J. Zool., Lond. (2004) **262**, 1–12 © 2004 The Zoological Society of London Printed in the United Kingdom DOI:10.1017/S0952836903004795

Mitochondrial sequences and karyotypes reveal hidden diversity in African pouched mice (subfamily Cricetomyinae, genus *Saccostomus*)

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(Accepted 14 October 2003)

Abstract

The African rodent genus *Saccostomus* is common and widespread in the savannas, scrubby areas and cultivated fields from South Ethiopia and Somalia through East Africa down to the Cape Province. Its systematics and taxonomy are however poorly known, with two species currently recognized, *S. mearnsi* and *S. campestris*, the former is typical of the Somali–Maasai domain and the latter is typical of Zambezian–South African. The karyotypic findings and the analysis of the mitochondrial gene cytochrome *b* for some populations from Somalia, Tanzania, Zambia and South Africa are presented showing that the diversity within the genus is much higher than previously reported and believed, and that *S. mearnsi* and *S. campestris* rather represent species complexes, still actively speciating. The times of cladogenetic events and the extent of chromosomal variability within the genus that may be coupled with speciation are discussed. Finally, the possible taxonomy is considered in the context of the wide karyotypic variation shown by each species, the extent and the boundaries which are still largely unknown, limiting therefore their definitive taxonomic assignment.

Key words: *Saccostomus*, systematics, taxonomy, cytochrome *b*, cytogenetics, Africa

INTRODUCTION

Pouched mice belonging to the genus *Saccostomus* Peters, 1846 are widespread and common in the savannas, scrubby areas, grassy places in open forests, cultivated fields and sandy plains from south Ethiopia through east Uganda and Kenya down to the Cape Province.

Despite its commonness, the taxonomy and systematics of the genus have been a source of much debate (Delany, 1975; Ellerman, Morrison-Scott & Hayman, 1953; Misonne, 1974). The most recent rodent check list (Musser & Carleton, 1993) includes two species only: *Saccostomus campestris* Peters, 1846 and *S. mearnsi* Heller, 1910. This conclusion was based upon the findings of Hubert (1978), who showed two different karyotypes and morphologies (although on a limited sample) corresponding to *S. mearnsi* and *S. campestris*; this was also supported by the analysis of dental and skull characters (qualitative as well as linear measurements) made by Denis (1988). *Saccostomus mearnsi* has a typical Somali–Maasai distribution, while *S. campestris* is an east Zambezian–South African taxon. The limits of their ranges occur somewhere in central south Tanzania, where the extent and the boundary of the contact area is still approximate.

It should be stressed, however, that there are findings suggesting the two recognized taxa are species complexes. Gordon (1986) found an extreme range of karyotype variation in *S. campestris* from South Africa, Namibia and Zimbabwe (both in diploid, fundamental number and arm morphology), with basically three groups, one with $2n = 28$–30 (the south-west arid biome), a second with $2n = 46$ (including at least 12 chromosomal variants) occurring in the savanna biome, and, finally, a third including $2n = 42$–50 forms that cannot be related easily to the latter. Although performed on a limited sample, multi-locus protein electrophoresis (Gordon, 1986) indicated that high and low diploid number groups do not share the same gene pool.

Saccostomus mearnsi also shows karyotypic variation. Apart for the $2n = 40$–42 karyotypes described by Hubert (1978) in the lower Omo Valley of Ethiopia, karyotypic variants occur in the Maasai Steppe (Tanzania) ($2n = 32$; Fadda *et al.*, 2001), and in Somalia ($2n = 44$; Capanna, Civitelli & Filippucci, 1985).

There is a clear need for taxonomic revision, as the distinction between the two accepted species based on morphology and karyology is an oversimplification as it is based on limited samples from the range with no reference

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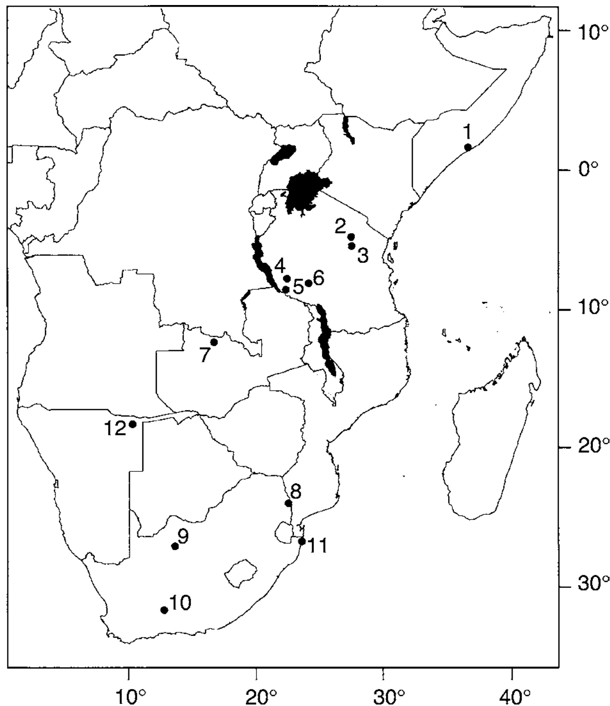


Fig. 1. The location of the samples of *Saccostomus* spp. studied. '*mearnsi*' species complex: 1, Afgoi; 2, Matongolo; 3, Ndaleta; '*campestris*' species complex: 4, Muze; 5, Chunya; 6, Kasanga; 7, Mutanda; 8, Kruger Nt. Park; 9, Kuruman; 10, Karoo Nt. Park; 11, Lalanek, KwaZulu-Natal; 12, Hereroland, Namibia.

to the karyotypic extreme variation. Here we present new karyotypic findings from Tanzania, Zambia and Somalia and the results of analysis of the mitochondrial gene cytochrome *b* for a number of populations from Tanzania, Zambia and South Africa, showing that diversity within the genus is much higher. We will discuss the evolution of the genus in Africa through time and present evidences that '*mearnsi*' and '*campestris*' are rather species complexes that are actively evolving and speciating.

Finally, we will discuss the taxonomic problems and the limits for taxonomic resolution of the species diversity shown by karyotypic and genetic variability.

MATERIAL AND METHODS

Pouched mice came from 13 localities in Somalia, Tanzania, Zambia and South Africa (see Table 1 and Fig. 1). For karyotyping and tissue-collecting simultaneously, the mice were live collected and transported alive to the Rodent Research Project, Sokoine University of Agriculture, Morogoro (Tanzania), Mutanda Research Station, Solwezi (Zambia), and to the Dipartimento di Biologia Animale e dell'Uomo, Università di Roma 'La Sapienza'. The specimens were killed and preserved in alcohol and deposited in the collections of the Royal Museum for Central Africa, Tervuren, Belgium and the Museo di Anatomia Comparata, Università di Roma 'La Sapienza', Italy.

Tissues of a number of non-karyotyped additional specimens from Tanzania and South Africa were collected in the field or obtained from the tissue bank of the former Transvaal Museum (Pretoria; see Table 1 and Fig. 1).

The South African samples were from localities for which there has been a previous karyotype description (Gordon, 1986) or were assigned to chromosomal types according to the karyotype's geographic distribution presented by Gordon (1986) (see Table 1).

