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Molecular Phylogenetics and Evolution 45 (2007) 142–157

MOLECULAR PHYLOGENETICS AND EVOLUTION

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Cytochrome *b* sequence analysis reveals differential molecular evolution in African mole-rats of the chromosomally hyperdiverse genus *Fukomys* (Bathyergidae, Rodentia) from the Zambezian region

Paul A.A.G. Van Daele [a,*], E. Verheyen [b], M. Brunain [a], D. Adriaens [a]

[a] *Ghent University, Evolutionary Morphology of Vertebrates, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

[b] *Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium*

Received 23 November 2006; revised 16 March 2007; accepted 9 April 2007

Available online 21 April 2007

Abstract

African mole-rats (Bathyergidae, Rodentia) of the (eu)social genus *Fukomys* are one of the most speciose mammal genera endemic to Sub-Saharan Africa. *Fukomys* distributed in the Zambezian phytochorion is characterized by extreme chromosomal variation (2n = 40–78). We inferred a molecular phylogeny of Zambezian *Fukomys* to resolve the interrelationships and the evolutionary history of the known chromosomal races. We sequenced the entire cytochrome b gene (1140 bp) for a total of 66 specimens representing 18 karyotypical races from Zambia. An additional 31 sequences were retrieved from GenBank including data on all other chromosomal races. The haplotypes belonging to a small chromosomal race from Salujinga cluster with the *Fukomys mechowii* (Giant mole-rat) haplotypes. Differential degrees of chromosomal variation are observed among the major mole-rat clades, which is most pertinent when comparing the central Zambezian *Fukomys micklemi* and the northern Zambezian *Fukomys whytei* clades. The karyotypically hyper-diverse (12 known chromosomal races) *Fukomys micklemi* clade shows low levels of cytochrome b sequence divergence. Within the *F. whytei* clade we find a more conservative pattern of chromosomal diversification (three known chromosomal races) while the levels of sequence divergence are much higher then in the *F. micklemi* clade. Our results suggest that chromosomal changes may drive phyletic divergence and, eventually, speciation. The observed cladogenetic events during the Plio-Pleistocene within the *F. mechowii*, *F. whytei*, *F. damarensis* and *F. micklemi* clades appear to coincide with climatically mediated speciation bursts in other savannah dwelling mammals, including hominids. Based on the molecular data presented, combined with morphological and chromosomal data, the taxonomic implication seems to be that *Fukomys* may contain several (undescribed) cryptic species.

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Keywords: *Cryptomys*; *Fukomys*; Mitochondrial DNA; Cytochrome *b*; Molecular phylogeny; Chromosome races

1. Introduction

1.1. A recently described genus of social mole-rats

Fukomys (Rodentia: Bathyergidae—African mole-rats) represents one of the most speciose rodent genera. The genus encompasses several species of subterranean rodents that are endemic to Sub-Sahara Africa. It is the most derived lineage within the Bathyergidae comprising several social and at least one eusocial species (*Fukomys damarensis*). The varying degree of sociality is negatively correlated with precipitation, which subsequently influences the distribution of geophytes, the animals' staple food (Jarvis et al., 1994). The causes of social evolution in bathyergids, however, are still debated (Bennett and Faulkes, 2000; Burda et al., 2000). While *Fukomys* has been vilified for damaging crops, it is an important food resource for humans in many parts of its geographic range. Recently, several research groups have succeeded in maintaining and breeding the

* Corresponding author. Fax: +32 9 264 53 44.
E-mail address: paul.vandaele@ugent.be (P.A.A.G. Van Daele).

1055-7903/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2007.04.008

animals in captivity, which will facilitate research in many different aspects of the biology of these mammals, including genetics, chromosomal evolution, evolution of sociality, ethology, biogeography, morphology, ecophysiology and sensory biology (e.g. Jarvis and Bennett, 1993; Bruckmann and Burda, 1997; Jarvis et al., 1998; Spinks et al., 2000a,b; Faulkes and Bennett, 2001; Nemec et al., 2001; Faulkes et al., 2004; Van Daele et al., 2004; Janse Van Rensburg et al., 2004; Scantlebury et al., 2006; Dammann and Burda, 2006). Furthermore, *Fukomys* is the subject of an increasing number of studies that investigate the mole-rats' behaviour and ecology (e.g. Hazell et al., 2000; Greeff and Bennett, 2000; Burland et al., 2002, 2004; Barnett et al., 2003; Ganem and Bennett, 2004). It may be expected that, in addition to the confamiliar naked mole-rat (*Heterocephalus glaber*), this group of African mole-rats will be of particular interest for gerontological research, taking into account longevity quotients similar to those of humans (Buffenstein, 2005; Dammann and Burda, 2006) combined with the differential levels of chromosomal and mitochondrial DNA sequence divergence, which is emphasized by the sequences available to date (Faulkes et al., 1997, 2004; Ingram et al., 2004; this paper).

1.2. Phylogenetic analyses, chromosomal diversification and classification

Given the fact that Bathyergidae are one of the best studied rodent groups in Africa, the systematics and phylogeny of this family have been addressed by other studies (reviewed in Van Daele et al., 2007). *Fukomys* is the most recently recognised member of the six genera that constitute the Bathyergidae. *Fukomys* (synonym *Coetomys* nomen invalidus; Kock et al., 2006) forms a northern clade within a phylogroup (*Cryptomys* sensu lato) that further contains the reciprocally monophyletic *Cryptomys sensu stricto* clade of southern Africa. Two recent phylogenetic analyses using nuclear and mitochondrial DNA sequences, combined with data on chromosomal variation, unambiguously support the taxonomic division of *Cryptomys* and *Fukomys*, although these data do not resolve the relationships within *Fukomys* (Faulkes et al., 2004; Ingram et al., 2004). To date no less than 12 *Fukomys* species are accepted as valid. In a taxonomic revision Honeycutt et al. (1991) retained 6 species: *Fukomys bocagei* (De Winton, 1897), *F. damarensis* (Ogilby, 1838), *F. foxi* (Thomas, 1911), *Fukomys mechowii* (Peters, 1881), *F. ochraceocinereus* (Heuglin, 1864), and *F. zechi* (Matschie, 1900). Subsequent allozyme and karyological studies (Aguilar, 1993; Macholán et al., 1998; Filippucci et al., 1994; Burda et al., 1999; Chitaukali et al., 2001; Van Daele et al., 2004; Burda et al., 2005) demonstrated the specific status of *Fukomys amatus* (Wroughton, 1907), *Fukomys anselli* (Burda et al., 1999), *Fukomys darlingi* (Thomas, 1895), *Fukomys kafuensis* (Burda et al., 1999), *F. micklemi* (Chubb, 1909) and *F. whytei* (Thomas, 1897). *Fukomys* mole-rats are a complex of morphologically very similar species that can not be readily diagnosed due to the low resolution of the known morphological markers.

However, variation in diploid number in this clade is known to be among the highest among mammals, as opposed to the karyotypically conserved sisterclade *Cryptomys sensu stricto* (Burda, 2001). Furthermore, Van Daele et al. (2004) recently described 9 new chromosomal races within the Zambezian clade (i.e. the *mechowi* (sic) group of Ingram et al., 2004), illustrating that south-central Zambia forms a "hotspot of karyotypical diversity". The evolutionary mechanisms behind chromosomal diversity within *Fukomys* remain poorly understood. The mainly allopatric distribution of the known chromosomal races that occur throughout the Zambezian phytochorion is consistent with a scenario of vicariance events, driven by major geomorphological reconfigurations in the Zambezian area (Van Daele et al., 2004; Cotterill, 2003). The observed absence of morphological differentiation accompanied with a high level of chromosomal diversity underpins the need for a systematic revision of these mammals, as well as a molecular phylogeographic approach to infer the evolutionary histories and interrelationships of these putative taxa and their populations.

Using mitochondrial cytochrome b (cyt b) sequences we aim to clarify the interrelationships and evolutionary history of the known chromosomal races. The combination of molecular and chromosomal data allows further exploration of the role of chromosomal evolution in the speciation of these small mammals. Are we to expect equal levels of genetic divergence between chromosomal races within different clades, as might be anticipated in view of the comparable range of diploid numbers in e.g. northern and southern Zambezian lineages? This study provides the most comprehensive sampling of this taxon so far, by increasing the number of studied chromosomal races and extending the sampling area in comparison with earlier studies. The choice of the mitochondrial cytochrome b locus was based on the facts that 1. due to its higher mutation rate compared with most nuclear DNA, it is known to be useful for the inference of relationships among closely related mammal species and populations, and 2. previously published sequences could be included in our analyses.

