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Original Investigation

Increased geographic sampling reveals considerable new genetic diversity in the morphologically conservative African Pygmy Mice (Genus *Mus*; Subgenus *Nannomys*)

Jennifer Lamb[a,*], Sarah Downs[a], Seth Eiseb[d], Peter John Taylor[a,b,c]

[a] School of Life Sciences, New Biology Building, University of KwaZulu-Natal, University Road, Westville, KwaZulu-Natal 3630, South Africa
[b] Department of Ecology and Resource Management, School of Environmental Sciences, University of Venda, Post Bag X5050, Thohoyandou 0950, South Africa
[c] Core Team Member, Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Post Bag X1, Matieland 7602, South Africa
[d] University of Namibia, Windhoek, Namibia

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ABSTRACT

African endemic pygmy mice (Genus *Mus*; sub-genus *Nannomys*) have considerable economic and public health significance, and some species exhibit novel sex determination systems, making accurate knowledge of their phylogenetics and distribution limits important. This phylogenetic study was based on the mitochondrial control region and cytochrome *b* gene, for which a substantial body of published data was available. Study specimens were sourced from eight previously unsampled or poorly sampled countries, and include samples morphologically identified as *Mus bufo*, *M. indutus*, *M. callewaerti*, *M. triton* and *M. neavei*. These analyses increase the known genetic diversity of *Nannomys* from 65 to 102 haplotypes; at least 5 unassigned haplotypes are distinguished by potentially species-level cytochrome *b* genetic distances. The monophyly of *Nannomys* is supported. *Mus musculoides*, *M. callewaerti*, *M. indutus*, *M. bufo*, *M. haussa*, *M. mattheyi*, *M. baoulei* and *M. sorella* are supported as discrete species. The range of *M. indutus* is extended to include Botswana. *M. setulosus* and *M. minutoides* appear to be species complexes. A south and east African *M. minutoides* clade was defined and includes 8 new haplotypes out of 15. *M. setulosus* sensu lato includes *M. setulosus* sensu stricto and a strongly-supported *M. bufo* clade. Two samples, morphologically identified as *M. triton* and *M. neavei*, fall within the *M. minutoides* clade.

Introduction

Mice have an enormous impact on the health and well-being of humans. In addition to their role as agronomic pests, African pygmy mice (subgenus *Nannomys*; genus *Mus*; Rodentia; Muridae) are known to be host reservoirs for Arenaviruses (Lecompte et al., 2007). Two *Nannomys* species, *Mus minutoides* and *M. triton*, have been shown to exhibit novel systems of sex determination (Jotterand-Bellomo, 1988; Veyrunes et al., 2010a). Britton-Davidian et al. (2012) point to the need for more comprehensive sampling throughout Africa to predict the limits and distribution of populations of interest by molecular typing, including cytochrome *b*. They predict that this may result in African pygmy mice becoming one of the most important biological models for the study of the evolution of sex chromosomes and sex determination in mammals.

This study focuses on the molecular systematics and evolution of *Nannomys*. This subgenus of *Mus* is thought to have originated 6.7–7.8 Mya and to have colonized Africa *via* Arabia through Miocene-era land bridges, subsequently radiating throughout the continent (Chevret et al., 2005; Lecompte et al., 2008a; Veyrunes et al., 2005). This is consistent with the earliest *Nannomys* remains found in east Africa and dated at 4.5 Mya (Winkler, 2002). The taxonomy of this group, representing 50% of the species richness of *Mus* (Veyrunes et al., 2005), is still a work in progress. Inference of phylogeny in this group is complicated by considerable morphological homoplasy (Michaux and Catzeflis, 2000), relatively high rates of molecular evolution and a fossil record which is not well-enough sampled to give accurate divergence times (Jansa et al., 2006; Honeycutt et al., 2007). The combination of morphological homoplasy and karyotypic diversity (Castiglia et al., 2002; Veyrunes et al., 2004) suggests that this sub-genus might harbor considerable cryptic genetic diversity.

We aimed to resolve phylogenetic relationships and species identities in a sample of *Nannomys* obtained opportunistically from eight African countries; Botswana, Namibia, Angola, Mozambique, South Africa, Swaziland, Tanzania and the DRC. This sample complements those used in previous molecular phylogenetic studies of *Nannomys*, most of which have been based on mitochondrial

* Corresponding author. Tel.: +27 31 260 3038; fax: +27 31 2602029.
E-mail addresses: lambj@ukzn.ac.za, alport.jennifer@gmail.com (J. Lamb).

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cytochrome *b* sequences (Kan Kouassi et al., 2008; Veyrunes et al., 2005, 2010b; Mboumba et al., 2011; Britton-Davidian et al., 2012), as it covers previously unsampled or poorly sampled areas. It also includes samples morphologically identified as *Mus bufo*, *M. callewaerti*, *M. triton* and *M. neavei*, which have not been clearly identified in molecular phylogenies. We provide a phylogenetic update on the most recent review of *Nannomys* (Britton-Davidian et al., 2012) by including all currently available sequences in our analysis. We note that while phylogenies based on mitochondrial data can be suggestive of species level differences, such hypotheses require verification from nuclear sequences and other data types (Britton-Davidian et al., 2012; Zachos et al., 2013).

Britton-Davidian et al. (2012) recognize 18 species of *Nannomys* and Happold and Veyrunes, 2013 recognize 20 species. Kan Kouassi et al. (2008) detected five *Nannomys* species in Guinea and four in Cote d'Ivoire, inclusive of *M. mattheyi*, *M. minutoides*, *M. setulosus*, *M. baoulei* and *M. musculoides*, and postulated that the *M. setulosus* clade may be a species complex. In addition, the *Nannomys* phylogenies presented by Mboumba et al. (2011) and Britton-Davidian et al. (2012) contain *M. indutus, M. haussa, M. sorella*, and an implied additional unknown species from Chad (associated with Genbank sequence AJ875085). Based on a combined cytochrome *b* and nuclear IRBP dataset, Veyrunes et al. (2005) recover a monophyletic *Nannomys* clade; they find support for *M. setulosus*, *M. mattheyi* sister to *M. haussa*, *M. indutus* basal to sister species *M. minutoides* and *M. musculoides*, and the abovementioned previously-unknown species from Chad.

M. minutoides, one of the smallest rodents, is found throughout southern Africa (de Graaff, 1997; Skinner and Chimimba, 2005; Musser and Carleton, 2005; Monadjem, 2008) extending to northwestern and northeastern Africa (Veyrunes et al., 2010a,b). It appears to be a well-differentiated monophyletic species that separated from other *Nannomys* species 1.17 Mya (Mboumba et al., 2011). This species is subdivided into three clades, from south and east Africa, west-central Africa and west Africa respectively (Britton-Davidian et al., 2012). The type locality of *M. minutoides* is Cape Town (Musser and Carleton, 2005), close to where the karyotype is $2n = 18$ (de Graaff, 1997), representative of *M. minutoides sensu stricto*. A karyotype of $2n = 34$ (Rb X.1) (de Graaff, 1997) has been reported for this species in KwaZulu-Natal (Veyrunes et al., 2010a,b).

Mus indutus (Thomas, 1910), the Desert pygmy mouse, is typically found in the drier northwestern parts of South Africa, Botswana, Namibia and western Zimbabwe (de Graaff, 1997; Skinner and Smithers, 1990; Chimimba and Bennett, 2005). This species may also be found in southern Angola (Crawford-Cabral, 1998 in Musser and Carleton, 2005), however this range could be limited to the morphologically similar *M. sybilla* (Thomas, 1918), which was previously included as a sub-species of *M. indutus* by Meester et al. (1986).