There is no available information on the karyotype for the Tanzanian localities of Muze and Kasanga and no tissues or voucher specimen available for the specimen from Afgoi, Somalia.

A direct comparison with the type specimens of the '*mearnsi*' and '*campestris*' groups allowed us to allocate our Tanzanian and Zambian samples (these localities are near the boundary between the ranges) to 1 of these 2 species complexes (see Discussion). Matongolo and Ndaleta fall into the '*mearnsi*' group (see also Fadda *et al.*, 2001), while Mbugani-Chunya, Kasanga, Muze, Mutanda, fall into the '*campestris*' division (Table 1).

Figure 1 presents the geographical location of the samples as well as the approximate ranges of the 2 species complexes. Table 1 summarizes the specimens for which chromosomes and tissues for mtDNA extraction were available.

Cytogenetic methods

Chromosome metaphases were obtained from the bone marrow following Hsu & Patton (1969). Cell suspensions in fixative were transported at the Dipartimento di Biologia Animale e dell'Uomo, Università di Roma 'La Sapienza' where slides were prepared. Metaphases were stained by the Giemsa standard method (pH7). G-bands have been enhanced with trypsin following the protocol of Seabrigth (1971). The heterochromatic portion of the genome was identified by C-banding using Barium hydroxide 5% (Sumner, 1972). Picture of metaphases were collected using a digital camera Photometrics Sensys 1600 and the software Iplab (Scanalytics, Inc, version 2.420).

DNA methods

Total DNA was extracted from muscle and liver tissues preserved in 80% ethanol and from museum specimens preserved in alcohol (Table 1). The DNA extraction was performed using Qiagen DNeasy kit. Complete sequences (1.140 bp) of the mitochondrial cytochrome *b* gene were obtained via PCR amplification using primers L14723 (5′-ACC AAT GAC ATG AAA AAT CAT CGT T – 3′), and H15915 (5′ – TCT CCA TTT CTG GTT TAC AAG AC – 3′), and the two internal primers L15408 (5′ – ATA GAC AAA TCC CAT TCC A – 3′) and H15553 (5′ – TAG GCA AAT AGG AAA TAT CAT TCT GGT). The entire gene was sequenced using the same primers.

PCR amplification was performed on a MJ Minicycler machine using the following conditions: a first cycle of an initial denaturation at 92° for 2 min, then 35 cycles

Table 1. Species, country, locality, latitude and longitude of the specimens analysed. The diploid (2*n*) and the autosomal fundamental number (NFa) are reported when available and the samples from which the entire sequence of the mitochondrial gene cytochrome *b* was obtained. The museum codes and the EMBL accession numbers are reported. (1) Jansa *et al.* (1999). (2) Royal Museum for Central Africa, Tervuren, Belgium. (3) Museo di Anatomia Comparata, Università di Roma 'La Sapienza', Italy. (4) Transvaal Museum, Pretoria, R. South Africa. (5) Gordon (1986)

Species and complex	Country	Locality	Latitude and Longitude	2*n*	NFa	Cyt. b	Museum code	EMBL code
Saccostomus 'mearnsi'	Somalia	Afgoi	02°08′N–45°07′E	44	42	–	No voucher specimen	
Saccostomus 'mearnsi'	Tanzania	Matongolo	04°31′S–36°28′E	32	48	+	50064[2]	AJ583918
Saccostomus 'mearnsi'	Tanzania	Ndaleta	05°12′S–36°30′E	32	48	+	50137[2]	AJ583919
Saccostomus 'campestris'	Tanzania	Mbugani-Chunya A	08°31′S–33°24′E	42	46	+	50709[2], 50664[2]	AJ583925, AJ583926
Saccostomus 'campestris'	Tanzania	Mbugani-Chunya B	07°58′S–33°18′E	44	46	+	TZ502[3]	AJ583936
Saccostomus 'campestris'	Tanzania	Kasanga	08°28′S–31°10′E	–	–	+	13822[4], 13823[4], 13830[4]	AJ583927, AJ583928, AJ583929
Saccostomus 'campestris'	Tanzania	Muze	07°42′S–31°34′E	–	–	+	13777 [4]	AJ583924
Saccostomus 'campestris'	Zambia	Mutanda Research Station	12°22′S – 26°16′E	44	48	+	ZM3[3], ZM9[3], ZM15[3], ZM35[3]	AJ583920, AJ583923, AJ583921, AJ583922
Saccostomus 'campestris'	South Africa	Kuruman	27°34′S–23°22′E	33–34[5]	48(Fn)	+	TM41392[4], TM41414[4]	AJ583930, AJ583931
Saccostomus 'campestris'	South Africa	Namibia, Hereroland	18°30′S – 20°15′E	32–36[5]	–	+	TM41623[4]	AJ583932
Saccostomus 'campestris'	South Africa	Kwazulu-Natal, Lalanek Insp. Qrts.	27°13′S – 32°46′E	46[5]		+	TM40441[4]	AJ583935
Saccostomus 'campestris'	South Africa	Karoo Ntl. Park	32°21′S–22°34′E	46[5]		+	TM41386[4]	AJ583934
Saccostomus 'campestris'	South Africa	Kruger Natl. Park; Satara	24°23′S–31°47′E	46[5]		+	TM42036[4]	AJ583933
Cricetomys emini [1]	Kenya					+		AF160610
Cricetomys gaqmbianus [1]	Tanzania					+		AF160614
Beamys hindei [1]	Kenya					+		AF160609

with denatutation at 92° for 1 min, annealing at 52° for 1 min, and extension at 72° for 1 min. These steps were followed by a 10 min extension at 72°. Each PCR reaction was obtained using 50–500 ng of template DNA, 200 ng of each primer, 0.2 mM of each dNTP and 2 μ of Taq polymerase (Promega), in a final volume of 50 μl.

Amplified products were purified using the Qiagen QIAquick purification kit and prepared for automated sequencing (M-Medical service, Roma).

Quantitative estimates of nucleotide divergence were evaluated by the number and proportion of nucleotide different sites and by the Kimura 2-parameters method (K2; Kimura, 1980). The level of saturation of base substitutions was estimated by comparing transitions and transversions for each codon position and the total number of substitutions.

Sequences were analysed though maximum parsimony (MP) and maximum likelihood (ML) using the program PAUP (2001 ver. 4.0b10, Sinauer Associates). The search for the shortest tree was performed through 1000 bootstrap replications.

The complete cytochrome *b* sequences of *Cricetomys emini*, *C. gambianus* & *Beamys hindei* (Jansa, Goodman & Tucker, 1999) were included into the analysis (the EMBL code in shown in Table 1). As outgroups, *Mus* and *Rattus* were used.