2. Materials and methods

2.1. Sampling strategy: topotypical approach

Specimens of *Fukomys* were sampled throughout their distribution in Zambia and Malawi between 1987 and 2002 (Fig. 1). All specimens collected at type localities are tentatively given the corresponding species name. However, species names are not provided for any of the other specimens due to the low resolution of the diagnostic morphological characters. The samples comprise the topotypes of all chromosomal races (underlined) that hitherto have been described as well as specimens collected from a

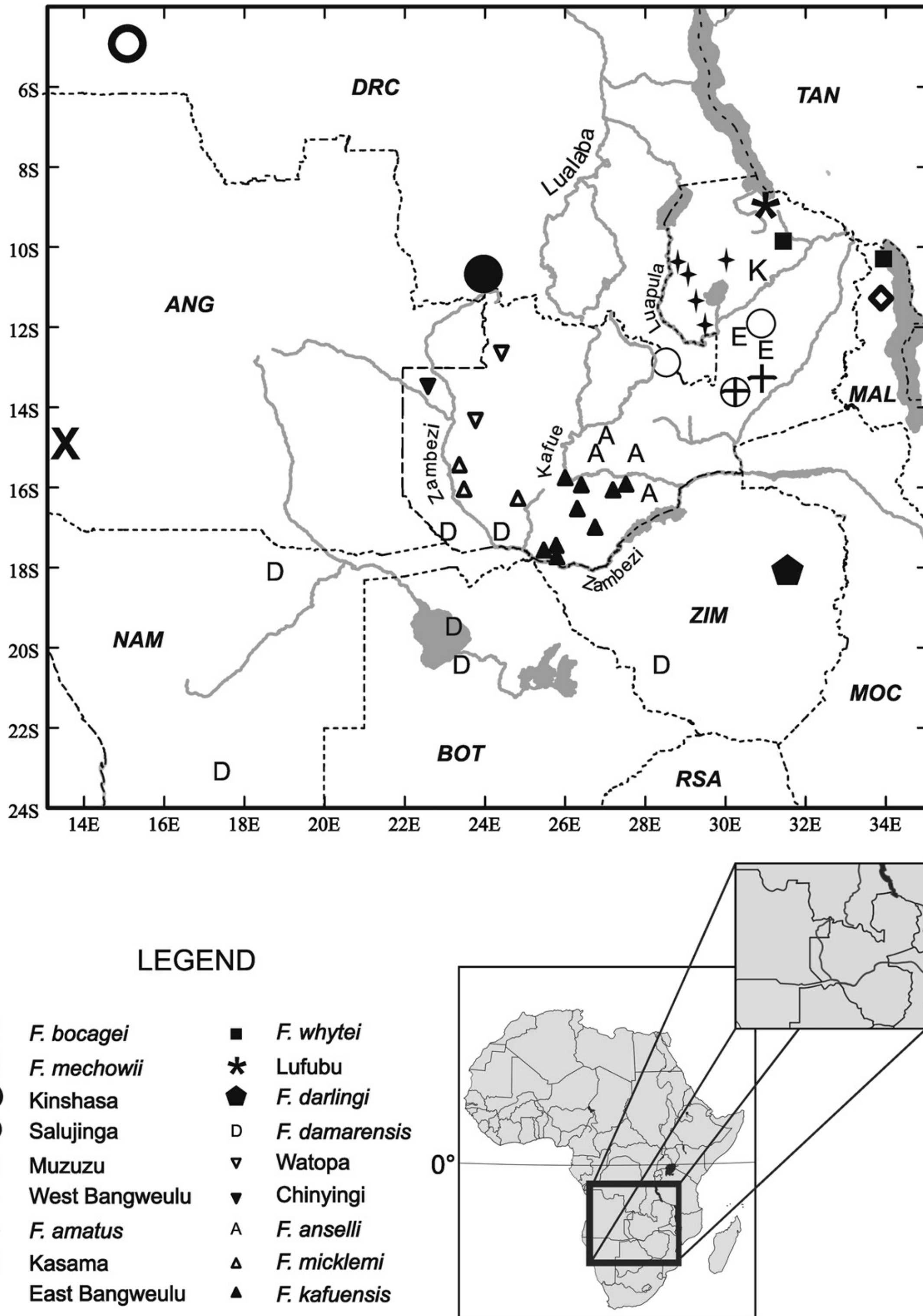

Fig. 1. Sampling localities in Zambia and neighbouring countries.

number of type localities (marked with *). Thus, our present data set includes data from all described species and chromosomal races in the genus (see above). From the total of 97 samples, sequence data of 66 samples are previously unpublished. This includes data from 27 new localities (Table 1).

2.2. DNA isolation, PCR amplification and nucleotide sequencing

Prior to DNA extraction, tissue (liver) was preserved in either 70% ethanol (University of Duisburg-Essen collection) or 95% ethanol (Ghent University collection) and stored at −20 °C.

Table 1
Specimens sequenced with their respective clade, geographic location and GenBank Accession Numbers

Clade	N	Location and country	Lat (S)	Lon (E)	GenBank No.
mechowii	1	Chiundaponde, Zambia*	12°14′	30°35′	EF043451
mechowii	1	Chibale, Zambia	13°35′	30°05′	EF043452
mechowii	1	Mbombo-Lumene NP (near Kinshasa), DRC	04°22′	15°27′	AF012231
mechowii	1	Chingola, Zambia	12°31′	27°51′	AF012230
mechowii	3	Salujinga, Zambia	10°58′	24°05′	EF043453–EF043455
bocagei	1	Lubango, Angola	14°56′	13°27′	AF012229
whytei	5	Kasanka, Zambia	12°08′	29°47′	EF043456– EF043460
whytei	1	Kambi, Zambia	11°30′	29°34′	EF043461
whytei	1	Kane, Zambia	10°33′	28°59′	EF043462
whytei	1	n. Kakululu River, Zambia	10°38′	29°04′	EF043463
whytei	1	Chief Tungati Local Forest, Zambia	10°27′	30°00′	EF043464
whytei	1	Kama, Zambia	12°24′	30°21′	EF043465
whytei	2	Chinsobwe, Zambia	13°23′	30°21′	EF043466– EF043467
whytei	1	Chibale, Zambia*	13°35′	30°05′	EF043468
whytei	1	Mushangashi, Zambia	12°28′	30°23′	EF043469
whytei	2	South of Lake Chiwakawaka, Zambia	12°32′	30°37′	EF043470–EF043471
whytei	1	Ndeba1, Zambia	12°26′	30°39′	EF043472
whytei	1	Ndeba2, Zambia	12°28′	30°38′	EF043473
whytei	1	Kasama, Zambia*	10°16′	31°00′	EF043474
whytei	2	Lufubu, Zambia*	09°15′	30°53′	EF043475–EF043476
whytei	1	Karonga, Malawi*	09°56′	33°56′	EF043477
whytei	3	Mbala, Zambia	09°50′	31°24′	AY425860–AY425862
whytei	1	Suma, Tanzania	09°10′	33°40′	AY425859
whytei	1	Mzuzu, Malawi	11°27′	34°03′	AY425863
darlingi	1	Goromonzi, Zimbabwe	17°52′	31°30′	AF012232
damarensis	2	Simungoma, Zambia	22°49′	38°57′	EF043478–EF043479
damarensis	2	Sioma Ngwezi N.P., Zambia	31°33′	19°36′	EF043480–EF043481
damarensis	1	Maun, Botswana	19°59′	23°21′	AF012221
damarensis	4	Okavango, Botswana	19°32′	23°11′	AF012220–AF12224
damarensis	1	Bulawayo, Zimbabwe	20°09′	28°38′	AY425857
damarensis	3	Dorbabis, Namibia	22°58′	17°41′	AF012225
damarensis	1	Rundu, Namibia	17°48′	19°32′	AY425858
damarensis	3	Hotazel, South Africa	27°17′	23°00′	AY425853–AY425855
micklemi	1	Chinyingi, Zambia	13°23′	23°00′	EF043482
micklemi	2	Mayau, Zambia	12°44′	24°20′	EF043483–EF043484
micklemi	2	Watopa, South of Kabompo River, Zambia	14°00′	23°47′	EF043485–EF043486
micklemi	1	Namwala N, Zambia	15°40′	26°25′	EF043487
micklemi	1	Namwala S, Zambia	NA	NA	EF043488
micklemi	1	Munali1, Zambia	15°57′	28°07′	EF043489
micklemi	1	Munali2, Zambia	15°58′	28°08′	EF043490
micklemi	1	Moono, Zambia	15°08′	26°57′	EF043491
micklemi	1	Kaindu, Zambia	14°29′	26°54′	EF043492
micklemi	1	Lusaka, Zambia*	15°19′	28°27′	AF012233
micklemi	2	Kataba, Zambia*	15°23′	23°23′	EF043493–EF043494
micklemi	1	Senanga, Zambia	15°58′	23°20′	EF043495
micklemi	1	Luampa, Zambia	22°46′	24°56′	EF043496
micklemi	1	Mazabuka, Zambia	16°12′	27°25′	EF043497
micklemi	1	Monze, Zambia	16°04′	27°32′	EF043498
micklemi	1	Lochinvar, Zambia*	16°06′	27°18′	EF043499
micklemi	2	Dongo, Zambia	16°38′	26°27′	EF043500–EF043501
micklemi	1	Kalomo, Zambia	16°58′	26°36′	AF012234
micklemi	2	Kavumba, Zambia	17°35′	25°21′	EF043502–EF043503
micklemi	2	Kalamba near Sekute, Zambia	17°38′	25°41′	EF043504–EF043505
micklemi	1	Sekute, Zambia	17°38′	25°41′	EF043506
micklemi	1	Libala near Sekute, Zambia	17°38′	25°41′	EF043507
micklemi	1	Kabala near Sekute, Zambia	17°38′	25°41′	EF043508
micklemi	1	Ndrevu, Zambia	17°38′	25°41′	EF043509
micklemi	1	Livingstone, Zambia	17°54′	25°53′	EF043510
micklemi	1	Itezhi-Itezhi, Zambia*	15°51′	26°03′	EF043516
micklemi	3	Mikata, Zambia	NA	NA	EF043511–EF043512
micklemi	3	Kajunika lila, Zambia	NA	NA	EF043513–EF043515
C. nimrodi	1	Hillside, Bulawayo, Zimbabwe	20°09′	28°38′	AF012237
C. hottentotus	1	Steinkopf, South Africa	29°17′	17°45′	AF12240