The Gray-bellied Pygmy Mouse, *Mus triton* (Thomas, 1909), is larger than most other *Nannomys* (except for *M. callewaerti*) and is typically found in highlands, grasslands, swamps and cultivated areas in central and eastern Africa (de Graaff, 1997; Chimimba and Bennett, 2005; Dieterlen and Agwanda, 2008). The taxonomy of *M. triton* is problematic (Chimimba and Bennett, 2005) as this species is morphologically similar to wild populations of *Mus musculus* and shares dental features with Indian *Mus cervicolor* (Chimimba and Bennett, 2005). *M. triton*, which displays novel sex determination systems (Jotterand-Bellomo, 1988), has not thus-far been included in published molecular phylogenies, but is distinguished by a chromosomal translocation, Rb (X.12) (Veyrunes et al., 2004).

The Toad Mouse, *Mus bufo* (Thomas, 1906), is endemic to the montane western Rift of Africa (Musser and Carleton, 2005). Morphologically similar to *M. triton*, it is distinguished by dentition and tail length (Petter and Matthey, 1975) and is also chromosomally distinct (Robbins and Baker, 1978). *M. bufo* is more closely related to *M. minutoides* than *M. triton* (Chimimba and Bennett, 2005). There is uncertainty regarding the identity of a sample from Burundi, lodged on Genbank as *M. bufo* (DQ789905), but regarded by Britton-Davidian et al. (2012) as potentially incorrectly assigned, as it nests within *M. setulosus*.

Although de Graaff (1997) regarded Neaves mouse, *M. neavei* (Thomas, 1910), as a sub-species of *M. sorella* (Thomas, 1909), it is also recognized as a valid species (Petter and Matthey, 1975; Meester et al., 1986; Musser and Carleton 1993; Bronner et al., 2003; Chimimba and Bennett, 2005). This species is thought to occur in southeast Zambia, the type locality, eastern DRC, southern Zimbabwe, northeast South Africa, southwestern Mozambique and southern Tanzania (Chimimba and Bennett, 2005).

Callewaert's pygmy mouse, *Mus callewaerti* (Thomas 1925), is a poorly known but distinctive species occurring in central and northern Angola and southern DRC. It is the largest member of the genus, having a head and body length of 84–97 mm (Crawford-Cabral, 1998; Happold, 2013).

Aims

The aim of this study, based on mitochondrial cytochrome *b* and control region sequence data, was to resolve phylogenetic relationships and species identities in a *Nannomys* sample obtained opportunistically from eight poorly sampled or unsampled African countries, and including morphologically-identified *Mus bufo*, *M. callewaerti*, *M. triton* and *M. neavei*, previously equivocal or absent in molecular phylogenies of *Nannomys*. We include all currently available sequences from DNA sequence databases and provide an updated cytochrome *b* based phylogeny for *Nannomys*, as well as estimates of the age of the major *Nannomys* clades.

We hypothesize that the following are discrete monophyletic species: *Mus minutoides*, *M. musculoides, M. indutus, M. haussa, M. mattheyi, M. bufo, M. callewaerti, M. baoulei, M. setulosus* and *M. sorella*.

We aimed to test the following hypotheses: (1) the monophyly of *Nannomys* is retained in an enlarged sample set derived from previously unsampled localities in Africa; (2) improved geographic sampling of *Nannomys*, which spans the African continent, will reveal new mitochondrial lineages which may be confirmed as cryptic species pending additional studies.

Material and methods

Sampling

This study was based on 36 *Nannomys* specimens obtained opportunistically from 14 localities within South Africa, Tanzania, Namibia, Botswana, Angola, Mozambique, Swaziland, and the DRC (Table 1). Reference samples downloaded from the NCBI GenBank website were also included in the dataset (Table 2). Specimens were obtained from PJT (South Africa), P. Kaleme (DRC), SE (Angola), A. Monadjem (Mozambique), M. McDonough (Botswana) and from the EcoRatProject (www.nri.org/ecorat/) (Namibia, Swaziland and Tanzania) under relevant collecting permits. Specimens were provisionally identified in the field and then confirmed to genus and species level where possible at the Durban Natural Science Museum (DNSM) and the National Museums of Namibia (NMN) by analysis of external characters or skull morphology. Skins and skulls of specimens, or whole bodies in 70% Ethanol (EtOH), are housed at the DNSM, BNM, NSRL, FMNH or the NMN for further reference (Table 1). Tissue samples of liver, kidney, muscle or skin were dissected and stored in 90% EtOH.

Table 1
Specimen details including external measurements, pelage characters and habitat information for voucher specimens. BNM = Botswana National Museum (un-numbered = pending accession); DM = Durban Natural Science Museum, Durban, South Africa; NMN = National Museum of Namibia; NMM = National Museum, Mammals, Namibia; NSRL = Natural Sciences Research Laboratory, Texas Tech University; FMNH = Field Museum of Natural History, Chicago; HB = head and body length (mm); TL = tail length (mm); E = ear length (mm); M = mass (g); Hap = haplotype; CR = control region; – = information not available.

Mus species	Museum number	Field code	Locality	HB	TL	Ear	M	Sex	Ventral color	Dorsal color	White marks	Habitat	Cyt*b* Hap	Cyt b Genbank	CR Hap	CR Genbank
minutoides	DM9367	NA173	Andara Hospital, Kavango, Namibia	49	39	7	6	M	White	Buffy	Dorsal tail & base of ear	Peri-urban	H20	KF484826	H2	KF484853
minutoides	DM9366	NA160	Diyogha village, Kavango, Namibia	53	42	11	5	M	White	Buffy	Dorsal tail & base of ear	Peri-urban			H1	KF484852
minutoides	DM9369	NA185	Diyogha village, Kavango, Namibia	60	47	11	7	F	White	Buffy	Dorsal tail & base of ear	Peri-urban	H20	KF484827	H3	KF484854
minutoides	NMN	NA216	Kake Village, Namibia	56	46	9	6	F	White	Light brown	Base of ear	Millet field	H22	KF484835		
minutoides	NMN	NA318	Kake Village, Namibia	61	59	11	6	F	White	Light brown	Base of ear	Inside house	H20	KF484828		
minutoides	NMN	NA183	Diyogha villiage, Kavango, Namibia	51	31	10	6	M	White	Light brown	Base of ear	Peri-urban	H21	KF484834	H16	KF484877
minutoides	NMM15370	ANG022	Longa village, Cuando Cubango, Angola	58	50	11	6	M	White	Buffy	None	In village	H96	KF484846	H4	KF484855
minutoides	NSRL	AWF226	Koanaka Hills, Northwestern Botswana	55	47	12	5.5	M	–	–	–	Savanna	H20	KF184311		
minutoides	NSRL	MMM294	Koanaka Hills, Northwestern Botswana	64	43	12	5.5	F	–	–	–	Savanna	H20	KF184308		
minutoides	NSRL	MMM210	Koanaka Hills, Northwestern Botswana	59	52	11	5.5	F	–	–	–	Savanna	H21	KF184312		
minutoides	NSRL	MMM293	Koanaka Hills, Northwestern Botswana	55	44	9	4	F	–	–	–	Savanna	H21	KF184313		
minutoides	BNM	AWF613	Koanaka Hills, Northwestern Botswana	49	47	10	3.7	F	–	–	–	Savanna	H21	KF184314		
minutoides	DM9888	SW15	Mahlanya, Swaziland	36	52	9	3.4	M	White	Brown	–	Peri-urban	H102	KF484850	H5	KF484856
minutoides	DM10288	GD3	uMkhuze, KwaZulu-Natal, South Africa	51	40	10	5	M	–	–	–	Savanna	H19	KF484825	H6	KF484857
cf. triton (skull=22.0 mm)	DM9126	N5	Mt Namuli, Muretha Plateau, Mozambique	67	54	13	–	M	Dark brownish-gray	Light gray	None	Montane grassland	H98	KF484847	H6	KF484858
minutoides	DM9128	N16	Mt Namuli, Malema Valley, Mozambique	54	48	9	–	F	White	Brown	None	Montane grassland			H7	KF484862
minutoides	DM9164	AR40	uMkhuze, KwaZulu-Natal, South Africa	55	35	9	6	F	White	Brown	None	Sandveld	H8	KF484824	H6	KF484859
cf. neavei (skull = 18.2 mm)	DM9174	AR169	uMkhuze, KwaZulu-Natal, South Africa	64	38	9	12	F	White	Rich buffy	–	Sandveld	H8	KF484824	H7	KF484863
	DM11467	–	uMkhuze, KwaZulu-Natal, South Africa	60	43	8	5	F	White	Brown	Base of ear	Riverine thicket			H6	KF484860
indutus	BNM	MMM208	Koanaka Hills, Northwestern Botswana	53	42	13	4.5	F	–	–	–	Savanna	H44	KF184315		
indutus	BNM	AWF614	Koanaka Hills, Northwestern Botswana	58	43	11	5.1	F	–	–	–	Savanna	H45	KF184317		