A relative rate test for the constancy of molecular clock was conducted using the 2-cluster test of Takezaki, Razhetsky & Nei (1995) through the program Phyltest (v. 2, Kumar, 1996). For calibration, 2 different outgroups were used, for which the divergence based on the fossil record has been estimated. These are: (1) *Tatera* and *Gerbillus*, for which the divergence has been estimated at 10–8 million years ago (Myr) (Tong, 1989), and (2) *Mus* and *Rattus*, for which Jaeger, Tong & Buffetaut (1986) have recorded a 12 Myr divergence. Divergence estimates have been computed on the *p*-distances for third base position, transversions only, following Ducroz, Volobouev & Granjon (1998), with *r* set as 1.53 and 2.275 for *Mus/Rattus* and *Tatera/Gerbillus*, respectively.

The sequence data from this study were deposited at the EMBL Nucleotide Sequence Database; their respective accession numbers are reported in Table 1.

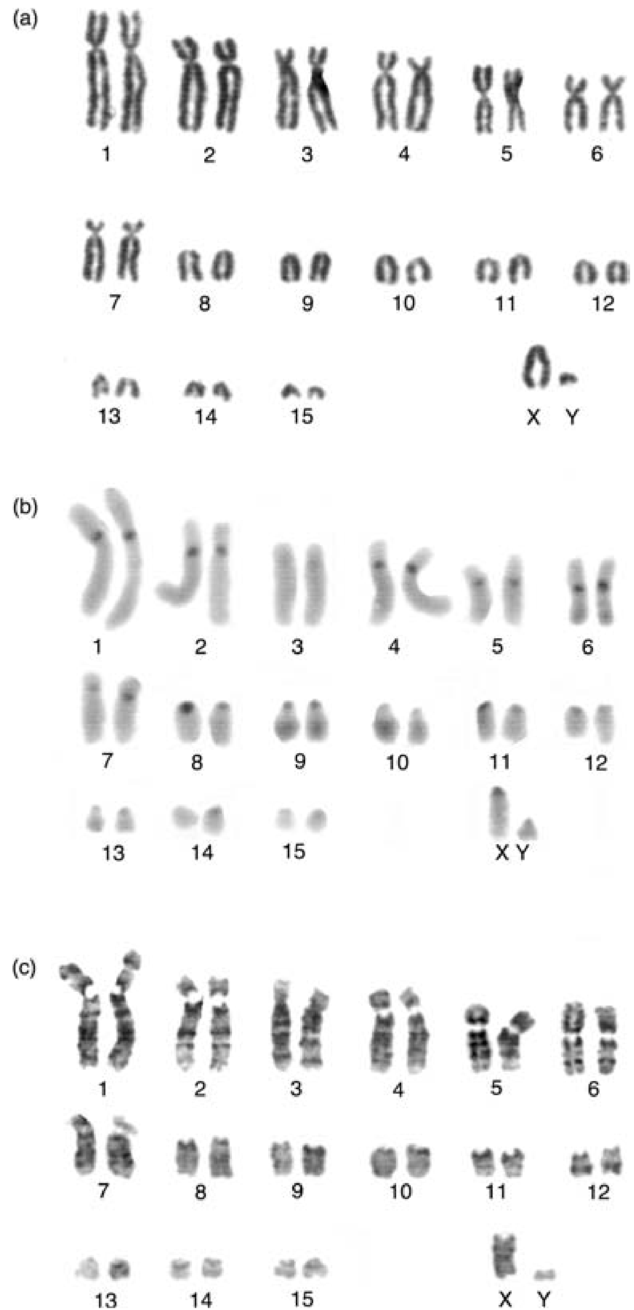


Fig. 2. The karyotype from Ndaleta, Tanzania; $2n = 32$, NFa = 44, X Y. (a) Giemsa stained; (b) C-banding; (c) G-banding.

RESULTS

Cytogenetics

Tanzania, Matongolo and Ndaleta, 2 ♂♂

The diploid number is $2n = 32$ and the autosomal fundamental number is NFa = 46 (Table 1). The karyotype is composed of seven pairs of large biarmed chromosomes, and by eight pairs of acrocentrics decreasing in size (Fig. 2a). Fadda *et al.* (2001) reported a different NFa (48) for these specimens because of the misidentification of two pairs of small chromosomes, which were erroneously considered as metacentrics rather than full acrocentrics. The X is a large acrocentric and the Y is a small acrocentric (Fig. 2a). The C-banding shows heterochromatic bocks at the centromeres of all the autosomes and on the X chromosome (Fig. 2b). The Y-chromosomes is entirely heterochromatic. Blocks of C-positive heterochromatine are visible along the arms of one pair of metacentrics (No. 6) and on three pairs of medium-sized acrocentrics (No. 9, 10, 13). G-banding (Fig. 2c) allowed the correct assignment of homologous chromosomes.

Somalia, Afgoi, 1 ♀

The diploid number is $2n = 44$ and the fundamental number is 46. The karyotype is composed by 21 pairs

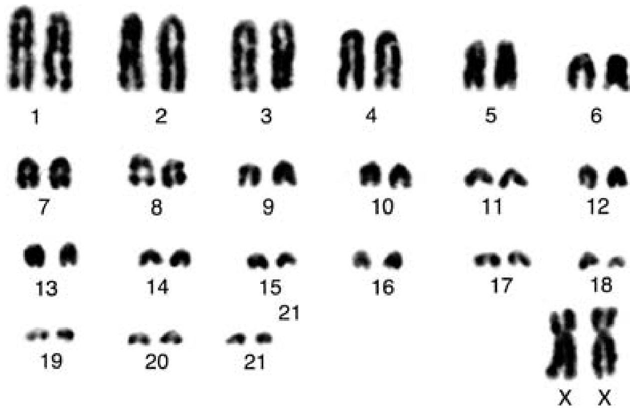


Fig. 3. The karyotype from Afgoi, Somalia; $2n = 44$.

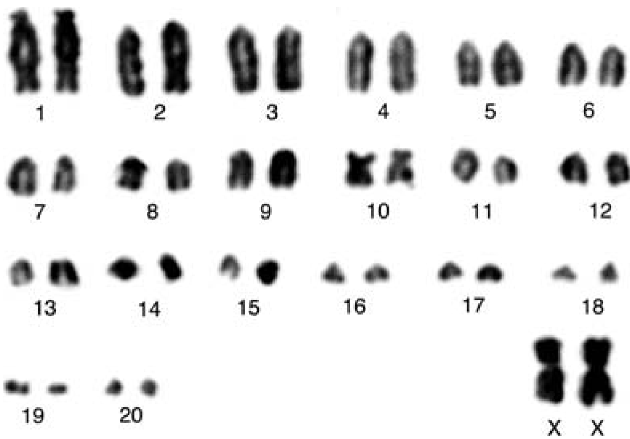


Fig. 4. The karyotype from Mbugani-Chunya A, Tanzania; $2n = 42$.

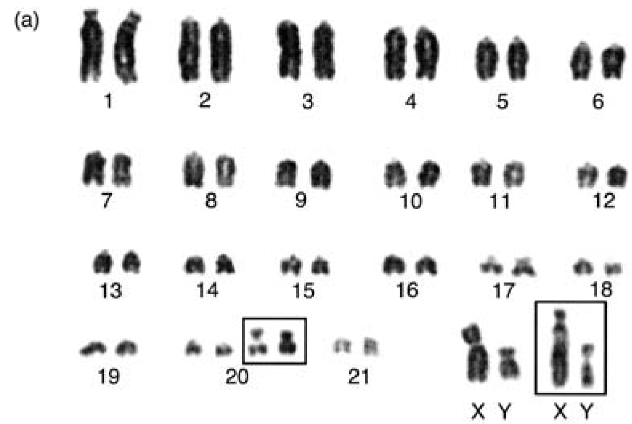
of acrocentrics decreasing in size and by one pair of large submetacentrics (Fig. 3). The sexual chromosomes cannot be identified. No banding was available for this karyotype.

