*N* = 17): C.A.R., Salo: R13536, R13374; Ngotto: **R13072**. C.R., Odzala National Park: **R23112**. Cameroon, Dja Reserve: R14025. Gabon, Doudou Mountains: **GA446**, GA666, GA1056, GA1629, GA2817, GA3019, GA3141, GA3280, GA3514, GA3703; Forêt Abeilles: **G10005**; Malounga: GA130.

P. rostratus (*N* = 19): Ivory Coast, Taï National Park: R24298, **R24476**, R24755, R25865, R25843, R25857; Niégré: **AKB136**, AKB144; Djidoubaye: AKB80. Guinea, Mont Nimba: P28, P29, P31, **P37**; Diécké: **DV5**, DV6, DV7, DV8, DV9, DV10.

P. jacksoni (*N* = 27): C.A.R., Bamingui-Brendja: **NC401**, NC425, NC472, NC494, NC502, NC577; Bohou: NC73, NC126; Bangoran: **NC642**, NC641; Ngotto: R12023, R12295, R12297, **R12408**, R12417, R12443. C.R., Léfini Reserve: **R16606**. D.R.C., Kisangani, right bank of the Congo River: DD9627, **DD9634**, DD9681, DD9761, DD9697, DD9789, R26125, **R26130**; Kisangani, left bank of the Congo River: **Z2570**, **Z2670**.

P. mutoni (*N* = 4): D.R.C., Kisangani, right bank of the Congo River: **DD9633**, R26114, R26117; Kisangani, left bank of the Congo River: **Z2684**.

APPENDIX 2

List of specimens used in the first canonical analysis on morphometrical data. All specimens will be deposited at the MNHN.

P. jacksoni ($N = 16$): C.A.R., Ngotto: R12295, R12297, R12408, R12417, R12443. C.R., Léfini: R16606. D.R.C., Kisangani, right bank of the Congo River: R26125, R26130, DD9627, DD9634, DD9681, DD9697, DD9761, DD9798; Kisangani, left bank of the Congo River: Z2570, Z2670.

Praomys 'MI' ($N = 30$): Gabon, Franceville: G10114, G10115, G10135; Mokekou: G10143, G10158; Carrière Four Place: GA77; Mvoum: GA14, GA39; Kili: GA283; Doudou Mountains: GA425, GA597, GA1217, GA1287, GA3137, GA3254, GA3698. C.A.R., Ngotto: R13004, R13035, R13036, R13040, R13837, R13852. Cameroon, Dja: R14034 R14221, R14311, R14355. C.R., Odzala National Park: R17513, R22004, R22010, R22061.

P. petteri ($N = 17$): Gabon, Forêt Abeilles: G10005; Malounga: GA130; Doudou Mountains: GA446, GA666, GA1056, GA1629, GA2817, GA3019, GA3141, GA3280, GA3514, GA3703. C.A.R., Ngotto: R13072; Salo: R13536, R13374. Cameroon, Dja: R14025. C.R., Odzala National Park: R23112.

APPENDIX 3

List of specimens used in the second canonical analysis on morphometrical data. All specimens will be deposited at the MNHN, except *P. tullbergi*, *P. rostratus* and *P. misonnei* specimens from Kisangani housed at Koninklijk Museum voor Midden Afrika, and *P. misonnei* specimens from Irangi (numbers 94.047-M-) housed at Staatliches Museum für Naturkunde.

Praomys 'MI' ($N = 37$): Gabon, Doudou Mountains: GA678, GA680, GA733, GA877, GA955, GA998, GA1050, GA1061, GA1157, GA1302, GA1330, GA1419, GA1453, GA1490, GA1505, GA1529, GA1334, GA1599, GA1696, GA1726, GA1956, GA2199, GA2287, GA2418, GA2538, GA2686, GA3058, GA3166, GA3344, GA3696, GA425, GA597, GA1217, GA1287, GA3137, GA3254, GA3698.

P. tullbergi ($N = 26$): Ivory Coast (Adiopodoumé): 80.009-M-0001, -0005, -0006, -0008, -0011, -0015, -0016, -0017, -0019, -0020, -0022, -0023, -0025, -0027, -0029, -0031, -0032, -0033, -0034, -0037, -0038, -0051, -0052, -0054, -0055, -0058.

P. rostratus ($N = 24$): Ivory Coast, Adiopodoumé: 80.009-M-0074, -0075, -0076, -0077, -0079, -0080, -0081, -0082, -0084, -0085, -0087, -0089, -0092, -0093, -0094, -0095, -0096, -0097, -0098, -0099, -0101, -0103, -0104, -0105.

P. misonnei ($N = 42$): D.R.C., Irangi: 10460, 10494, 10504, 10505, 10507, 10511, 10518, 10549, 10552, 10554, 10562, 10565, 10601, 10609; Kisangani: 94.047-M-0001, -0002, 95.051-M-1152, -1156, -1157, -1159, -1161, -1162, -1163, -1164, -1166, -1167, -1168, -1488, -1489, -1491, -1492, -1493, -1494, -1495, -1496, -1497, -1498, -1499, DD9640, D9661, DD9712, Z2703.