bufo	FMNH188837	DRC1	KahuziBiega, Albertine Rift, eastern DRC	–	–	–	–	–	–	–	–	–	H71	KF484840	H8	KF484864
	FMNH188838	DRC2	KahuziBiega, Albertine Rift, eastern DRC	–	–	–	–	–	–	–	–	–	H72	KF484841	H9	KF484867
	FMNH188835	DRC3	KahuziBiega, Albertine Rift, eastern DRC	–	–	–	–	–	–	–	–	–	H73	KF484842	H10	KF484868
	FMNH188836	DRC4	KahuziBiega, Albertine Rift, eastern DRC	–	–	–	–	–	–	–	–	–			H11	KF484869
	FMNH189036	PK931	KahuziBiega, Albertine Rift, eastern DRC	–	–	–	–	–	–	–	–	–	H99	KF484848	H8	KF484866
	FMNH189035	PK966	KahuziBiega, Albertine Rift, eastern DRC	–	–	–	–	–	–	–	–	–	H100	KF484849	H8	KF484865
Mus sp?	DM10455	TE6146	Berega, Tanzania	60	45	10	8	F	White	Brown	Base of ear	Inside house	H42	KF484836	H12	KF484870
Mus sp?	DM10554	TE6273	Berega, Tanzania	43	–	10	9	F	White	Brown	Base of ear	Around houses	H42	KF484837	H13	KF484871
Mus sp?	DM10609	TA450	Berega, Tanzania	58	49	10	6	M	White	Brown	None	Fallow land			H14	KF484872
triton	FMNH	TLC2542	KahuziBiega, Albertine Rift, eastern DRC	–	–	–	–	–	–	–	–	–	H105	KF484851		
cf. indutus	DM9265	AR84	uMkhuze, KwaZulu-Natal, South Africa	60	30	11	8.5	M	White	Pink buffy	Base of ear (left)	Sandveld			H6	KF484861
callewaerti	NMM15371	ANG012	Mbala Nondolo village, Huambo, Angola	80	57	11	15	M	Light gray	Dark brown, mottled	None	Banks of river in village			H15	KF484873
callewaerti	NMM15374	ANG014	Mbala Nondolo village, Huambo, Angola	85	55	11	14	F	Light gray	Dark brown, mottled	None	Banks of river in village	H94	KF484843	H15	KF484875
callewaerti	NMM15373	ANG018	Mbala Nondolo village, Huambo, Angola	87	54	13	18	M	Light gray	Dark brown, mottled	None	Banks of river in village	H95	KF484845	H15	KF484874
callewaerti	NMM15375	ANG019	Mbala Nondolo village, Huambo, Angola	80	54	12	18	M	Light gray	Dark brown, mottled	None	Banks of river in village	H94	KF484844	H15	KF484876

Table 2
Accession codes and haplotype numbers for cytochrome *b* sequences downloaded from NCBI GenBank.

Haplotype	Genbank accession number
1	AJ875076
2	AJ875077
3	AJ875078 FN985223
4	AJ875079 FN985221 FN985224
5	AJ875080
6	AJ875081
7	AJ875084 AY057816
9	EU603925
10	EU603926
11	EU603927 EU603928
12	EU603929 EU603930 EU603931 EU603933 EU603934 EU603935 EU604003
13	EU603932 EU604004
14	EU603938 EU603939 EU603940 EU603941 EU603942 EU603943 EU603944 EU603945
15	EU603946 EU603947
16	EU603950 EU603951 EU603952 EU603953 EU603954 EU603955
17	EU603961
18	FN985222
24	AJ875075
25	DQ789901
26	DQ789902
27	HM635856
28	Z96069
29	DQ789906 DQ789907 DQ789909 DQ789910 DQ789911 DQ79912 DQ789913 DQ789914 DQ789916 DQ789917 DQ789918 DQ789919 DQ789920 DQ789922 DQ789923 DQ789924 DQ789925 DQ789926 DQ789927 DQ789928 DQ789929 DQ789930 DQ789931 DQ789932 DQ789933 DQ789934 DQ789935 DQ789936 DQ789937 DQ789940 DQ789941 DQ789943 DQ789945 DQ789946 DQ789947 DQ789950 DQ789951 DQ789952 DQ789955 DQ789956 DQ789957 DQ789958 DQ789959 DQ789960 DQ789961 DQ789962 DQ789963 DQ789964 DQ789965 DQ789966 DQ789967 DQ789968 DQ789969 DQ789970
30	DQ789908
31	DQ789915 DQ789944
32	DQ789921 DQ789948 DQ789949 DQ789953
33	DQ789938
34	DQ789939
35	DQ789942
36	DQ789954
37	DQ789971
38	EU603967
39	EU603968
40	EU603969
41	HM635855
43	AJ698875
46	AJ698876
47	AJ875066
48	AJ875067
49	AJ875068
50	AJ875069
51	AB12578
52	EU6039
53	EU603971
54	EU603972
55	EU60397
56	AJ698877
57	AJ875071
58	AJ875072
59	AJ875073
60	AJ875074
61	EU603991
62	EU603992
63	EU603993
64	EU603994
65	EU603995
66	EU603998
67	AJ698873
68	AJ875082
69	AJ875082
70	DQ789900 EU603989
74	EU603974
75	EU603975
76	EU603976
77	EU603977
78	EU603978
79	EU603979
80	EU603980 GU830867
81	EU603981
82	EU603982
83	EU603983
84	EU603984
85	EU603986
86	EU603987 EU603988
87	EU603990
88	EU603997
89	GU830865 GU830865
90	GU830869
91	DQ789903
92	DQ789905
93	AJ875085
115	DQ789904

DNA isolation, PCR and sequencing

DNA isolation, PCR amplification, sequencing and alignment of mitochondrial cytochrome *b* gene and control region sequences were carried out as in Lamb et al. (2011). BLAST searches of the NCBI Genbank sequence database were carried out in order to obtain closest matches for reference or as outgroups. This facilitated the species-level identification of samples. Samples used as outgroups included the following: *Mus musculus molossinus* NC 006915.1, *Mus musculus musculus* AB205275.1, *Mus caroli* AF520637.1, *Mus musculus castaneus* AB205280.1, *Mus musculus* EF605390, *Mus musculus* EF605403.1, *Mus musculus bactrianus* HQ148567.1, *Mus spicilegus* AY057809.1, *Mus macedonicus* AY057808.1, *Mus spretus* AY057810.1, *Mus cookii* AY057813.1, *Mus famulus* AJ698872.1, *Mus terricolour* AB125777.1, *Mus fragilicauda* AB125780.1, *Mus crociduroides* AJ698878.1, *Rattus rattus* AB211039, *Apodemus sylvaticus* AJ511937, *Otomys sloggetti* AF141223, *Mastomys natalensis* HM130519 and *Praomys rostratus* GU397446.