Tanzania, Mbugani-Chunya A, 1 ♀

The diploid number is $2n = 42$ and the NFa is 46. The X chromosome is a medium-size submetacentric. The karyotype includes two pairs of large submetacentrics and two pairs of very small metacentrics, the remaining chromosomes being telocentrics decreasing in size (Fig. 4). No banding is available for this specimen.

Tanzania, Mbugani-Chunya B, 2 ♀♀, 3 ♂♂

The diploid number is $2n = 44$ and NFa = 46. The karyotype is composed of three pairs of biarmed autosomes, one of which is a large subtelocentric and two are small metacentrics; the remaining autosomes are telocentrics decreasing in size (Fig. 5a). The X and Y chromosomes are a large metacentric and a medium-size submetacentric, respectively. Heterochromatin occurs at the centromeric area of all the autosomes and in the short arms of the two small metacentrics (Fig. 5b). Also the short arm of the X and the entire Y chromosome are heterochromatic. Figure 5c reports the complete G-banding and the identification of the homologous pairs.



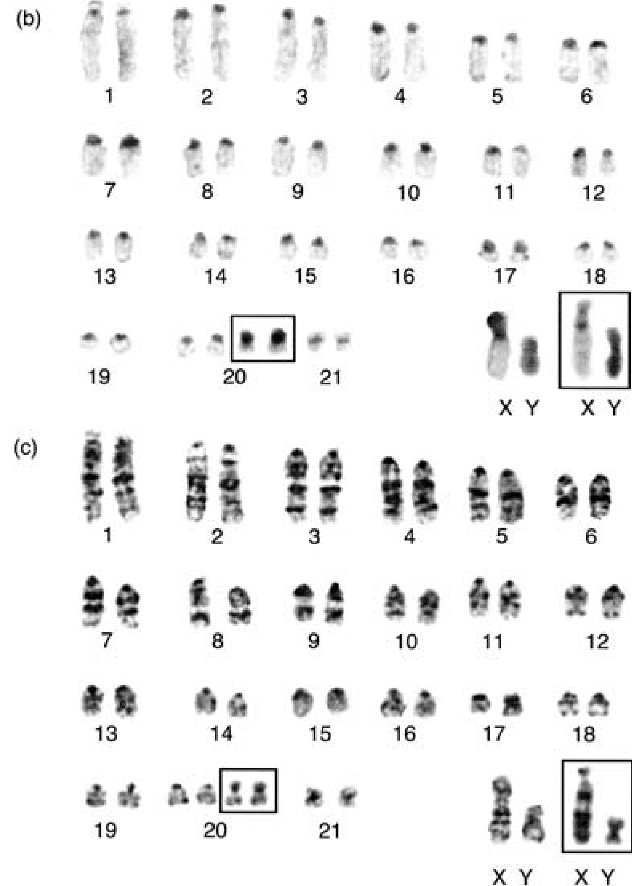


Fig. 5. The karyotype from Mbugani-Chunya B; $2n = 44$, NFa = 48, X Y; (a) Giemsa staining; (b) C-banding; (c) G-banding. This karyotype is identical to that of Mutanda, Zambia, except for the small couple of metacentrics (No. 20) and the heterochromosomes, shown in the inboxes.

Zambia, Mutanda Research Station, 4 ♂♂

The diploid number is $2n = 44$ and the NFa is 48. The autosomal complement is identical to the one described

for Chunya B, except for a couple of small metacentric chromosomes (No. 20; Fig. 5a,b,c) that in Chunya B are acrocentrics. Furthermore, the X chromosome is a large submetacentric and the Y is a medium-size metacentric (Fig. 5a,b,c). Apart from these differences, the karyotype has the same C- and G-banding pattern of Chunya B (Fig. 5b,c). Constitutive heterochromatine is present at the centromere of all the autosomes (Fig. 5b). The short arms of the pair of small metacentrics (No. 20) are completely heterochromatic. The short arms and the proximal portion of the long arms of the X chromosome are heterochromatic. The Y chromosome is entirely heterochromatic.

Chromosomal comparison

The karyotypes from Tanzania (Chunya A, $2n = 42$ FNa = 46; Chunya B, $2n = 44$, NFa = 46), from Somalia $2n = 44$ (FN = 46) and the one from Zambia ($2n = 44$; NFa = 48; FN = 52) are, according to the diploid number and the morphology of the chromosomes, apparently very similar (Figs 3, 4 & 5), while the $2n = 32$ from central-north Tanzania ($2n = 32$, NFa = 46, FN = 48; Matongolo and Ndaleta) is the most different (Fig. 2).

A direct comparison in G- and C-banding patterns is available only for the specimens from Tanzania (Maasai Steppe and Chunya B) and those from Zambia. The G-banding comparison among the karyotypes from Chunya B and Zambia reveals a high conservativeness with nearly a complete homology for the morphology of autosomes and banding pattern. The comparison between these two karyotypes and that from Ndaleta reveals greater changes. Their partial G-banded karyotypes are shown in Fig. 6. Chromosome number 1 of the Ndaleta specimens corresponds to acrocentric pairs 2 and 8 of the Zambian and Chunya ones after Robertsonian fusion; moreover, the long arm of submetacentrics 2, 3, 4, 5, 6, 7 of Ndaleta are homologous to acrocentrics 3, 4, 6, 9, 7, respectively, of Zambia and Chunya. Furthermore, the short arm of submetacentrics 2 of the Ndaleta is clearly different from the short arm of subtelocentric 1 of Zambia and Chunya; this suggests the occurrence of a monobrachial homology condition.

These observations, together with the comparison of the C-banding pattern, allow us to hypothesize a considerable differentiation between the autosomes of these two karyotypes, which involves at least six Robertsonian fusions with the occurrence of two biarmed chromosomes in monobrachial condition, one inversion in a small chromosome, and a deletion/addition of heterochromatin in two chromosomes.

Cytochrome b

Nineteen individuals have been studied (Table 1). Sequences obtained were all 1140 nucleotide long. The ratio between transitions and transversions for first, second, and third position are 1.7, 1 and 1.8, respectively. There were 431 informative sites.