(*continued on next page*)

Table 1 (*continued*)

Clade	N	Location and country	Lat (S)	Lon (E)	GenBank No.
C. pretoriae	1	Pretoria, South Africa	25°47′	28°13′	AF12235
C. mahali	1	Patryshoek, Pretoria, South Africa	25°40′	28°02′	AY524870
C. natalensis	1	Kokstad, South Africa	31°32′	29°38′	AF12236
B. suillus	1	Rondawel, South Africa	30°47′	17°53′	AY425913

Genomic DNA was isolated by proteinase-K digestion followed by extraction over Qiagen DNAEasy spin columns (Hilden, Germany) according to the manufacturers' instructions. PCR amplification of the complete cyt b (1140 base pairs) was carried out using primers (L14723 and H15915) and reaction conditions described previously for African mole-rats (Faulkes et al., 1997). Sequencing was carried out in both directions using the PCR primers to obtain partially overlapping strands. Sequencing reactions were performed on an ABI 377 automated sequencer using BigDye Terminator v3.1 chemistry (Applied Biosystems, Foster City, CA). All new sequences (including voucher references) have been deposited in NCBI with Accession Nos. EF043451–EF043516 (Table 1).

2.3. Sequence and phylogenetic analyses

Previously published cytochrome b sequences from *Fukomys* specimens from the Zambezian area (Faulkes et al., 2004) were obtained from NCBI and included in the analyses (although many are partial cyt b sequences; Table 1). The compiled sequences were subsequently aligned using ClustalW (Thompson et al., 1994) in MEGA v3.0 (Kumar et al., 2004). Based on previous studies (Faulkes et al., 2004; Ingram et al., 2004), other bathyergids, five *Cryptomys species* and *Bathyergus suillus* (Table 1) (Faulkes et al., 2004) were included as outgroup taxa. Calculation of genetic distances and both maximum parsimony (MP) analysis and two probabilistic approaches, maximum likelihood (ML) and Bayesian analyses, were performed on the complete dataset. Both for reasons of clarity and to limit computing time, we used a subset of representative haplotypes in the final analyses. Datasets were examined for substitution saturation (transversions and transitions separately) plotting matrices of patristic against adjusted character distances calculated by PAUP* v4.0b11. For ML and Bayesian analysis we included *C. nimrodi* as the only outgroup taxon. Prior to ML and Bayesian analyses Akaike information criterion (AIC) tests of different models of evolution were performed with Modeltest v3.07 (Posada and Crandall, 1998). MP and ML analyses were performed using PAUP* v4.0b11 (Swofford, 2002). For the MP analyses we used the heuristic search option with characters having an equal weight. In a second round of MP analyses characters were then weighted a posteriori (Farris, 1969) according to their rescaled consistency index (RC; Farris, 1989). Under these MP criteria bootstrap proportions (BP) were calculated, using 1000 pseudoreplicates, random addition of taxa and TBR branch swapping with the steepest descent option not in effect. For ML analyses we employed the heuristic search option with TBR branch swapping, where starting trees were obtained via random stepwise addition. Robustness of the ML tree was determined in a bootstrap analysis using the stepwise addition option and the heuristic search option with 500 pseudoreplicates (Felsenstein, 1985). Bayesian posterior probabilities (PP) were estimated using MrBayes v3.01 (Ronquist and Huelsenbeck, 2003). The analyses were initiated with random starting trees and were run for 1×10^6 generations, sampling every 100th generation. Likelihood values were checked graphically to see if stationarity had been reached. Four separate independent searches were run, each consisting of three heated chains and one cold chain. The burn-in value was set at 500. Thus the first 500 trees (50,000 generations) were discarded in the approximation of posterior probabilities. The trees of the various runs were combined, producing a 50% majority rule consensus tree. All trees were visualised with Treeview 1.6.6 (Page, 1996).

2.4. Estimation of divergence time

Phylogenetic dating was applied to genetic distances inferred from a ML tree. A likelihood ratio test (Felsenstein, 1981), as well as Tajima's relative rate tests (RRT; Tajima, 1993), indicated that the cytochrome b dataset departs slightly from true clocklike evolution. To identify significant differences in substitution rate between lineages we used the two-cluster test of the LINTRE package (Takezaki et al., 1995) Substitution rate varies among lineages (see below). Therefore, we applied two different methods which relax the stringency of the molecular clock assumption to estimate time of divergence: (1) a semiparametric rate smoothing method, using the penalised likelihood smoothing (PLS) approach; and (2) a non-parametric rate smoothing (NPRS) method. Both were implemented in R8s version 1.7 (Sanderson, 1997, 2003). For the penalised likelihood smoothing approach, an optimal (=lowest) smoothing value was determined by a cross-validation procedure (Sanderson, 2004). Following the recommendations of the author of that application, we trimmed the original ML phylogeny, retaining only unique haplotypes ($N = 29$). There are only a few reliable calibration points to estimate divergence time in the derived lineages of the Bathyergidae (for a review of fossil data, see Faulkes et al., 2004). Following Ingram et al. (2004), who estimated the divergence of *Cryptomys* and *Fukomys* between approximately 10 and 11 Mya, using NPRS on a combined data set of the mitochrondrial 12s ribosomal

RNA and the nuclear transthyretin (TTR) gene, we used 10 and 11 Mya as calibration points in our analyses.

3. Results

3.1. Relationships between major clades

Maximum parsimony analysis recovered three trees of equal length (1068 steps). Out of 1140 sites which were examined, 695 were constant, 320 were parsimony informative and 125 were uninformative. MP analysis after reweighting characters with their rescaled consistency index (RI) resulted in 256 characters not retaining a character weight of one and recovering three trees (consistency index = 0.495 retention index = 0.799 rescaled consistency index = 0.396 homoplasy index = 0.505). Fig. 2 shows the consensus tree based on the reweighted character set. Initial analysis (not shown) demonstrated that the inclusion of previously published sequences did not affect the overall topology compared with previously published phylogenies (Ingram et al., 2004; Faulkes et al., 2004). The major phylogroups that were also recovered in previous studies (op. cit.) formed well-supported monophyletic groups: *F. bocagei*/*mechowii* clade, *F. whytei* clade, *F. darlingi* clade, *F. damarensis* clade and *F. micklemi* clade. According to the AIC tests of model evolution, the TIM model corrected for invariable sites and among-site rate variation using the discrete gamma distribution was found to best fit the data. A manual selection procedure (Swofford and Sullivan, 2003) resulted in GTR+I+Γ as the best choice. Both models produced ML trees with the same topology. The inferred ML tree (Fig. 3) differs slightly from the MP tree, the ML tree being two steps longer than the single most parsimonious tree (TL = 1070 vs. 1068, respectively). The only difference between the MP and ML trees concerns the placement of *F. bocagei*, while it forms a monophyletic group with the *F. mechowii* clade in the MP tree, it is basal to all other major clades in the ML tree. Note that in all analyses the enigmatic *F. cf. whytei* haplotype from Mzuzu forms a divergent, basal lineage to the *F. whytei* clade (BP = 72, PP = 0.97). The Bayesian analysis yields the same topology as the ML tree and generated high posterior probabilities for all the major phylogroups (Fig. 3).