Haplotype and diversity analyses were carried out in DnaSP 4.10.9 (Rozas et al., 2003). The GTR+I+G and HKY+G substitution models were selected in jModeltest version 3.7 (Posada, 2008) for the cytochrome *b* and control region datasets respectively. Genetic distance, maximum parsimony (MP), neighbor-joining (NJ) and maximum likelihood (ML) analyses were carried out in PAUP 4.0b10 (Swofford, 2000), as described in Lamb et al. (2011). Nodal support was estimated by bootstrap resampling analysis (1000 replicates, MP and NJ; 200 replicates, ML). SplitsTree version 4.8 (Huson, 1998), was used to generate a Neighbor-net network based on haplotypes. Program defaults were applied.

Dating

Divergence dating was carried out using Bayesian inference in Beast 1.6.1 (Drummond and Rambaut, 2007). Analyses were based on clades identified in the phylogenetic analysis (Figs. 1a and b). Estimations of divergence times were based on four calibration points derived from paleontological data (Mboumba et al., 2011). These were (i) the *Mus*/*Rattus* divergence 11–12.3 Myr ago (Benton and Donoghue 2007), (ii) the appearance of *Otomys* 6 Myr ago (Jansa et al. 2006), (iii) the divergence 7 Myr ago between the big *Apodemus mystacinus* and the small species of the subgenus *Apodemus* (*Sylvaemus*) (*A. flavicollis*, *A. sylvaticus*) (Michaux et al. 2005) and (iv) the divergence 4 Myr ago between *A. sylvaticus* and

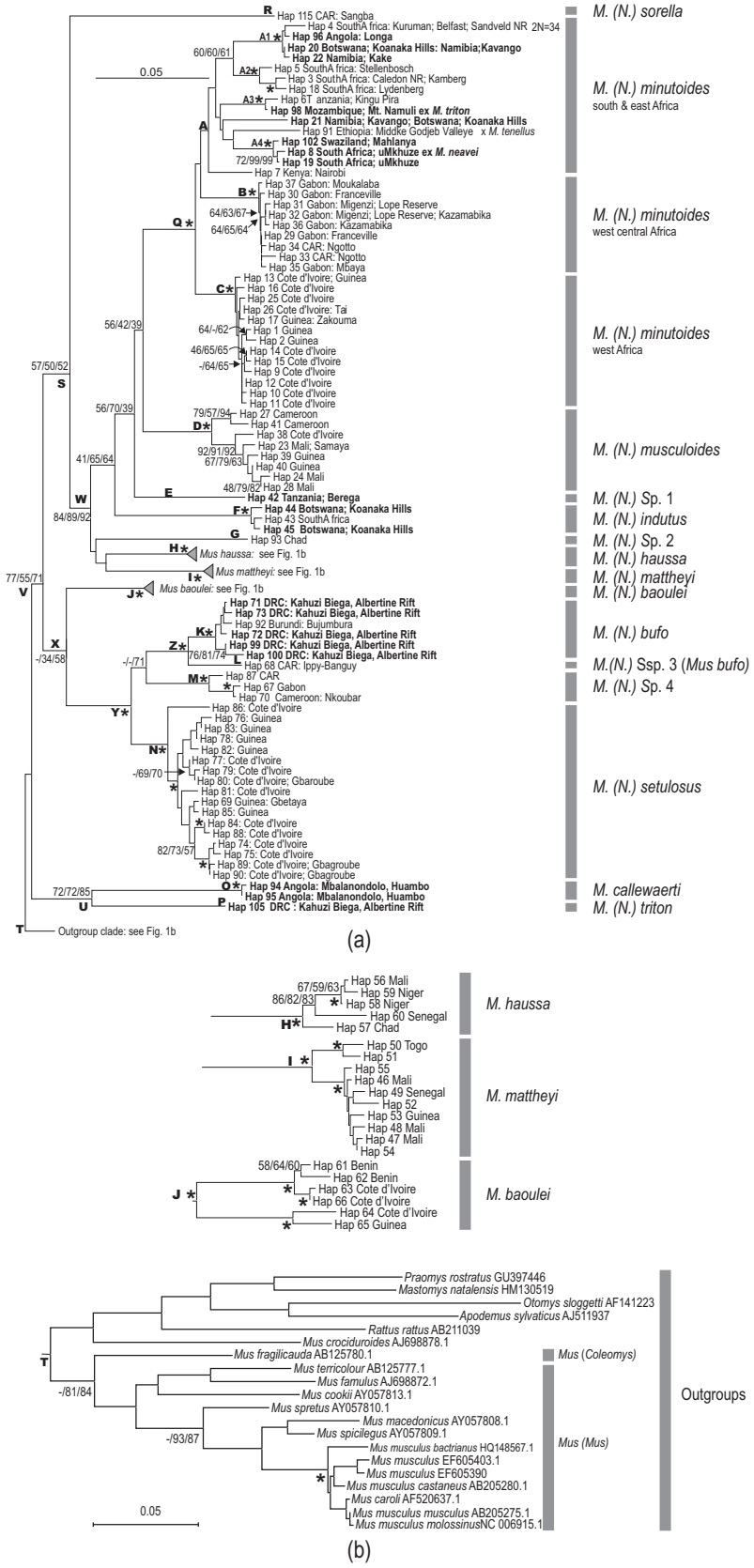

Fig. 1. (a) Phylogram of relationships among *Nannomys* haplotypes based on analysis of 623 nucleotides of the mitochondrial cytochrome *b* gene using the neighbour-joining, maximum parsimony and Bayesian methods. Bold text denotes haplotypes derived from experimental samples. Node support levels are indicated as [maximum parsimony%/maximum likelihood%/neighbour joining%]; where bootstrap support from all three methods was greater than 95%, the node is labeled with the symbol *; where support is less than 50% for all methods, support levels are not indicated. Sp. = species; Ssp. = subspecies. (b) Phylograms of relationships among *Nannomys* haplotypes belonging to Clades H (*Mus haussa*), I (*M. mattheyi*), J (*M. baouli*) and T (outgroups) from (a), based on analysis of 623 nucleotides of the mitochondrial cytochrome *b* gene. These are presented separately due to space constraints in (a). Node support levels are indicated as in (a).

A. flavicollis (Michaux et al. 2005). When applied to the *Nannomys* dataset, this results in a substitution rate of 2.83% per million years.

Bayesian analyses were carried out using the GTR substitution model using a Yule process as the tree prior. Divergence times and their credibility intervals were estimated using a relaxed clock model with branch rates drawn from an uncorrelated lognormal distribution to account for lineage-specific rate heterogeneity. Two independent runs of 10 million generations each with burn-ins of 1 million were performed. TreeAnnotator v1.6.1 (Drummond and Rambaut, 2007) was used to select the maximum clade credibility tree, which was viewed in FigTree v1.3.1 (Rambaut, 2009).

Results and discussion

This study provides further insights into the systematics of the African pygmy mice, *Nannomys*, by contributing data from 36 samples sourced from countries or areas not previously represented in *Nannomys* phylogenies. We also provide an updated cytochrome *b* based phylogeny and information on the timing of the major divergences within *Nannomys*. We recover a monophyletic *Nannomys* clade (Fig. 1a). We also find support for *Mus bufo* and *Mus callewaerti*, which have not previously been conclusively identified based on DNA sequence data, and identify a number of previously unreported lineages within *Nannomys*.