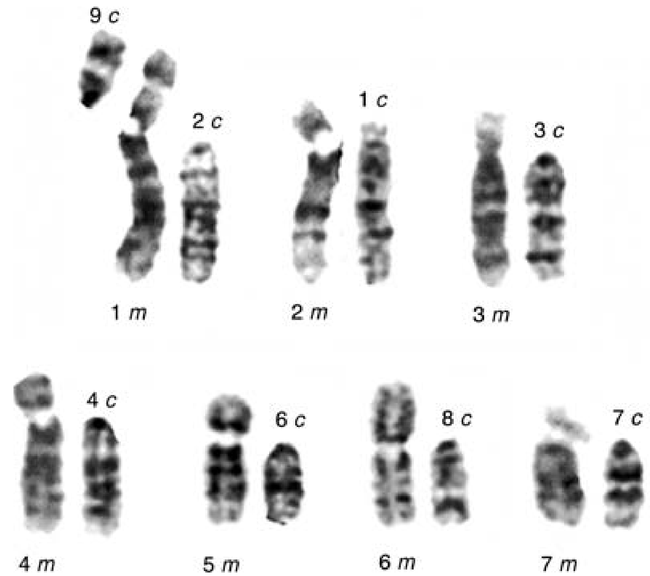


Fig. 6. G-banding comparison between selected chromosomes of *Saccostomus* from Ndaleta (*m*; $2n = 32$, '*mearnsi*' group) and Mbugani-Chunya B (*c*; $2n = 44$, '*campestris*' group). The comparison is limited to the largest autosomes of the two complements for which homologies are identifiable without ambiguity. Chromosome numbers correspond to the numbering used for the karyotypes of the two species in Figs 2c & 5c.

The comparison of transitions versus transversions showed some saturation for transitional changes in third base position. For this reason, different hypotheses have been tested, by giving the same or different weight to transversions in third codon position (2, 5, and 10 times than transitions). All maximum parsimony analyses produced trees with the same topology. The consistency indexes of these trees are 0.658, 0.654, 0.647 and 0.643 (unweighted and weighted parsimony), with differences in bootstrap values for some of the in-groups. In particular, there is a contrasting effect between the south Tanzanian and Zambian group and the South African one, in which higher weights given to transversions in third position increase bootstrap values in the latter and vice versa, evidently suggesting different rates of cytochrome *b* evolution in the two clades. One of these most parsimonious trees (transversions in third base weighted two times transitions) is shown in Fig. 7, together with the bootstrap values. The length of the tree is 1446 and its consistency index is 0.654.

The ML analysis was performed using the GTR+G+I model with a 1000 bootstrap replicates, and the likelihood (-Ln) is 6853.478. The tree is shown in Fig. 8 together with the bootstrap values and has the same topology of the MP ones.

Both the MP and ML trees indicate the occurrence of two monophyletic clades, one formed by the Maasai Steppe populations, and the second including all the other populations (MP bootstrap values: 100 and 99.9%, and ML 100% and 96%, respectively). These two clades correspond to the two species complexes '*mearnsi*'

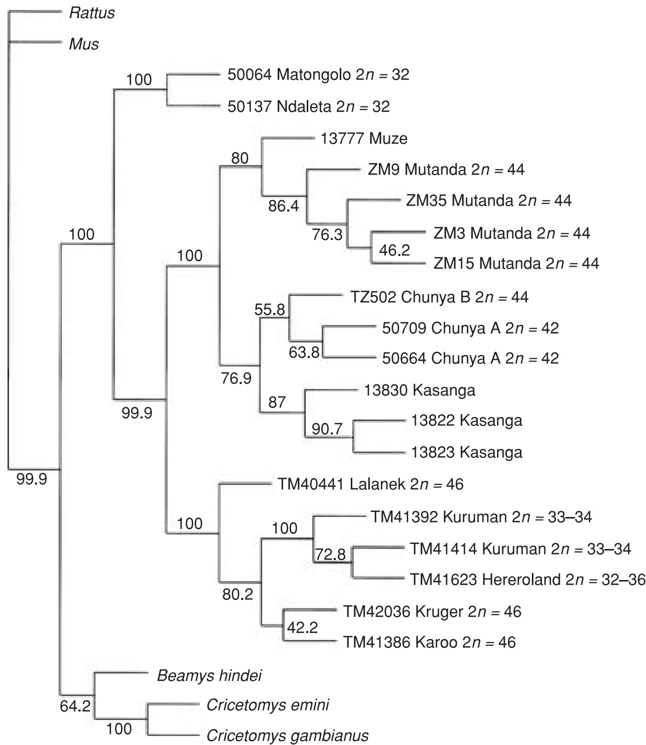


Fig. 7. Maximum parsimony bootstrap consensus tree (1000 replicates, branch and bound search; transversions in third base weighted two times transitions), with bootstrap values. The tree has been construed on the entire cytochrome *b* sequences and it has been outgroup rooted on *Mus* and *Rattus*. The diploid numbers characterizing lineages are indicated when available.

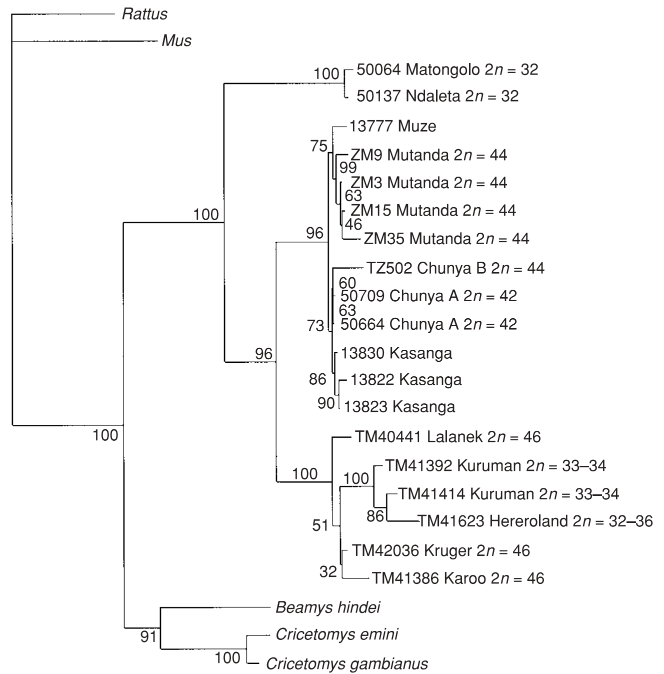


Fig. 8. Maximum likelihood tree (1000 replicates), with bootstrap values. The tree has been construed on the entire cytochrome *b* sequences and it has been outgroup rooted on *Mus* and *Rattus*. The diploid numbers characterizing lineages are indicated when available.

and '*campestris*', respectively. The in-group to *the 'campestris*' species complex is represented by two other clades, one formed by the South African and the other by the south Tanzanian and Zambian populations.

Within the south Tanzanian and Zambian group two clades occur, i.e. Chunya–Kasanga and Muze–Zambia, supported by moderately high bootstrap values.

Concerning the South African forms, Lalanek (Natal) represents the most ancestral population. The MP bootstrap support of the dichotomy between the low and high diploid numbers increases from 67.6% to 90.9% in dependence of the weight given to transversions in third position, i.e. highest values correspond to high weights, and in the ML tree it is high (86%) for the low chromosomal forms.

Monophyly and the derived condition of the low diploid number populations (Kuruman, Hereroland) are supported by high bootstrap values. However, it is not so for the high diploid number populations that do not constitute a monophyletic group.