3.2. Relationships within major phylogroups

In comparison with previous studies, the clades for which the sampling range was extended provide a detailed phylogeographic picture for the various groups. In each case similar topologies were recovered in all analyses.

3.2.1. Fukomys mechowii clade (Fig. 4)

In all analyses two reciprocal monophyletic subclades were recovered. MP analysis, however, gave strong support for both subclades as opposed to both probabilistic analyses, which provide only weak support for the monophyly of the Salujinga/Kinshasa subclade (BP = 66; PP = 0.72). The other subclade consists of all *F. mechowii* haplotypes, including the haplotype from near the type locality of *Georychus mellandi* (Thomas, 1906).

3.2.2. Fukomys whytei clade (Fig. 5)

Six subclades can be distinguished within the *whytei* clade (making abstraction of the *F. cf. whytei* haplotype from Mzuzu, Malawi (MZU)—see above). A first divergence contains the Western Bangweulu subclade (WBA), which forms a strongly supported monophyletic group with the *F. amatus* subclade. Both subclades are strongly supported in all analyses and are characterised by long branches leading to the subclades relative to branch lengths observed within the subclades. The monophyly of a second divergent lineage is weakly supported (BP = 67; BP = 0.76). It comprises, on the one hand, a well supported clade containing the Kasama (KAS), Eastern Bangweulu (EBA) and Lufubu (LUF) subclades. On the other hand, there is a basal clade with the topotypical *F. whytei* haplotype (Karonga) and the Mbala haplotype. Results from the initial analyses (not shown) indicate that this clade also contains the Tanzanian haplotype (Suma; excluded from later analyses because the obtained sequence was shorter).

3.2.3. Fukomys damarensis clade (Fig. 3)

The divergence pattern within the group of the eusocial Damaraland mole-rat is shallow. In all analyses the two Southwest Zambian haplotypes cluster with the geographically proximate haplotypes. The Simungoma haplotype from North of the Zambezi River is sister to this subclade. This is the first time a member of the *F. damarensis* clade has been discovered north of the Zambezi River. Interestingly, *F. damarensis* are morphologically very similar to the mole-rats of the *F. micklemi* clade, to which they are closely related.

3.2.4. Fukomys micklemi clade (Fig. 6)

This sister group to the *F. damarensis* clade is also characterised by relatively short branches. In all analyses we recovered a subtree with three main lineages, although their interrelationships remain unresolved: 1. the Watopa (WAT) subclade, which is sister to all other taxa in the clade; 2. the Chinyingi (CHI) subclade from West of the Zambezi River; and 3. a major radiation that includes all chromosomal races from South Central Zambia. In turn, this South Central Zambian subclade contains three monophyletic lineages including, respectively, topotypical *F. kafuensis* (Itezhi-Itezhi), *F. anselli* (Lusaka) and. *F. micklemi* (Kataba). Although the monophyly of the Kataba and Itezhi-Itezhi groups is well supported, the interrelationships among the members of these karyotypically hyper-diverse groups remain partly unresolved. The bootstrap support for the Lusaka group, containing all haplotypes of animals with 2n = 68, is weak (BP = 63; PP = 0.64). In the MP analysis the

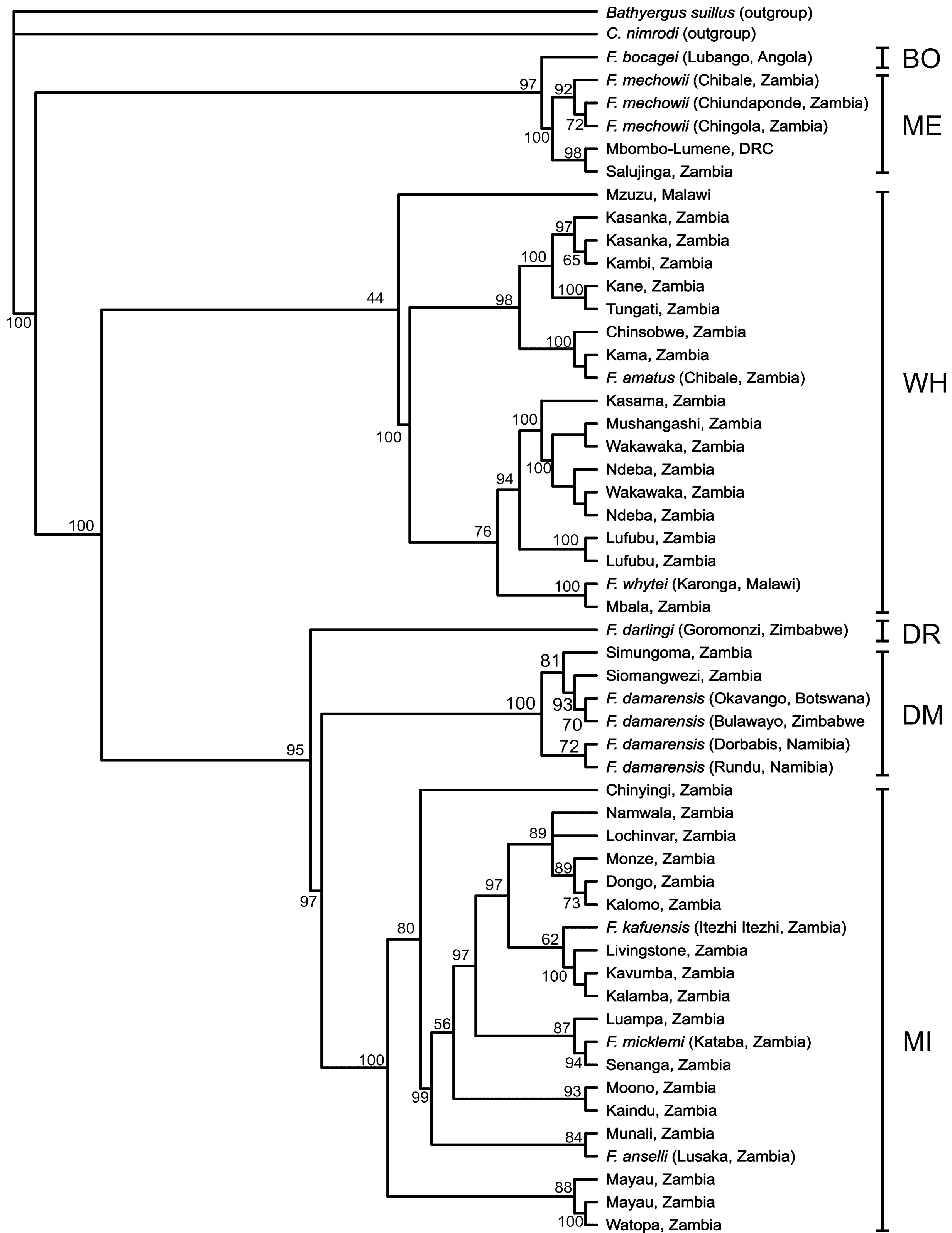

Fig. 2. Maximum parsimony estimate of the Zambezian *Fukomys* phylogeny based on cytochrome b gene sequences: 50% majority rule consensus tree using the heuristic search option (tree length = 1068, consistency index = 0.495, rescaled consistency index = 0.396). For all branches values above the branches refer to bootstrap proportions after weighting sites with their rescaled consistency index. Major clades: BO, *F. bocagei* clade; ME, F. mechowii clade;, WH, *F. whytei* clade; DR, *F. darlingi* clade; DM, *F. damarensis* clade; MI, *F. micklemi* clade.