In defining species, we considered field identifications based on morphology (Table 1), karyotypes and molecular phylogenies. A species is typically separated from other taxonomic units by a genetic distance indicative of genetic isolation (Baker and Bradley, 2006). Based on a study of 27 different rodent taxa, these authors report a mean intraspecific genetic distance of 1.50% (range 0.0–4.7%); a mean distance between sister species of 7.3% (1.3–13.0%) and a mean inter-generic distance of 10.9% (4.9–16.9%). While these distances overlap considerably and cannot be used prescriptively, they provide a guide to the level of distinctiveness of a clade. Although members of a species often form a monophyletic clade, phenomena such as incomplete lineage sorting and introgression of mitochondrial DNA due to past hybridization events can create the appearance of poly-or paraphyly (Zachos et al., 2013). Thus while the presence of a clade distinguished by species level genetic distances may lead us to hypothesize the existence of a new species, this would need confirmation from studies based on multi-locus nuclear DNA and/or other forms of evidence.

Sequence analysis

Two cytochrome *b* alignments were created, trimmed to 623 bp and 1013 bp respectively. The longer dataset contained 62 sequences and yielded 47 haplotypes, whereas the shorter dataset, which included more Genbank sequences, contained 219 sequences and 101 haplotypes. As phylogenetic analyses yielded congruent trees, we present results from the shorter dataset only, which contained 195 parsimony-informative sites and 17 singletons. The haplotype diversity (Hd) was 0.941 ± 0.0005. The D-loop dataset yielded 16 in-group haplotypes from 29 experimental sequences. The trimmed length of 294 bp contained 129 variable sites, of which 1 1 1 were parsimony-informative. The haplotype diversity (Hd) was 0.962 ± 0.008.

For both datasets, the topologies of the maximum parsimony, maximum likelihood and neighbor joining trees were congruent and are presented as one tree (cytochrome *b*, Figs. 1a and b; control region, Fig. 2). Species relationships based on analysis of the cytochrome *b* gene are also presented as a Neighbor-network (Fig. 3). Discussion on *M. sorella*, *M. musculoides*, *M. haussa*, *M. mattheyi* and *M. baoulei* is limited, as our dataset did not include new samples of these species.

Monophyly of Nannomys

All *Nannomys* form a moderately to weakly supported monophyletic clade (V) (bootstrap support = 77% maximum parsimony/55% maximum likelihood/71% neighbor joining %). This includes *Mus sorella*, *M. minutoides*, *M. musculoides*, *M. indutus*, *M. haussa*, *M. mattheyi*, *M. baoulei, M. bufo, M. setulosus, M. callewaerti, M. triton* and four previously undescribed clades/haplotypes genetically distinct by greater than intraspecific distances described for rodents by Baker and Bradley (2006) (Table 3; Fig. 1a). These are labeled in the text as *M. (N.)* sp. 1?, 2?, 3?and 4? to indicate that they may represent new species (or possibly subspecies), if confirmed as such by other data sources. Clade P (H105) from the DRC, unambiguously morphologically identified by Prince Kaleme as *Mus triton* (Table 1), has a moderately supported sister relationship with clade O (*M. callewaerti* based on large body size of voucher specimens: Table 1, Fig. 4).

Deeper level relationships among Nannomys species

Our dataset provides support for some deeper level relationships among *Nannomys* species. There is good support for a clade (W) comprising *Mus haussa*, *M. indutus*, *M. mattheyi*, *M. musculoides, M. minutoides*, and lineages E (H42) and G (H93), which last shared a common ancestor 4.96 Mya (Fig. 5). Members of clade W diverged from sister group *Mus sorella* 6.13 Mya. *M. sorella* is separated from all other groups by a minimum genetic distance of 13.5% (Table 3), consistent with its status as a distinct species. There is essentially no support for the clade comprising sister species *M. musculoides* (D) and *M. minutoides* (Q), which last shared a common ancestor 3.22 Mya (Fig. 5). This contrasts with the strong support reported by Britton-Davidian et al. (2012), and may be due to the inclusion of additional taxa in our dataset. Thus our data do not support a *M. minutoides*/*M. musculoides* species complex as proposed by Aniskine et al. (1998), Veyrunes et al. (2005) and Musser and Carleton (1993), especially as each of these species clades, separated by a genetic distance of 9.45%, is strongly supported. Consistent with Veyrunes et al. (2005) and Britton-Davidian et al. (2012), we recover support for *M. indutus* basal to *M. minutoides*, *M. musculoides* and Tanzanian Haplotype 42 (Fig. 1a), from which it diverged 4.13 Mya (Fig. 5).

Support for distinct species

Our analyses provide strong support for the following species as currently described: *Mus minutoides* (Crown age 2.22 Mya), *M. musculoides* (1.18 Mya), *M. callewaerti* (0.11 Mya), *M. indutus* (0.35 Mya), *M. haussa* (1.12 Mya), *M. mattheyi* (1.34 Mya), *M. baoulei* (3.11 Mya) *and M. setulosus* (1.09 Mya). *M.* sp. 1? appears to have diverged 2.78 Mya (Fig. 5).

The *M. minutoides* clade (Q) appears to have radiated 2.22 Mya, considerably earlier than the 1.17 Mya reported by Mboumba et al. (2011). Similarly, our estimate of the age of *M. minutoides* clade A, from southern and eastern Africa, is older (1.78–2.22 Mya) than theirs (0.79 Mya), most likely due to the presence of additional taxa and therefore greater diversity in our dataset. *M. haussa* and *M. mattheyi* last shared a common ancestor 4.96 Mya. *M. setulosus* diverged from *M. bufo* 2.53 Mya, and these taxa diverged from *Mus* sp. 4? 3.16 Mya.

Mus setulosus

M. setulosus sensu lato (Clade Y) is sister to *M. baoulei*, and comprises four strongly supported subclades. Clade N, *M. setulosus sensu stricto*, comprises 17 haplotypes from Guinea and Cote d'Ivoire. Within N, H86 from Cote d'Ivoire is separated from a strongly

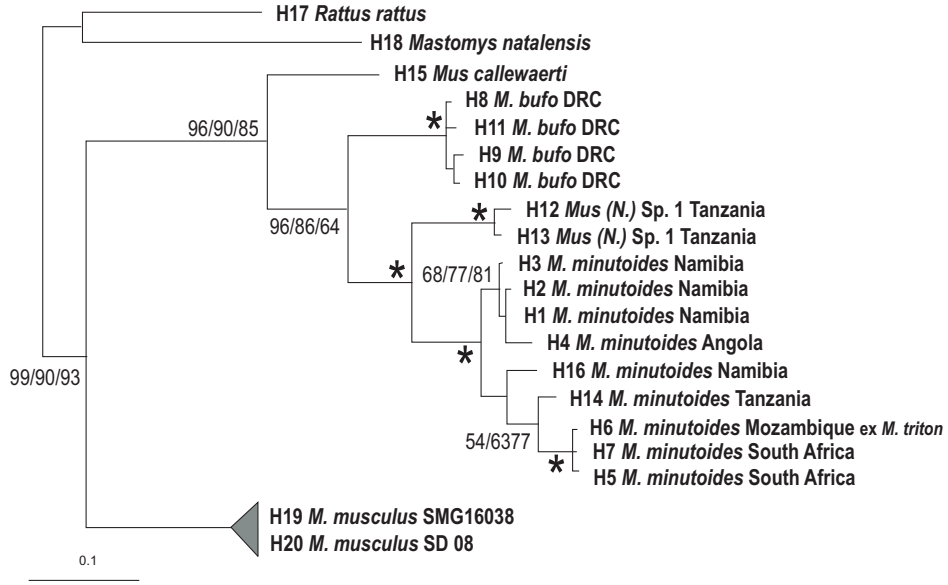

Fig. 2. Phylogram of relationships among *Nannomys* haplotypes based on analysis of 294 nucleotides of the mitochondrial control region. Bold text denotes haplotypes derived from experimental samples. Node support levels are indicated as for Fig. 1a. Sp. = species.