Kimura two-parameters between-groups distances and the proportion of nucleotide differences are shown in Table 2. Groups represented in that distance matrix table have been defined on a geographical, chromosomal, and tree topology basis as follows: Maasai Steppe (Matongolo, Ndaleta, $2n = 32$), Chunya A ($2n = 42$), Chunya B ($2n = 44$), Kasanga, Zambia (Mutanda, $2n = 44$), south and central South Africa (Kuruman, Hereroland, $2n = 32$–36), Karoo ($2n = 46$), Kruger ($2n = 46$), Lalanek (KwaZulu-Natal, $2n = 46$). The two localities A and B of the Chunya area are left separated because their chromosomal differences, although geographically proximal (15 km). Because Muze is the sister group of the Zambian clade, although geographically proximal to Kasanga (approximately 60 km), it was decided to leave it as an independent group without including it into Zambia. All these groups are supported by bootstrap values that in most cases are high (see previously).

Within the genus *Saccostomus*, the highest proportion of nucleotide differences occur between the two species complexes '*mearnsi*' and '*campestris*' (17.5%; min. 18.7%, max. 23.7%). The lowest proportion occurs between Chunya A and Kasanga (south Tanzania); these two localities are separated by a 150 km distance. In comparison, the number of substitutions between the two localities A and B of the Chunya area, distant 15 km each other are remarkably high (3.2%). Low proportions occur amongst all the Zambian and south Tanzanian samples. Slightly larger proportions occur amongst the South African samples, with the higher values characterizing the differences between the low and high diploid numbers.

Nucleotide differences between *Saccostomus* and the other two Cricetomyinae are slightly higher than those between the '*mearnsi*' and '*campestris*' groups, i.e. 21% between *Saccostomus* and *Cricetomys*, and 20.3% between *Saccostomus* and *Beamys*. On the contrary, the value between *Cricetomys* and *Beamys* is

Table 2. Below the diagonal: Kimura two-parameter distances between groups. Above the diagonal: the percentage of nuleotide differences

	Masaai Steppe	Muze	Kasanga	Chunya A	Chunya B	Zambia	Kuruman	Hereroland	Kruger	Karoo	Natal	*Beamys*	*Cricetomys*
Masaai Steppe	–	0.166	0.168	0.162	0.174	0.165	0.200	0.205	0.171	0.186	0.176	0.203	0.210
Muze	0.191	–	0.024	0.023	0.047	0.027	0.145	0.149	0.118	0.127	0.123	0.212	0.206
Kasanga	0.194	0.024	–	0.012	0.039	0.029	0.141	0.146	0.113	0.119	0.116	0.210	0.202
Chunya A	0.187	0.024	0.012	–	0.031	0.024	0.139	0.146	0.108	0.114	0.114	0.206	0.199
Chunya B	0.202	0.049	0.040	0.032	–	0.050	0.154	0.160	0.121	0.125	0.128	0.203	0.193
Zambia	0.190	0.028	0.030	0.025	0.052	–	0.142	0.149	0.115	0.124	0.121	0.215	0.206
Kuruman	0.237	0.165	0.159	0.157	0.175	0.161	–	0.046	0.053	0.070	0.065	0.229	0.230
Hereroland	0.243	0.169	0.165	0.165	0.182	0.169	0.047	–	0.058	0.072	0.074	0.239	0.236
Kruger	0.198	0.131	0.125	0.120	0.134	0.128	0.055	0.060	–	0.032	0.032	0.198	0.201
Karoo	0.220	0.142	0.132	0.126	0.139	0.139	0.074	0.076	0.033	–	0.046	0.208	0.210
Natal	0.206	0.138	0.130	0.126	0.143	0.135	0.069	0.078	0.033	0.047	–	0.195	0.205
Beamys	0.239	0.253	0.250	0.244	0.240	0.257	0.277	0.292	0.233	0.247	0.228	–	0.155
Cricetomys	0.249	0.242	0.237	0.233	0.225	0.242	0.277	0.285	0.236	0.249	0.242	0.176	–

comparatively lower (15.5%) than between the '*mearnsi*' and '*campestris*' groups.

The relative rate tests showed constancy in molecular evolution for all comparisons between clades, with the exception of the low/high diploid numbers in the South African forms and within the Chunya–Kasanga and Mutanda–Muze groups. The test has been performed using the *p*-distances for third base position, transversions only. Divergence estimates for the molecular clock calibrated on *Mus–Rattus* and on *Tatera–Gerbillus* are shown in Table 3. There are evident differences in the estimates of the two calibrations. Older divergence times resulted when based on the *Mus–Rattus* split than on the *Tatera–Gerbillus* split. Differences are higher at the subfamily (*c.* 5 Myr) and at the genus (3 Myr) level, and decrease at the species (2–1 Myr) and population (0.3 Myr) level. The divergence between *Beamys* and *Cricetomys* was of 9.54 ± 1.17–6.41 ± 0.79 Myr. The split between *Beamys–Cricetomys* and *Saccostomus* was much older, i.e. between 13.79 ± 1.11 and 9.27 ± 0.75 Myr; and that between the '*mearnsi*' – '*campestris*' groups was of 7.9 ± 0.98–5.32 ± 0.53 Myr. Within '*campestris*', the estimate divergence between the Tanzanian–Zambian and the South African groups was of 3.79 ± 0.65 – 2.55 ± 0.44 Myr; that between the Chunya–Kasanga and Mutanda–Muze is much more recent, i.e. between 0.72 ± 0.26 and 0.48 ± 0.17 Myr. No constancy was observed in the comparison between the low and high diploid numbers in the South African group, with the former being significantly more quickly evolving ($P < 0.0001$).

DISCUSSION

Diversity within the genus *Saccostomus* is larger than previously reported and believed in the past. This should not be surprising, as most of the African rodent genera show the typical pattern of the order, i.e. high species diversity associated with or coupled to chromosomal mechanisms of speciation (see, for example, in *Arvicanthis*, Corti *et al.*, 1995; Capanna *et al.*, 1996; Ducroz *et al.*, 1998, Volobouev *et al.*, 2002; Castiglia *et al.*, 2003). This obviously creates confusion for taxonomy and systematics, due to the occurrence of several cryptic species complexes, which represents the norm rather than an exception. For this reason, the number of species of the entire order (representing 43.65% of the entire mammalian class, including monotrems and marsupials, according to the 1993 checklist by Musser & Carleton, 1993) is going to increase consistently when more detailed analyses on African rodents are carried out.

Data presented here however are not definitive for the taxonomy of the genus as well as for the subfamily, as large areas of the distributional range are not included and comparisons with a greater number of Cricetomyinae taxa are needed before an accurate picture can be drawn.

The findings of this study are discussed below in an evolutionary and taxonomic framework.