Fig. 3. Maximum likelihood estimate of the *Fukomys* phylogeny based on cytochrome b gene sequences. The ML analysis using a TIM+I+Γ model of sequence evolution ($R = 1.0000$ 30.3522 1.5304 1.5304 17.4605; Pinvar = 0.5192 gamma shape parameter = 2.1328) generated a tree with $-\ln L = 7774.03$ and tree length = 1070. Values above the branches indicate ML bootstrap proportions (1000 replicates), while values under the branches refer Bayesian posterior probabilities. The scale bar represents the number of substitutions per site. Major clades: BO, *F. bocagei*; ME, F. mechowii clade; WH, *F. whytei* clade; DR, *F. darlingi* clade; DM, *F. damarensis* clade; MI, *F. micklemi* clade.

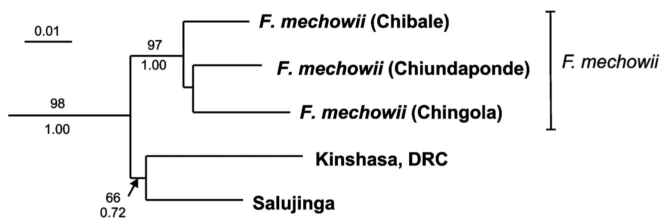

Fig. 4. ML subtree of *F. mechowii* clade (cf. Fig. 3).

haplotypes of the Lusaka group are paraphyletic (Moono and Kaindu haplotypes vs. Munali and Lusaka haplotypes).

3.3. Intra- and interspecific sequence differences

Both uncorrected and TIM+I+Γ corrected distances are listed in Table 2. Average corrected (TIM+I+Γ) pairwise sequence differences between outgroup and ingroup taxa ranged from 21.5 to 28.4% (mean = 26.3%). The average corrected pairwise distances among and within the ingroup taxa (subclades) were 9.6 % (Range: 1.7–15.5) and 1.0% (Range: 0.3–2.5), respectively. The highest distances are found between the *F. bocagei* clade (basal lineage in the ML phylogeny) and the other clades (range: 12.8–15.5%)

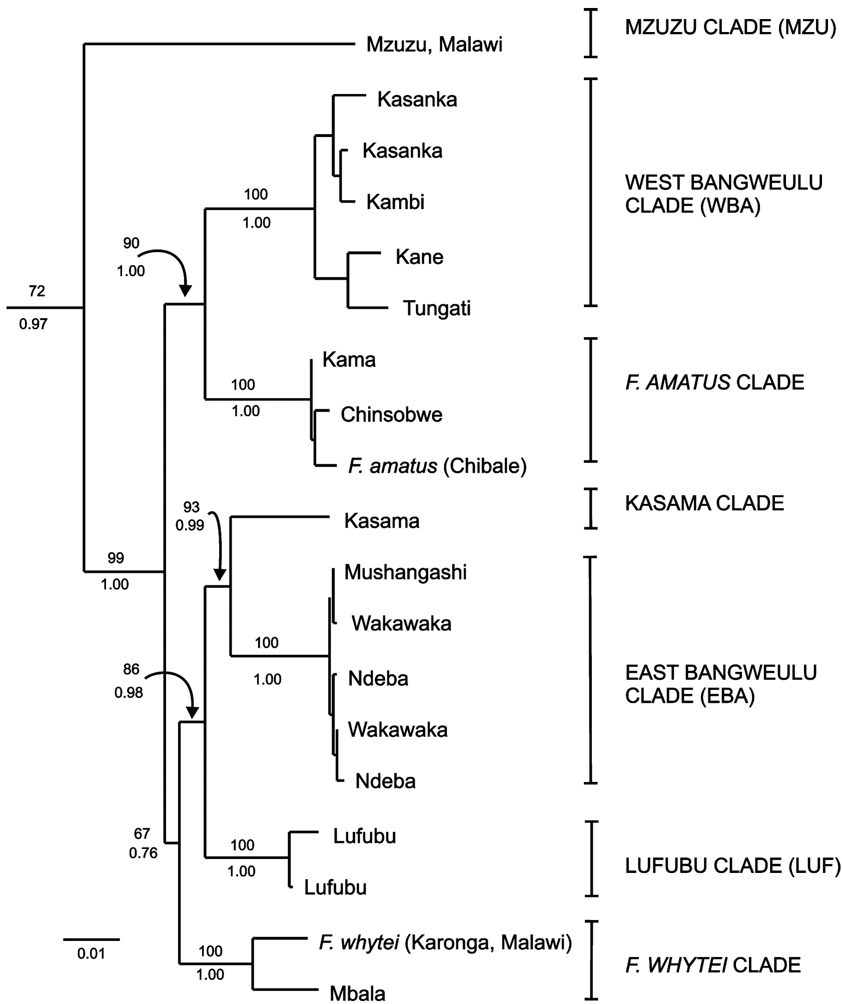

Fig. 5. ML subtree of *F. whytei* clade (cf. Fig. 3).

with a maximum between the Mzuzu and *F. bocagei* haplotypes. The lowest distances are observed among the subclades of the *F. micklemi* clade (Range: 1.7–3.6), with particularly low values between the *F. anselli* subclade and *F. micklemi* subclades (1.7%) and between the *F. micklemi* and *F. kafuensis* subclades (1.8%). The highest TIM+I+Γ corrected distances within the F. *micklemi* clade were found between the Chinyingi and Watopa subclades on the one hand and all remaining clades on the other hand (respective ranges: 2.5–4.1 and 2.8–4.7). A remarkably low level of genetic divergence is found within the chromosomally hyper-diverse *F. micklemi* subclade. The average TIM+I+Γ corrected distance within this phylogroup is 0.6%, with an interhaplotype range of 0.1 to 1.6%, excluding the Kalomo haplotype, which appears to be considerably more dissimilar from the other haplotypes (range 1.6–3.0%). Within the *F. whytei* clade, the Mzuzu haplotype is an outlier with TIM+I+Γ corrected distances that differ on average 8.4% from all other haplotypes in this subclade. The two Zambian haplotypes show genetic divergence levels that are similarly low as in other haplotypes of this clade. Among major clades average corrected TIM+I+Γ distances correlate well with geographic distance, while within there is no apparent trend in any of the major clades.

3.4. Estimates of divergence times

For this analysis we used a subset of the data. Graphical saturation analysis indicates a linear relationship up to 20% p-distance, while beyond that level slight substitution saturation occurred in transition substitutions only. These values correspond to comparisons with outgroups. Therefore, both substitution types were used to calculate estimates of divergence times (Table 3 and Fig. 7). The value of the PL models' optimal smoothing value was set to 1, illustrating a poor fit to a molecular clock and allowing for considerable rate heterogeneity across branches. Significant departures from rate constancy were observed in comparisons between the major lineages (Table 4) Using the calibration of 10–11 Mya for the divergence of *Fukomys* and *Cryptomys* we were able to estimate key cladogenetic events. The obtained results suggest that all major clades radiated during the Pleistocene. A northern Zambezian lineage (containing the *F. whytei* clade) evolved into separate subclades from the Late Pliocene on (Fig. 7, node E). In contrast, two

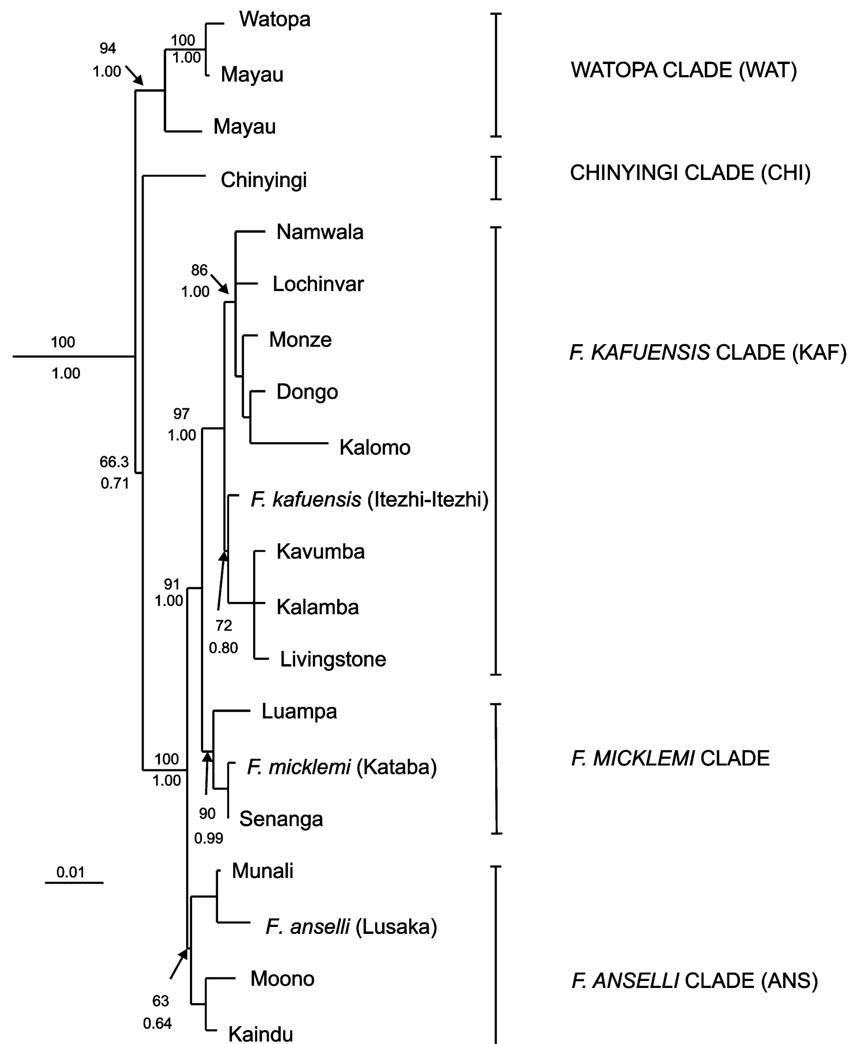

Fig. 6. ML subtree of *F. micklemi* clade (cf. Fig. 3).