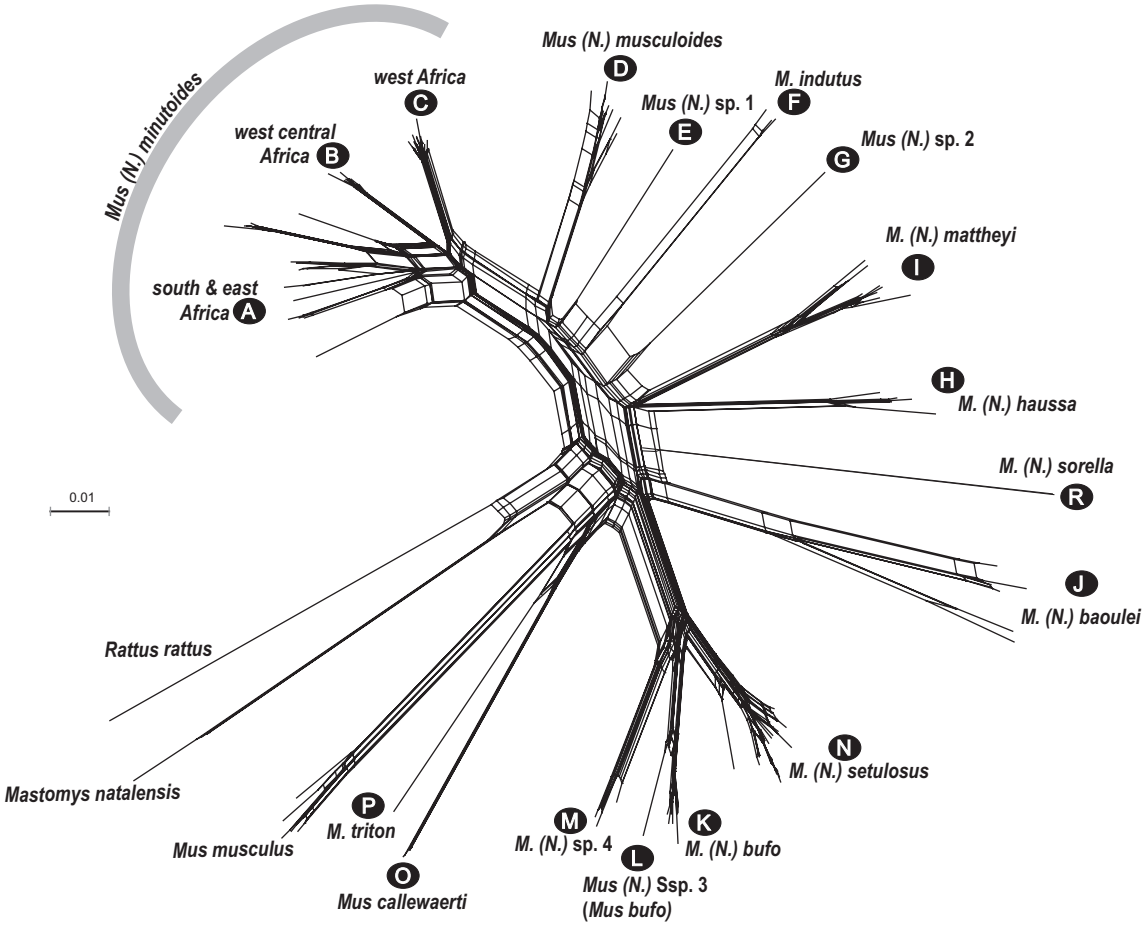

Fig. 3. Neighbor net illustrating evolutionary relationships among *Nannomys* lineages. Sp. = species; Ssp. = subspecies.

Table 3
GTR genetic distances (%) between clades listed in Fig. 1. M. = *Mus* (subgenus *Nannomys*).

	Clade	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	R
M. minutoides S & E Africa	A																	
M. minutoides W C Africa	B	7.0																
*M. minutoides*W Africa	C	7.1	4.6															
M. musculoides	D	10.5	9.0	8.1														
M. sp. 1?	E	10.1	9.2	8.8	8.7													
M. indutus	F	12.7	11.0	10.7	12.0	10.4												
M. sp. 2?	G	14.0	12.0	12.0	10.8	13.7	11.9											
M. haussa	H	12.9	12.0	11.0	11.9	11.3	11.8	11.4										
M. mattheyi	I	13.3	12.2	12.8	12.2	11.0	13.0	11.3	9.8									
M. bayoulei	J	16.3	16.0	15.4	15.7	14.1	15.8	15.3	12.7	13.5								
M. bufo	K	16.7	15.3	15.9	14.9	14.1	15.1	14.2	13.4	12.8	13.3							
M. ssp. 3?	L	17.1	15.8	16.0	14.9	14.0	14.4	14.3	14.7	13.4	13.8	3.6						
M. sp. 4?	M	16.3	15.9	15.3	15.9	13.4	15.0	14.6	13.8	13.0	14.2	7.0	6.3					
M setulosus	N	15.5	13.6	14.1	13.4	13.1	15.1	14.0	13.2	12.5	13.5	6.1	7.0	7.0				
*M.*callewaerti	O	17.7	17.2	16.6	16.3	17.5	17.5	16.3	16.4	17.2	15.4	15.4	14.4	14.4	14.5			
M. triton	P	16.3	16.0	15.6	17.1	15.4	16.3	17.0	15.1	15.8	15.3	15.5	15.5	13.7	13.8	10.9		
M. sorella	R	15.2	14.8	14.9	14.2	14.3	14.2	13.6	13.5	14.1	15.8	13.8	14.6	13.9	13.8	16.7	15.0	
Mus musculus (subgenus *Mus*)	Mm	17.2	17.9	18.9	17.8	19.0	18.7	17.0	17.8	16.5	18.3	15.9	16.2	16.9	16.1	15.9	14.7	19.3

supported clade comprising 16 haplotypes by a mean genetic distance of 3.0%, consistent with an intraspecific distance (Baker and Bradley, 2006).

Mus bufo

Haplotype 92 from Burundi was originally identified as *Mus bufo* (Mboumba et al., 2011). Britton-Davidian et al. (2012) reported that this was probably an incorrect species assignment, as this single *M. bufo* haplotype nested within their *M. setulosus* clade. However our four experimental haplotypes from the Albertine Rift, DRC, morphologically identified as *M. bufo* by Prince Kaleme, form a strongly supported monophyletic clade (K) with H92. Thus clade K from the DRC and Burundi, separated from *M. setulosus sensu stricto* by a mean genetic distance of 6.1%, is likely to represent *M. bufo*.

Mus (N.) ssp. 3? and sp. 4?

Within strongly supported clade Z, clade L (H68 from Ippy-Bangui, CAR) was separated from *Mus bufo* (clade K) by a genetic distance of 3.6% (Table 3). This distance, between the mean intraspecific (1.5%) and interspecific (7.3%) distances reported by Baker and Bradley (2006), leads us to speculate that H68 may be a cryptic species, *Mus (N.)* sp. 3?, although it is perhaps more likely to be a sub-species of *M. bufo*, and is referred to as *Mus (N.)* Ssp. 3 (*Mus bufo*) in Figs. 1a, 3 and 5. Strongly supported clade M (CAR, Gabon and Cameroon) was distant from *M .bufo* and L by 7.0% and 6.3%, respectively, again possibly representative of a cryptic species, *M. (N.)* sp. 4? These phylogenetic hypotheses need to be tested using evidence from other sources.

Mus minutoides

Kan Kouassi et al. (2008) suggested that *M. minutoides* is a complex of morphologically cryptic species. We recovered three clades within strongly supported *M. minutoides* (clade Q). These include strongly-supported clades from west-central Africa (B) (Mboumba et al., 2011) and west Africa (C), and an unsupported and diverse clade (A) from south and east Africa (Britton-Davidian et al., 2012). Clade A, widely distributed over southern and eastern Africa, is separated from clades B and C by apparently species-level genetic distances of 7.0% and 7.1% respectively, and may represent a species complex in its own right (see below). Clades B and C, from west central and west Africa respectively, are 4.6% divergent, suggestive of subspecies or possibly species status if supported by nuclear markers or other data.