Table 3. Date estimates for each major cladogenetic event. The molecular clock has been calibrated on the split between *Mus* and *Rattus*, and between *Tatera* and *Gerbillus*

Cladogenetic event (nodes as in the trees in Figs 2 & 3, except for *Tatera* and *Gerbillus*)	Estimated date of divergence in *Myr*		
	Proportion of nucleotide divergence (third base position)	*Mus–Rattus* split at 12 Myr	*Tatera–Gerbillus* split at 8 Myr
Mus/Rattus – Cricetomynae	0.248 ± 0.018	16.2 ± 1.17	10.9 ± 0.79
Tatera/Gerbillus – Cricetomynae	0.256 ± 0.018	16.73 ± 1.17	11.25 ± 0.79
Beamys – Cricetomys	0.146 ± 0.018	9.54 ± 1.17	6.41 ± 0.79
Beamys/Cricetomys – Saccostomus	0.211 ± 0.017	13.79 ± 1.11	9.27 ± 0.75
'*mearnsi*' – '*campestris*' species groups	0.121 ± 0.015	7.9 ± 0.98	5.32 ± 0.53
Tanzania/Zambia – South Africa	0.058 ± 0.01	3.79 ± 0.65	2.55 ± 0.44
Chunya/Kasanga – Zambia/Muze	0.0153 ± 0.0044	1 ± 0.28	0.67 ± 0.19

Evolution and speciation

The time estimations of the cladogenetic events occurring within the lineages of Muroidea constitute a continuous source of debate. Except for 12 Myr split between *Mus* and *Rattus* (Jaeger *et al.*, 1986) and that of 8 Myr between *Tatera* and *Gerbillus*, both based on the fossil record, there are no other valid sources to calibrate a molecular clock. One should recall however, that alternative estimates situated the *Mus–Rattus* divergence much earlier, i.e. 23 Myr (Adkins *et al.*, 2001) and 41 Myr (Kumar & Hedges, 1998), although both have been criticized as overestimates (Michaux, Reyes & Catzeflis, 2001).

Our estimates for the separation events (Table 3) show important differences when either based on the *Mus–Rattus* or the *Tatera–Gerbillus* split. Barome *et al.* (2001) used a slightly different approach in their study on *Acomys*, where the *Mus/Rattus* divergence was based alternatively on 10 or 14 Myr. Clearly, a 10 Myr interval would reduce the gap between our two alternative estimates making them apparently more reliable; but since they constitute an approximation, the chronological framework has been left as it is. However, it should be remembered that the *Saccostomus* first appearance date (FAD) has been recorded at 10.5 Myr (Denis, 1999), which means: (1) it is the oldest rodent genus within eastern and southern Africa, second only to *Anomalurus* (15.5 Myr; Denis, 1999); (2) its FAD would thus favour the 12 Myr *Mus–Rattus* calibration clock hypothesis.

The cladogenetic event at the base of the splitting between *Beamys–Cricetomys* and *Saccostomus* is quite old (Table 3), even when based upon the *Tatera–Gerbillus* dichotomy. It occurred just after the divergence of the entire Dendromurinae–Cricetomyinae–Mystromyinae–Nesomyinae 'African' clade, for which the origin has been dated within the range of 16.1 ± 0.5 and 18.8 ± 1 Myr (Michaux *et al.*, 2001). Within the Cricetomyinae, the split between *Beamys* and *Cricetomys* is more recent, occurring approximately 9–6 Myr later. The relative rate test suggests constancy in molecular evolution, so that these two genera are, comparatively, younger.

Even if the origin of *Saccostomus* were to be overestimated, it has occurred apparently between the Middle and Late Miocene, in a period just following the emergence of most living rodent subfamilies (Michaux & Catzeflis, 2000). This represents a striking difference with the first appearance of the majority of the other East and South African rodent genera, for which the FAD is between 5 and 3.3 Myr (see Denis, 1999, for an overview).

Furthermore, although not corroborated by complete G-banding, the karyotypes occurring within *Saccostomus* show diploid numbers not exceeding $2n = 50$, with a clear tendency towards reduction. On the contrary, the known karyotypes for *Cricetomys* have remarkably higher diploid numbers ($2n = 78–80$; Matthey, 1954; Granjon *et al.*, 1992; Codja *et al.*, 1994).

These findings would put a question mark on the strict monophyly of the Cricetomyinae, so that the eventual re-evaluation of the Saccostomurinae *sensu* Roberts (1951) needs to be tested.

Concerning the evolution within *Saccostomus*, there is no doubt that two main species complexes corresponding to '*campestris*' and '*mearnsi*' exist. There is a high proportion of nucleotide differences testifying for a Late Miocene, possibly Messinian separation between the two lineages (Table 3). This period is associated with the complex rifting-uplifting of Eastern Africa that has made the area a true island, allowing evolution in allopatry. Unfortunately, the chromosome banding limited to three localities only does not allow the identification of synapomorphies characterizing the evolution of the karyotype within each species group.

Furthermore, the proportion of nucleotide differences between '*campestris*' and '*mearnsi*' is even higher than that observed between different other allied muroid genera, such as *Arvicanthis* and *Lemniscomys* (Ducroz *et al.*, 1998). It should be mentioned also that other species with a typical Zambezian domain, such as *Acomys spinosissimus*, had even an older split (15.6–11.1 Myrs) from their close congeneric relatives (Barome *et al.*, 2001).

Saccostomus is known from the Early to the Late Pliocene of East and South Africa (Denis, 1999). The separation event within the '*campestris*' group in a 'northern' Zambezian (Tanzania and Zambia) and a 'southern' (South Africa) lineage has been traced back to $3.79 \pm 0.65 – 2.55 \pm 0.44$ Myr (Table 3). Denis (1999) noted that the highest frequency in the occurrence of modern South African rodent species occurs at 5 Myr, later followed (3.7–3.3 Myr) by the East African ones,

with a strong difference between the South and East African rodent faunas occurring between 3.5 and 3 Myr. It is therefore likely that the divergence between these two clades has been driven by the same factors that affected more recent Pliocene rodents.

One of the main observations from this work is the striking karyotype variability shown by the genus *Saccostomus*. This was already remarked by Gordon (1986) in the southern part of the continent, but as new populations are being sampled, it becomes more and more evident that karyotype variability constitutes the norm during evolution, and that chromosomal polymorphism occurs even within populations, such as the South African ones.

Unfortunately, there are limited data on the *mearnsi* group, apart from those reported here and by Hubert (1978), so it is impossible to evaluate the extent of chromosomal variability in that group, but these findings suggest the occurrence of at least three species. On the contrary, we have shown karyotype variability in the south Tanzanian–north-west Zambia area, for which Kimura distances fall in the intra-specific/sibling species range (see Bradley & Baker, 2001).

There are, however, questions open on this concern that cannot receive definitive answers. First of all is the maintenance over a long period of time of the ability to keep a karyotype prone to quick changes, including Robertsonian fusions/fissions and pericentric inversions. This must have been preserved after the divergence between the South African and the south Tanzanian–Zambian clades. There is no available information at the molecular level on the mechanisms allowing this striking ability of the karyotype to undergo rearrangements of several kinds. This obviously is not a unique feature among rodents, but other similar phenomena characterize single and recent lineages, for example *Mus musculus domesticus* (see Capanna, 1982 for *Mus*; and King, 1993 for a general discussion). Second, the role and amount of fertility reduction needs to be tested in structural heterozygote hybrids. This is essential to evaluate if karyotype variation represents a mechanism *per se* favouring and promoting speciation (see King, 1993; Rieseberg, 2001) or if genetic incompatibilities only act in speciation events.