southern Zambezian lineages—the *F. damarensis* clade and the *F. micklemi* clade—constitute more recent radiations dating back to the middle Pleistocene. The chromosomal radiation within the *F. micklemi* subclade is dated at 0.56–1.2 Mya (Fig. 7, nodes L and M).

4. Discussion

4.1. Phylogenetic relationships

Within Zambezian *Fukomys* our cytochrome b analyses recovered the same overall topology as in previous molecular sequence studies (Ingram et al., 2004; Faulkes et al., 2004). At the base of the Zambezian *Fukomys* trees the exact position of the F. *bocagei* clade remains unclear. To address this problem, denser sampling in the presumably exclusively Angolan distribution range of *F. bocagei* clade will be necessary. Earlier studies showed that small mole-rats (i.e. representatives from the *F. whytei*, *F. darlingi*, *F. damarensis* and *F. micklemi* clades), do not constitute a monophyletic assemblage of haplotypes (op. cit.). For example, the Salujinga haplotypes cluster within the *F. mechowii* clade, implying that the representatives of this small race are closely related to the giant mole-rats (*F. mechowii* clade), a finding that is supported by the similarity of their karyotypes (Van Daele et al., 2004). This chromosomal race is congruent in external morphology with the robust mole-rats from the geographically close Watopa phylogroup (a group within the derived *F. micklemi* clade). In view of the poor support in the ML for the monophyly of the Salujinga and Kinshasa haplotypes, the latter presumably forms a separate divergent lineage. The *F. mechowii* clade encompasses robust to giant forms, which are uniformly brown in colour (Fig. 3). The monophyly of the *whytei* clade is not well supported. In spite of our extended sampling in the Zambian distribution range of the *F. whytei* clade, the affinities of the Mzuzu haplotype remain unclear (cf. low bootstrap values), though it appears to have a basal position in relation to the different *F. whytei* subclades. All these subclades form well supported monophyletic groups that are genetically well differentiated (3.1–5.8%). This finding may be a result of

Table 2
Mean cytochrome b distances between sequences (%)[a]

Clade	MECHOWII clade			BOC	WHYTEI clade							DAM	DAR	MICKLEMI clade					CRY	BAT
Subclade	F. mec	KIN	SAL	F. boc	WBA	F. ama	EBA	KAS	LUF	F. why	MZU	F. dam	F. dar	CHI	WAT	KAF	ANS	F. mick		
n/N	4/4	1/1	2/3	1/1	6/9	3/4	5/5	1/1	2/2	3/5	1/1	16/17	1/1	1/1	4/4	18/23	4/5	4/4	5/5	1/1
F. mec	***2.4***	5.3	4.6	10.5	11.5	11.1	11.3	10.9	10.9	12.3	12.4	13.2	11.8	13.0	13.0	13.3	13.4	13.2	28.4	25.0
KIN	4.9	***NA***	4.8	12.4	12.4	11.6	11.8	12.3	12.5	13.4	12.7	14.5	13.0	13.7	13.8	14.2	14.6	14.6	27.0	24.8
SAL	4.3	4.5	<***0.1***	10.8	11.5	12.4	11.7	11.7	11.8	12.9	12.3	13.5	11.1	11.8	13.0	12.9	13.0	12.5	28.1	26.4
F. boc	9.0	10.4	9.2	***NA***	13.1	12.8	13.6	12.8	12.9	12.8	15.5	13.2	15.1	13.5	13.2	14.5	13.4	13.8	24.5	24.7
WBA	9.6	10.3	9.6	10.9	***1.4***	5.0	5.1	4.6	4.9	5.8	8.0	10.1	8.9	9.5	9.7	9.7	9.5	9.4	27.2	24.2
F. ama	9.4	9.7	10.2	10.7	4.6	***0.3***	5.6	4.3	5.2	5.5	8.9	9.6	9.5	9.2	9.0	10.5	9.9	10.0	25.6	23.7
EBA	9.4	9.8	9.8	11.2	4.7	5.0	***0.3***	3.1	3.8	5.0	9.0	9.8	9.2	9.8	9.8	9.8	10.1	10.1	26.1	22.4
KAS	9.2	10.2	9.8	10.7	4.2	4.0	3.0	***NA***	3.4	4.5	8.5	8.8	8.8	8.7	9.1	9.8	9.4	9.3	24.8	21.6
LUF	9.3	10.4	9.9	10.8	4.5	4.7	3.5	3.1	***0.7***	4.4	7.2	8.7	8.3	9.5	9.6	10.1	10.0	9.8	25.2	21.5
F. why	10.2	11.0	10.6	10.7	5.3	5.0	4.6	4.2	4.1	***1.5***	9.1	8.7	8.9	9.3	9.5	10.4	9.8	9.8	26.0	22.4
MZU	10.2	10.5	10.1	12.4	7.0	7.7	7.7	7.4	6.4	7.8	***NA***	9.1	9.3	10.1	10.1	10.1	10.4	10.0	25.8	23.8
F. dam	10.8	11.7	11.0	10.9	8.6	8.3	8.5	7.7	7.6	7.6	7.9	***1.3***	8.0	6.6	6.5	7.0	6.4	6.8	24.7	22.3
F. dar	9.9	10.7	9.4	12.2	7.7	8.2	8.0	7.7	7.4	7.8	8.1	7.1	***NA***	7.9	8.3	7.9	7.9	8.0	27.1	22.9
CHI	10.5	11.1	9.8	11.1	8.1	7.9	8.4	7.6	8.2	8.1	8.6	5.9	7.0	***NA***	2.8	3.6	3.0	3.0	25.9	23.9
WAT	10.6	11.1	10.5	10.9	8.3	7.8	8.4	7.9	8.2	8.2	8.6	5.9	7.3	2.6	***0.7***	3.7	3.1	3.1	26.4	23.7
KAF	10.8	11.5	10.5	11.7	8.4	9.0	8.4	8.4	8.7	8.9	8.6	6.3	7.0	3.4	3.5	***1.0***	2.2	1.8	26.2	23.6
ANS	10.9	11.7	10.6	11.1	8.1	8.5	8.7	8.1	8.6	8.4	8.8	5.8	7.0	2.9	3.0	2.1	***1.1***	1.7	26.7	23.5
F. mick	10.7	11.7	10.3	11.3	8.1	8.6	8.7	8.0	8.4	8.4	8.5	6.1	7.1	2.8	3.0	1.8	1.6	***0.6***	27.0	23.8
CRY	19.0	18.5	19.0	17.6	18.6	17.8	18.2	17.6	17.8	18.1	18.1	17.4	18.4	17.9	18.2	18.0	18.2	18.3	***9.7***	24.6
BAT	17.6	17.6	18.2	17.6	17.4	17.3	16.7	16.1	16.1	16.5	17.2	16.5	16.9	17.1	17.1	17.0	16.9	17.1	17.4	***NA***

[a] Below diagonal TIM+I+Γ corrected distances, above diagonal uncorrected p distances, along diagonal within clade uncorrected p distances. The following taxa are included: F. mec, *F. mechowii*; KIN, Kinshasa; BOC/F. boc, *F. bocagei*; WBA, western Bangweulu group; F. ama, *F. amatus*; EBA, eastern Bangweulu group; KAS, Kasama group; LUF, Lufubu group; F. why, *F. whytei*; MZU, Mzuzu group; DAR/F.dar, *F. darlingi*; DAM/F. dam, *F. damarensis*; CHI, Chinyingi group; WAT, Watopa group; KAF, containing topotypical *F. kafuensis* clade ($2n = 42$–58); ANS, containing topotypical *Fukomys anselli* ($2n = 68$); F. mick, *F. micklemi* ($2n = 60$); CRY, *Cryptomys species.*; BAT, *Bathyergus suillus*.