Mus minutoides Clade A comprises four strongly supported subclades (A1–A4), as well as unaffiliated haplotypes 21, 91 and 7. The inclusion of our experimental samples increased the number of haplotypes in Clade A from 7 to 15. Strong support for the specific status of at least some of these subclades and affiliated haplotypes comes from cases of sympatry at Koanaka Hills (Botswana) and Kavanga (Namibia) involving unaffiliated H21, which differs from divergent H20 and H22 (subclade A1) by at least 7%. There is no discernible difference in morphology between vouchers assigned to these divergent haplotypes occurring sympatrically in Namibia and Botswana (Table 1).

Three of our experimental samples, derived from Angola, Botswana and Namibia, fell within subclade A1, which also includes H4 from mid-South Africa, with a karyotype of 2N = 34 (Veyrunes et al. 2010b). Subclade A3 comprised H6 from Selous Game Reserve in Tanzania (with $2n = 35–36$ and X:1 translocation; Veyrunes et al., 2005) and our sample, H98 from the Muretha plateau (elevation 1650 m), Mt Namuli, Mozambique, morphologically identified as *M. triton* (based on larger body and skull size and grayish belly: Table 1). Strongly supported clade A4 is previously unreported, and comprises our experimental samples from Swaziland and uMkhuze in South Africa. Phylogenetic analyses place haplotype 8 from uMkhuze, whose voucher is morphologically identified by PJT as *Mus neavei* based on the presence of proodont incisors, distinctive, bright tawny dorsal color, mandible and incisor length exceeding 12 mm and two-rooted M2 (Skinner and Chimimba, 2005; Table 1), within *M. minutoides.* Both of the above disparities between morphological and molecular species assignments based on mitochondrial markers may be attributed to introgression due to past hybridization between the species concerned. However, in the case of *M. cf triton* from subclade A3, it is more parsimonious to suppose that this clade represents a recently-evolved, distinctive, large, gray-bellied species within the *minutoides* complex. The voucher specimen, collected in montane grassland on Mt Namuli (Mozambique) has a skull length of 22.0 mm, which places it well above the maximum value of 20.4 mm given for *minutoides* by Happold and Veyrunes, 2013 and within the range given by these authors for *M. triton* (20.5–22.7 mm). However, the head and body length (67 mm) is slightly smaller than the range given for *M. triton* (69–80 mm).

Also present within *M. minutoides* Clade A are three unaffiliated haplotypes, including: H21, our experimental samples from Namibia and Botswana; H91 from Ethiopia (ex. *M. tenellus*, Britton-Davidian et al., 2012); and H7 from Kenya. These samples are separated from other clades within A by 4.27–5.87%, generally above the range of intraspecific distances reported by Baker and Bradley (2006).

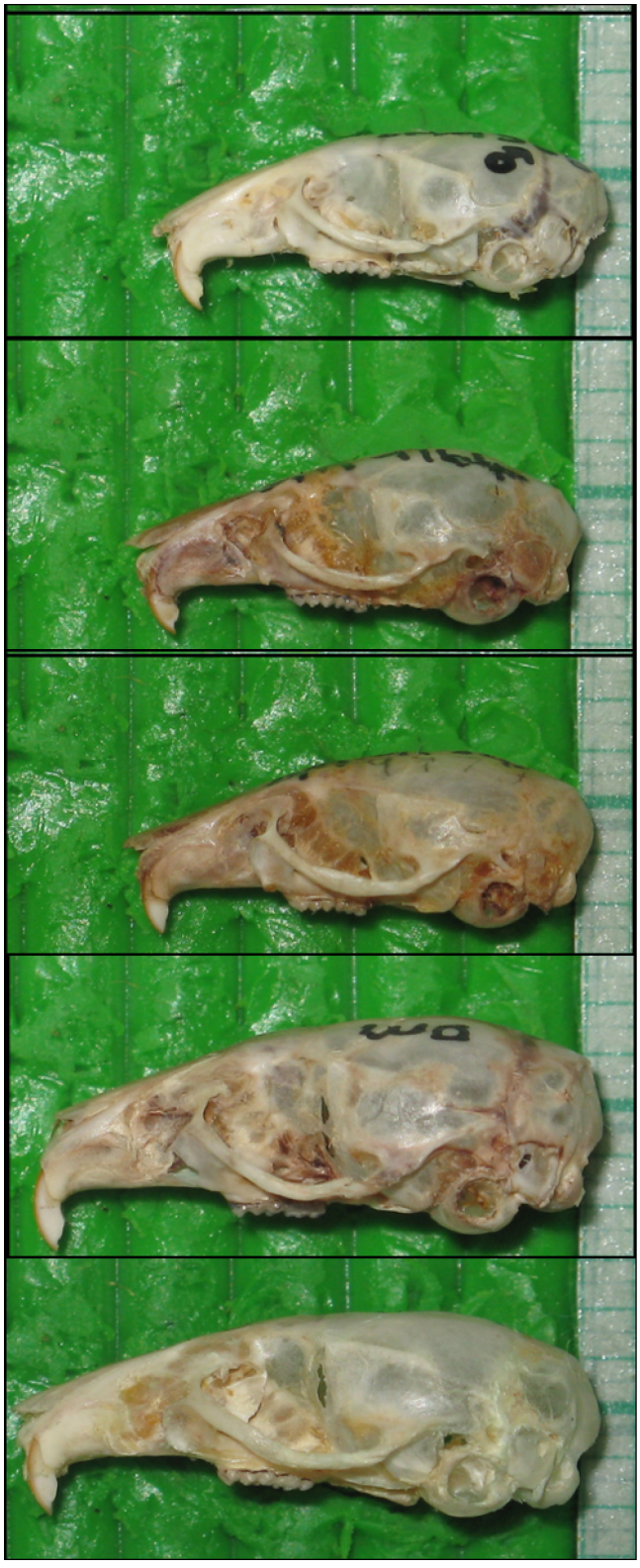

Fig. 4. Lateral skull views of selected *Mus* (*Nannomys*) voucher specimens included in this study, indicating key differences between species as discussed in text. Specimens arranged from top to bottom as follows: (1) *Mus cf. indutus* DM9366 (NA160 – Namibia: Kavango); (2) *Mus minutoides* DM9164 (KZN: Mkuze GR); (3) *Mus cf. neavi* DM9174 (KZN: Mkuze GR); (4) *Mus cf. triton* DM9126 (Mozambique: Mt Namuli); (5) *Mus callewaerti* NNm15373 (ANG018-Angola: Village Mbala Nondolo).

Mus (N.) sp. 1?

Our experimental haplotype 42 from Berega in Tanzania, basal to *M. minutoides* and *M. musculoides*, is separated from all other *Nannomys* haplotypes by genetic distances of 8.8–17.5%. We hypothesize that further studies based on nuclear markers may support its description as a distinct genetic species, referred to here as *Mus (N.)* sp. 1?

Mus indutus

Mus indutus (Clade F) is separated from all other *Nannomys* species by genetic distances of 11.8–16.3%. By including two haplotypes from the Koanaka Hills in northwestern Botswana, we extend the range of genetically described *M. indutus*, which previously only included South African haplotypes. We found no evidence of *M. indutus* in our sample set in Namibia, where our samples were allocated to *M. minutoides*, or Angola, from where our samples were either *M. minutoides* or *M. callewaerti*.

Mus (N.) sp. 2?

Sister to *Mus haussa* and *M. mattheyi* and basal to *M.indutus* is a single haplotype (93, *Mus (N.)* sp. 2?) from Chad (Veyrunes et al. 2005; Britton-Davidian et al., 2012). As it is distinct from these species by a minimum genetic distance of 11.3%, we hypothesize that future studies may confirm this as a distinct species.