The occurrence in Somalia, i.e. in the north of the *mearnsi* range, of a karyotype that should be the ancestor of the *campestris* group, i.e. all telocentrics with high chromosomal number, cautiously suggests that the split between *campestris* and *mearnsi* may not have had a chromosomal basis.

On the other hand, the presence of extreme karyotype diversity from the ancestral one characterizing Ndaleta and South Africa would not exclude the possibility that, within the two major clades, chromosomal diversification had a role as a post-mating mechanism giving rise to chromosomal raciation or full speciation. For these reasons it is difficult to identify biological species boundaries both within the Zambezian and the South African clade.

Within the Zambezian clade, the proportion of nucleotide differences between the Mutanda–Muze and the Chunya–Kasanga groups varies between 1.2% and 5.2%. Furthermore, Mutanda and the two localities of the Chunya area show differences in the diploid and fundamental numbers (Table 1; Figs 3 & 4), although not comparable with those characterizing the southern part of the continent. However, it is impossible without proper experimental design on the fertility of structural heterozygote hybrids to ascertain whether the process of speciation is still in action or is completed.

The same is true in part for the South African clade, where the K2 distances and the proportion of nucleotide differences between the low and high diploid number forms are higher (Table 2), corresponding, according to Gordon (1986), to the absence of gene flow. According to the tree topologies (Figs 7 & 8), the low diploid number forms constitute a monophyletic group that is derived from a higher diploid number ancestor, therefore supporting a general idea of a direction of karyotype evolution towards reduction in diploid numbers.

All these lineages are recent, so that the occurrence of several karyotypic variants and the moderate levels of genetic differentiation would suggest a speciation process *in fieri* that is not yet completed.

Taxonomic resolution and limits

A comparison of adequate series of voucher and type specimens will prove to be necessary to assess correctly the taxonomic status of the populations investigated in the present publication.

A multivariate approach based upon cranial morphometric measurements, but with a relatively limited number of samples and type specimens (not shown), supports the division of the genus *Saccostomus* into '*campestris*' and '*mearnsi*' species groups. However, this analysis is still in progress and for the moment does not allow us to solve the taxonomic status of all the samples involved. It is equally important to stress that in view to the high chromosomal variability, adequate information on the karyotypes occurring at the type localities will be required before definitive species identification will become possible.

The following discussion is also based upon craniological comparisons of most of the *Saccostomus* type material. However, we were unable to include in our study the types of *S. lapidarius* Peters 1852, *S. fuscus* Peters 1852, *S. streeteri* Roberts 1914 and *S. limpopoensis* Roberts 1914.

S. mearnsi *species group*

The direct comparison of the Maasai Steppe specimens with the type specimens of *S. mearnsi* Heller 1910, *S. umbiventer* Miller 1910, *S. isiolae* Heller 1912, and *S. cricetulus* G. M. Allen and Lawrence 1936 has shown that, while undoubtedly belonging to the '*mearnsi*' species complex, these specimens are morphologically sufficiently differentiated to be considered to represent an undescribed new taxon.

The karyotype described from Afgoi (southern Somalia) could be representative for the typical *S. mearnsi*. However, since a voucher specimen for this individual is not available, we are unable to assess its taxonomic status.

S. campestris *species group*

The south Tanzanian specimens (Muze, Chunya and Kasanga) occur north (230 km) of Karonga, the locality on the northern shores of Lake Nyasa from which *S. elegans* Thomas 1897 has been described. It would therefore seem reasonable to ascribe for the moment all these populations to *S. elegans*, of which they could represent two subspecies. However, a craniological comparison of our specimens with the type of *S. elegans* (BMNH 97.18.1.207) and of two 'co-types' of *S. campestris* (BMNH 7.1.1.181 and 58.6.18.19) reveals that Chunya and Kasanga could also be allocated to typical *campestris*, whereas Muze and northern Zambian specimens resemble closely to the *elegans* type skull.

Concerning the South African populations, the low diploid numbers forms occurring in the south-west arid biotic zone suggest a monophyletic group (Figs 7 & 8). *S. anderssoni anderssoni* Winton 1898 has been described for Damaraland, later followed by *S. anderssoni hildae* Schwann 1906, from the locality of Kuruman, southern Bechuanaland, and then by *S. anderssoni pagei* Thomas and Hinton 1923 from northern Bechuanaland. All these localities fall well within the range of the low diploid numbers karyotypic forms, and for priority they should be ascribed to *S. anderssoni* Winton 1898. It should be reminded however, that *S. campestris* Peters 1846 has been described from the locality of Tette (Mozambique) and that Gordon (1986) has shown that in Zimbabwe there are high diploid karyotypic forms occurring in the south and low diploid numbers in the north (Tette being northern to the latter). Consequently, *S. mashonae* De Winton 1897, described from the locality of Mazoe (north of the Zambezi) and resembling craniologically rather well to typical *anderssoni*, could eventually also make part of this low diploid numbers species group, in which case *mashonae* would have taxonomic priority over the former. However, since there are no adequate data available covering properly the areas of Mozambique and Zimbabwe, the proposed taxonomy remains provisional.

The taxonomic attribution of the high diploid number South African savanna biome populations is even more difficult, as there is no clear evidence they constitute a natural group or a single species, either for the cytochrome *b* gene sequences as for their chromosome variability. Lalanek and Kruger are 140 and 70 km distant from Hector Spruit, Transvaal, the locality respectively for which *S. streeteri* Roberts 1914 has been described. Although specimens from these localities share the same diploid number, they are not characterized by the same FNa (Gordon, 1986). Furthermore, *S. fuscus* Peters 1852 (type locality Inhambane, Mozambique), of which we have not seen the type skull, has been described from this area and especially this taxon could play an important role in the taxonomic priority discussion. Unfortunately there is no karyological or craniometric information available to characterize the *Saccostomus* populations of this region so that it is presently impossible to clarify the taxonomical situation in the south eastern part of Africa.

CONCLUSIONS

For its antiquity, the genus *Saccostomus* constitutes within the African muroids a remarkable example of evolution throughout the major geological events that characterized the history of African savannas from the end of the Middle Miocene to the present. Speciation is still in progress as new species are currently being formed. Chromosomal evolution plays an important role, and constitutes the main challenge to understanding mechanisms acting during speciation. The scenario depicted, however, is intricate so that the taxonomy, which should reflect unambiguous natural units, remains in part still uncertain.

Acknowledgements

Several people have collaborated in field collections. Among these, Ernesto Capanna (La Sapienza, Rome), Rhodes Makundi (Sokoine University, Tanzania), Alfred Sichilima (Mutanda Res. Station, Zambia). Comments and suggestions on the manuscript were made by Paolo Colangelo (La Sapienza, Rome) and Erik Verheyen (Royal Belgian Institute for Natural Sciences, Brussels). To all of them goes our gratitude. This work has been supported by grants 'Diversità genetica e microevoluzione nei mammiferi' (Programmi d'Ateneo 60%) and 'STAPLERAT: Protecting staple crops in eastern Africa: integrated approaches for ecologically based field rodent pest management' (European Union).

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