Table 3
Estimated dates of divergence (Mya) for nodes in Fig. 7 using NPRS and PLS[a]

NODE	PLS 10&11	NPRS 10&11
CRY	10–11	10–11
A	3.8–4.18	5.45–6
B	1.41–1.55	2.98–3.28
C	3.33–3.66	4.88–5.37
D	2.15–2.37	3.19–3.51
E	1.84–2.02	2.75–3.02
F	1.28–1.41	1.92–2.11
G	1.01–1.11	1.47–1.61
H	1.11–1.22	1.7–1.88
I	1.7–1.87	2.58–2.84
J	1.3–1.43	2.04–2.25
K	0.35–0.39	0.77–0.84
L	0.61–0.67	1.09–1.2
M	0.56–0.62	1–1.1

[a] Dates obtained from non-parametric rate smoothing (NPRS) and semiparametric rate smoothing (penalized likelihood smoothing: PLS). The ages are in million years before present (Mya).

accelerated cytochrome b sequence evolution rather than of ancient divergences, since cladogenesis would have occurred ca 1–2 Mya (see Table 4; similar results are obtained with Tajima's RRT—results not shown). All forms in this clade are uniformly brownish rats, which lack clear headspots (Fig. 3). The chromosomal *F. micklemi* radiation is characterised by extremely low levels of cytochrome b sequence divergence. The *F. micklemi* subtree topology basically consists of a trichotomy with three morphologically distinct groups. Firstly, there is the small, fawn-grey race with only an indication of a white mark on the head, collected at Chinyingi in the western Kasiji Plains. Second, there is a cluster of robust, brownish mole-rats from Watopa and Mayau, which are seemingly similar in size and shape to the Salujinga specimens. A third large cluster contains typical blesmoles (a misnomer for *Cryptomys* and *Fukomys* in view of the variation in external morphology) with clear headspots and brownish to black pelage (Fig. 3). The observed pattern of divergence appears to be congruent with several consecutive evolutionary bursts, which seem to have started in Northwestern Zambia (see below).

4.2. Taxonomic implications

Bathyergids exhibit considerable geographically structured morphological and genetical variation (Faulkes et al., 2004; Van Daele et al., 2006). In the past this fact triggered the description of many species and subspecies of bathyergids. Although this is not the place to revive the discussion about species concepts, it seems that the various species concepts can't be neatly applied to bathyergids and subterranean rodents in general (see e.g. Lessa, 2000). We advocate that species limits should be established using multiple sources of evidence which includes data from the molecular, cytogenetical and organismal levels (as opposed to e.g. Roberts, 1951). Traditional morphology based systematic studies of *Fukomys* (earlier synonym *Cryptomys*) yield no clear results because of the lack of clear morphological differences between species. This problem is best illustrated by Haymans' struggle to classify the species of both *Fukomys* and *Cryptomys* (in Ellerman et al., 1940).

The presently available information indicates that *Fukomys* contains more species than the 12 that are currently recognised. Within all major clades there are multiple examples that provide support for the hypothesis that a number of described chromosomal races represent valid sibling species, of which several with limited parapatric distributions. Unpublished experiments attempting to crossbreed different chromosomal races indicate that the chromosomal races tested so far appear to have developed both (ethological) premating and postmating mechanisms, which isolate these (presumptive) species (P. Van Daele, unpublished data; H. Burda personal comment). Within the *F. mechowii* clade, at least the Salujinga lineage may represent a so far undescribed species. These two potential species not only differ in body size but also have different skull shapes and a different karyotype (Van Daele et al., 2004), and represent separate cyt b lineages. Chromosomal data are missing for the ambiguous Kinshasa (DRC) haplotype (originating from the Mbombo-Lumene National Park—DRC), but the external morphological features of the specimens of this population are typical *F. mechowii* (R.K. Kisasa, personal comment). The large mole-rat specimen from Chiundaponde (Zambia), collected in the vicinity of the type-locality of *Georychus mellandi* (Thomas, 1906) resembles *F. mechowii* in all aspects studied so far (external morphology, karyotype, cyt b) and should, therefore, be considered a synonym. Our data, combined with geometric morphometrical data (skull shape and size, Van Daele et al., 2006) reveal that the *F. whytei* clade contains genetically well differentiated taxa that are morphologically very similar. Therefore, the two currently recognised species within the northern Zambezian *F. whytei* clade, *F. amatus* and *F. whytei* may represent as many as 7 species (including Mzuzu). Species descriptions of these Northern Zambian, Tanzanian and Malawian taxa will have to await the analysis of topotypical material from the geographically proximate *C. h. occlusus* (Allen and Loveridge, 1951) described from Kigogo, Tanzania. Overall the *F. whytei* clade is characterised by a brown pelage colour and the absence of clear headspots or the infrequent occurrence of small headspots. Therefore, future studies should take into account that many museum specimens (typically from outside the distribution range of the *F. whytei* clade) that were assigned on morphological grounds to *F. amatus* or *F. whytei* probably belong to taxa in the parapatrically distributed *F. micklemi* clade. Within the *F. micklemi* clade the taxonomical picture is muddled. *F. micklemi* is the senior specific name available for representatives of that clade. Further systematic studies are required to determine the specific status and the validity of taxonomic assignment of *F. anselli* and *F. kafuensis*.