Control region

Analysis of the more limited control region dataset (Fig. 2), which was congruent with the cytochrome *b* dataset, is included for completeness. *Nannomys* was moderately supported with respect to the outgroup *Mus musculus.* Basal within *Nannomys* was a new haplotype from Huambo, Angola (H15), representative of *M. callewaerti* based on cytochrome *b* sequences. In a derived position was *M. bufo*, *M. minutoides* and a well supported clade from Tanzania (H12, H13), representative of *M. (N.)* sp. 1? based on cytochrome *b* sequences.

Biogeographical synthesis

Two central and east African, predominantly montane sister taxa, *M. callewaerti* (from Angola and DRC) and *M. triton* (from central and east Africa) are situated basally within the *Nannomys* radiation, suggesting that *Nannomys* may have originated in the montane regions of central and east Africa and diversified in the late Miocene 6.33 Mya, somewhat earlier than the first known African fossil records for the subgenus (4.5 Mya), and earlier than the date of 5–6.5 Mya given by Lecompte et al. (2008a) for the first appearance of the subgenus *Nannomys* in Africa. Subsequent radiation seems to have resulted in clades restricted to central-west Africa (Clade X) and more widespread in west, central, east and southern Africa (clade S). Within the latter, radiations occurred into lowland forest (*M. sorella*), savanna (*M. mattheyi*, *M. minutoides*, *M. musculoides*) and semi-arid habitats (*M. haussa*, *M. indutus*). Many of these species-level divergences occurred in the late Pliocene between 2.5 and 3.5 Mya. Palaeo-climatic and tectonic forcing in the late Pliocene leading to the conversion of forests into open woodland (Partridge, 2010; Cotterill & de Wit, 2011) have been invoked as drivers of speciation in African mammals including antelope (Moodley & Bruford, 2007), bats (Taylor et al., 2012) and rodents (Lecompte et al., 2005; Taylor et al., 2009).

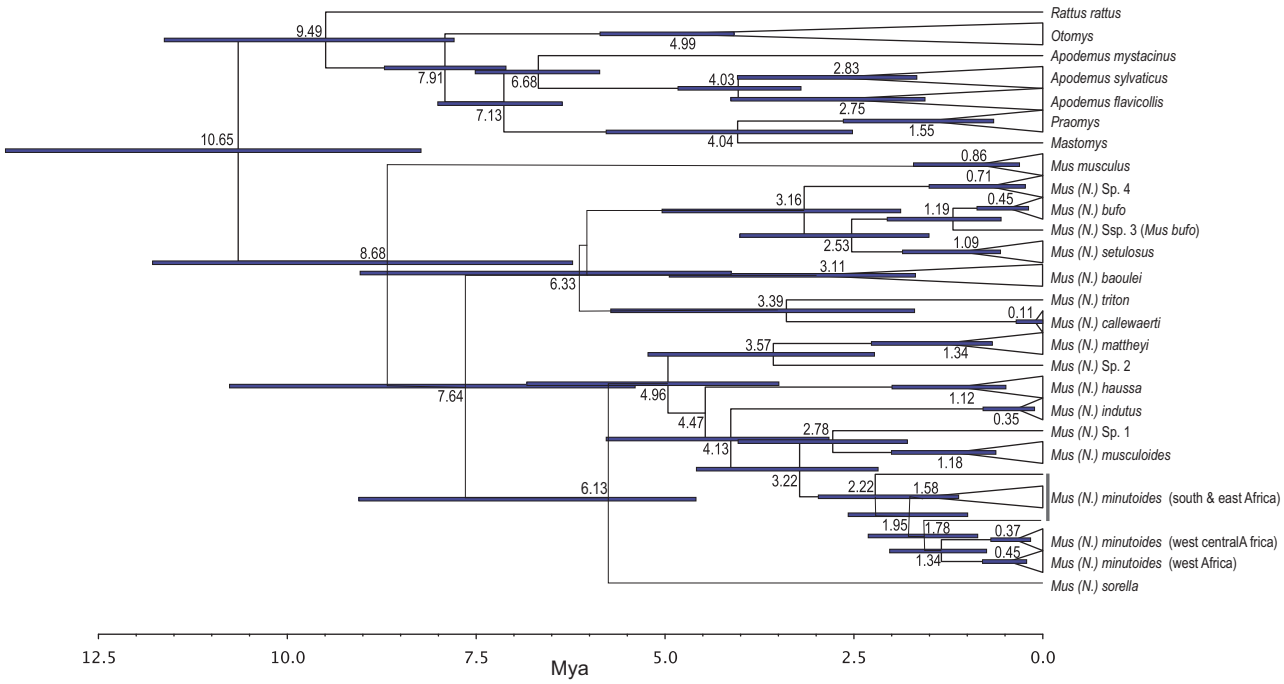

Fig. 5. Chronogram illustrating the evolution of major *Nannomys* clades. Mya = million years ago; Sp. = species; Ssp. = subspecies.

Taxonomic synthesis

Molecular phylogenetic analyses provide invaluable insights into evolutionary history, but without morphological and other characters from voucher specimens, formal taxonomic revision is impossible, particularly given the large number of Genbank sequences which are incorrectly identified (Pleijel et al., 2008). This is particularly true in the case of cryptic species-groups such as many African rodent genera (Kan Kouassi et al., 2008; Lecompte et al., 2008b; Taylor et al., 2009, 2011). In the case of *Mus*, studies such as that of Kan Kouassi et al. (2008) and Veyrunes et al. (2010a,b) have shown that several species defined on molecular grounds can often be distinguished by subtle morphological and/or karyological differences. In our study, morphological characters from voucher specimens (Table 1) allowed us to assign accurate identifications to new material representing *M. callewaerti*, *M. triton* and *M. bufo*. However, due to small samples sizes and morphological conservatism, our morphological data failed to allow discrimination between distinct clades such as the A1, A2, A3 and A4 sub-clades of *M. minutoides sensu lato*. Detailed multivariate morphometric and karyological analysis beyond the scope of this study may be able to resolve these taxa in the future. Conflicts between morphological and molecular designations meant that the taxonomic status of morphologically-identified specimens of *M. cf. neavei* (from uMkhuze Game Reserve in South Africa) and *M. cf. triton* (from Mt Namuli in Mozambique) could not be resolved. Past introgression of mitochondrial genes could plausibly explain the position of *M. neavei* within the *minutoides* A4 clade. In the case of gray-bellied *M. cf. triton* from Mt Namuli, it is more probable that this divergent haplotype represents an un-described species which shows morphological convergence with *M. triton* (particularly given that both these taxa are from montane forests).

Conclusions

This report considerably increases the known genetic diversity of *Nannomys*, from 65 to 102 haplotypes. The monophyly of *Nannomys* (Hypothesis 1) is supported by our mitochondrial data. Our findings do not conflict with the null hypothesis that *Mus musculoides, M. indutus, M. callewaerti, M. haussa, M. mattheyi, M. baoulei* and *M. sorella* are discrete monophyletic species. However our results suggest that the *M. minutoides* and *M. setulosus* clades may be species complexes. The widely distributed south and east African *M. minutoides* clade also appears to be a species complex, thus the null hypothesis that *M. minutoides* is a discrete species is not supported. The *M. setulosus* clade consists of *M. setulosus sensu stricto*, a potentially species level clade (*Mus (N.)* sp. 4?), *M. bufo* and a clade which is likely to represent a subspecies of *M. bufo* (*Mus (N.)* Ssp. 3). We also hypothesize that two other genetically distinct clades (*Mus (N.)* sp. 1? and 2?) may represent new species, pending confirmation from nuclear markers or other types of evidence. For samples identified as *M. triton* and *M. neavei*, incongruence between morphological identification and molecular phylogenetic position may be due to introgression, and underscores the need for species assignment based on multiple lines of evidence.

Acknowledgements

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