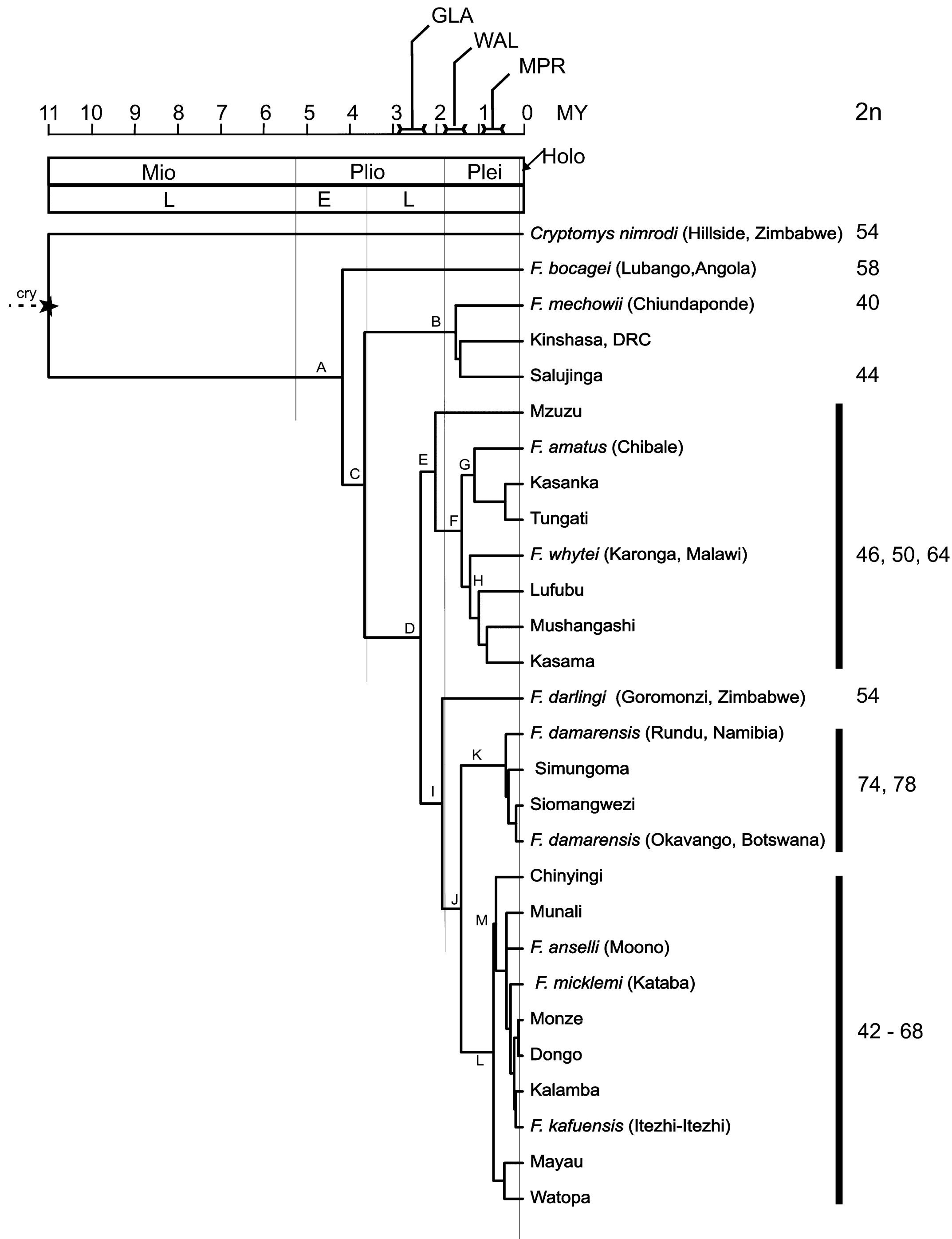

Fig. 7. Dated phylogenetic tree of Zambezian *Fukomys* obtained after semiparametric rate smoothing (penalized likelihood smoothing: PLS) in R8s. Letters on the nodes refer to Table 3. The time scale shows ages in million years before present Mya. Mio, miocene; Plio, pliocene; Plei, pleistocene; Holo, holocene; L, late; E, early; GLA, onset of northern glaciations; WAL, development of Walker circulation; MPR, mid pleistocene revolution.

Table 4
Results of a Two Cluster test showing significant heterogeneity between major clades

Cluster		delta	*Z*
A	B		
F. damarensis clade	<*F. micklemi*	0.006243	5.08339**
F. darlingi clade	<*F. damarensis*, *F. micklemi* clades	0.00725	2.685171**
F. whytei clade	>*F. darlingi*, *F. damarensis*, *F. micklemi* clades	0.01785	9.395536**

** Values are significant at the 1% level.

No topotypical material has been analysed for *C. molyneuxi*, possibly a senior synonym of *F. anselli*. There is only limited evidence that supports splitting *F. micklemi* (the senior synonym) and *F. kafuensis* (Burda et al., 1999). It should be stressed that the diagnostic value of chromosomal markers for the separation and identification of putative *Fukomys* species remains to be tested.

4.3. Biogeographical and temporal implications

The current distribution of the different phylogroups correlates well with the geomorphological structuring and vegetational division of the Zambezian area (Fig. 11 in Van Daele et al., 2004; Fig 1). In spite of our efforts to collect on the Zaire River/Zambezi River watershed in Zambia, no haplotypes from the *F. bocagei* clade were recorded. *F. bocagei* seems to be the earliest divergence in the Zambezian mole-rats. This could be an indication of a western origin of the Zambezian group. This is further supported by both the coincidence of our divergence time estimate and the geomorphological history of South-central Africa. The *F. mechowii* clade is widespread along the Zaire River/Zambezi River watershed and in the Guineo-Congolean savannahs bordering the Guineo-Congolean forest. Based on our calibration of the molecular clock, we estimate that the clade diverged at the end of the Pliocene when dispersion along the Zaire/Zambezi watershed and across the Guineo-Congolean belt would have been possible. (See Fig. 5 in Van Daele et al., 2007). If our molecular clock is correct then the radiations within the *F. micklemi* and *F. damarensis* clades are surprisingly young, which implies that they are the result of rapid consecutive cladogenetic events since the early Pleistocene. Elsewhere we explained the coincidence of the distribution of chromosomal races with the geomorphological repatterning of the Zambezian area (Van Daele et al., 2004). That historical model, based mainly on riversystem reconfigurations, helps to explain the divergence between the major phylogroups. We suggest that climatic fluctuations may have played a substantial role in the diversification within each phylogroup. Associated vegetation shifts, leading to spatial fragmentation of populations, would have favoured the fixation of chromosomal rearrangements (Wang and Lan, 2000; Rieseberg, 2001; Veyrunes et al., 2005). Major steps in the evolution of *Fukomys* appear to coincide with shifts to more arid, open habitat conditions near 2.7–2.5, 1.9–1.7, and 0.95–0.7 Mya with alternating climatic shifts that lead to humid conditions and habitat fragmentation (see, respectively, Haug and Tiedemann, 1998; Ravelo et al., 2004; Berger and Jansen, 1994). The chronogram (Fig. 7) reveals that the observed cladogenetic events during the Plio-Pleistocene within the *F. mechowii*, *F. whytei*, *F. damarensis* and *F. micklemi* clades appear to coincide with climatically mediated speciation bursts in other savannah dwelling mammals, including hominids (Ducroz et al., 1998; Bobe and Behrensmeyer, 2004; Trauth et al., 2005; Veyrunes et al., 2005).

4.4. Chromosomal radiations

Although we find several cytotypes within each major clade for which several topotypes could be karyotyped, we do not detect a clear pattern of chromosomal evolution. It is remarkable that within the *F. whytei*, *F. damarensis* and *F. micklemi* clades, we observed more chromosomal races and subdivisions of chromosomal races in areas with a particularly affected geomorphology, such as capture elbows of the Zambezi, Kafue and Chambeshi rivers. Possibly the geomorphological disturbances in these areas have fuelled cladogenesis in these mole-rats. The combination of suitable alluvial grounds for the dispersal of mole-rats and fluctuating water levels in these streams (correlated with climatic shifts) may have resulted in the fragmentation of mole-rat populations and their differentiation through allopatric and peripatric speciation. Similar events have been suggested to have affected the speciation rates in small antelopes (Cotterill, 2003). However, when we plot the karyotypical data on the molecular phylogeny, a differential pattern of chromosomal evolution between the clades emerges. This difference is most striking when we compare the *F. micklemi* and *F. whytei* clades. The karyotypically hyper-diverse *F. micklemi* clade shows low levels of cytochrome b sequence divergence in agreement with the results from more limited datasets of other genes (op. cit.). Within the *F. whytei* clade we find a more conservative pattern of chromosomal diversification, while the levels of sequence divergence are much higher then in the *F. micklemi* clade. There are different plausible explanations for this phenomenon. Firstly, structural chromosomal rearrangements may play an important role in initiating species divergence (White, 1978), while mutations in mitochondrial DNA sequences will accumulate over longer periods of time. In addition, it has been suggested that chromosomal rearrangements may have a disturbing influence on the functioning of co-adapted genome complexes, leading to subtle, non-lethal disturbance of developmental processes (Graham, 1992). According to that scenario, directional selection would optimize gene groups' function and filter out unfit chromosome combinations over time. It is tempting to suggest that the older *F. whytei* lineage may represent a more stable chromosomal state, and that in the process a number of unfit diploid combinations have disappeared. In contrast, the more recent *F. micklemi* radiation possibly represents a younger stage with a high number of chromosomal rearrangements, of which many will be selected against over a longer time span. Similar patterns have been observed in other taxa (cf. Nevo et al., 2001 for *Spalax*; Veyrunes et al., 2005 for *Nannomys*; Castiglia et al., 2006 for *Arvicanthis*).

Until karyotypical evolutionary trends are better understood, it is premature to decide whether demographic, historical or ecological models (see e.g. Patton, 1990; Nevo et al., 1995; Nevo, 1999) are more suitable to explain the observed differential evolutionary trends in different clades. While we have discussed the (historical) geographic setting

in which cladogenesis of *Fukomys* took place, we lack evidence on demographic processes that have shaped the evolutionary trajectories of these populations. Nor do we have reliable evidence on the possible role of hybridisation in raciation and speciation of mole-rats, as has been suggested before (Harrison, 1993). This may be important in *Fukomys* as evidenced by the odd diploid ($2n = 45$) race originating from the vicinity of Lochinvar, Zambia (Van Daele et al., 2004). However, in order to understand the evolutionary mechanisms that shape the chromosomal evolution within each phylogroup, we will have to develop and use differential staining techniques (cf. Deuve et al., 2006) to map the observed chromosomal rearrangements, and fathom their functional, genomic consequences. In addition, it is clear that more sequences, including other genes, are required to obtain more detailed phylogeographical patterns within each of the major clades.

Acknowledgments

For assistance in collecting specimens in the field the authors are extremely grateful to E. Sikaboa, G. Sikaulu, S. Stoops and F. Van Daele. We are especially indebted to H. Burda, P. Dammann, R. Honeycutt and C. Ingram for sending specimens. Special thanks to V. Katanekwa and ZAWA for field support and providing permits. We like to thank Tine Dillen, G. Borgonie, A. Huysseune and A. Vierstraete for help with the molecular logistics.

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