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A new cytotype of the African pygmy mouse *Mus minutoides* in Eastern Africa. Implications for the evolution of sex-autosome translocations

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Abstract The African pygmy mice (genus *Mus*, subgenus *Nannomys*) are recognized for their highly conserved morphology but extensive chromosomal diversity, particularly involving sex-autosome translocations, one of the rarest chromosomal rearrangements among mammals. It has been shown that in the absence of unambiguous diagnostic morphological traits, sex-autosome translocations offer accurate taxonomic markers. For example, in *Mus minutoides*, irrespective of the diploid number (which ranges from 2n=18 to 34), all specimens possess the sex-autosome translocations (X.1) and (Y.1) that are unique to this species. In this study, we describe a new cytotype that challenges this view. Males are characterized by the translocation (Y.1) only, while females carry no sex-autosome translocation, the X chromosome being acrocentric. Hence, although sex-autosome translocations (X.1) and (Y.1) are still diagnostic when one or both are present, their absence does not rule out *M. minutoides*. This cytotype has a large distribution, with specimens found in Tanzania and in the eastern part of South Africa. The nonpervasive distribution of Rb(X.1) provides an opportunity to investigate different evolutionary scenarios of sex-autosome translocations using a phylogenetic framework and the distribution of telomeric repeats. The results tend to support a scenario involving a reversal event, i.e., fusion then fission of Rb(X.1), and highlighted the existence of a new $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system, confirming the remarkable diversity of neo-sex chromosomes and sex determination systems in the African pygmy mice.

KEYWORDS African pygmy mouse · *Mus minutoides* · Sex-autosome translocations · $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system · Neo-sex chromosomes

Abbreviations

AIC	Akaike information criterion
DAPI	4′,6-Diamino-2-phenylindole
DNA	Deoxyribonucleic acid
EMBL	European molecular biology laboratory

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FISH	Fluorescence in situ hybridization
GTR + G + I model	General-time reversible + Gamma + proportion invariant model
ML	Maximum likelihood
PCR	Polymerase chain reaction
Rb fusion	Robertsonian fusion

Introduction

The African pygmy mice are a group of very small-sized rodents (4–15 g) endemic to sub-Saharan Africa. They form a vast complex of morphologically similar species, constituting the most species-rich subgenus (*Nannomys*) of the genus *Mus* with about 18 recognized species (Happold 2013). In contrast to the species' highly conserved morphology, chromosomal studies have uncovered extensive karyotypic diversity within this group (e.g., Matthey 1966; Jotterand-Bellomo 1986, 1988; Castiglia et al. 2002, 2006; Veyrunes et al. 2004, 2007, 2010a; Britton-Davidian et al. 2012). The most frequently encountered rearrangements are Robertsonian (Rb) fusions between autosomes (consisting of the fusion by the centromere of two nonhomologous acrocentric autosomes that produces a single metacentric chromosome), but more unexpectedly, a large number of chromosomal changes also involve the sex chromosomes (Veyrunes et al. 2004, 2007, 2010b; Britton-Davidian et al. 2012). Among these, Rb fusions between an autosome and a sex chromosome, also called sex-autosome translocations, are a prominent feature of the African pygmy mice. Sex-autosome translocations are notable for generating important perturbations of gametogenesis and gene expression due to (i) conflicting replication requirements between the sex chromosome and the autosomal components, (ii) intrusion of autosomal material into the sex body with subsequent interference with X-inactivation, and finally, (iii) the spreading of X-inactivation into the adjacent autosome causing the silencing of the autosomal genes (e.g., White et al. 1998; Ashley 2002; Dobigny et al. 2004; Barasc et al. 2012). They are, thus, considered as one of the rarest and most deleterious chromosomal rearrangements in mammals, albeit, they are relatively common in the African pygmy mice. These mice are indeed the mammalian lineage with the greatest diversity of this type of rearrangement known so far (Veyrunes et al. 2004, 2007). Recently, the combination of cytogenetic and molecular phylogenetic analyses has shown that contrary to autosomal Rb fusions (which are prone to convergence), sex-autosome translocations are rare genomic changes and offer accurate diagnostic characters to discriminate the different species of African pygmy mice (Veyrunes et al. 2004, 2005). Hence, whereas *Mus minutoides* and *Mus musculoides* constitute a pair of sibling species and are often referred to as the species complex *M. minutoides*/*M. musculoides* in the literature, they can easily be discriminated by G-banded karyotypes since the first is characterized by the sex-autosome translocations Rb(X.1) and (Y.1), and the second by Rb(X.7). In the same manner, Rb(X.12) is specific to *Mus triton*, and Rb(X.15) and (Y.15) to *Mus oubanguii* (Veyrunes et al. 2004; Britton-Davidian et al. 2012). *M. minutoides* is of special interest since it harbors a particularly variable karyotype owing to the accumulation of Rb fusions. In South Africa for example, the two extremes of the karyotypic range are recorded, some populations having 2n=34, and others only 2n=18, an instance where all autosomes are involved in Rb fusions (Veyrunes et al. 2010a). All populations analyzed so far, spanning the large distribution range of this species (i.e., Ivory Coast, Guinea, Zambia, Kenya, Botswana, and South Africa), and irrespective of the diploid number, possess the diagnostic Rb(X.1) and (Y.1) sex-autosome translocations (Jotterand-Bellomo 1986; Castiglia et al. 2002, 2006; Kan Kouassi et al. 2008; Veyrunes et al. 2010a; McDonough et al. 2013). The sole exception is one South African population where the sex-autosome translocation Rb(X.16) is present in some specimens following a whole-arm reciprocal translocation (WART) between the chromosomes Rb(X.1) and Rb(13.16) (Veyrunes et al. 2007).

In this study, we describe a new cytotype of *M. minutoides* from Tanzania and South Africa that challenges this view and provides new insights into the evolution of sex-autosome translocations in this species.

Material and methods

Animals, chromosome preparation, and karyotype analyses

Four males and one female near Morogoro in Tanzania (S6 51.071 E37 38.378) and two males in the vicinity of Durban, South Africa (S29 54.342 E30 56.233) were lived-trapped and karyotyped. Chromosome

preparations were made from fibroblast cell cultures established from skin biopsies following standard procedures; identification of the chromosomes was performed by G-banding (Seabright 1971) or inverted DAPI-banding (equivalent to a G-banding; Bayani and Squire 2004), and karyotypes were reconstructed according to the nomenclature proposed by Jotterand-Bellomo (1986).

Since G-banding identification of the small autosome pairs (i.e., 16 and 17) is sometimes difficult when they are involved in rearrangements, we confirmed the chromosomal assignment by chromosome painting. For this, we hybridized the paints of the chromosomes 16 and 17 of *Mus indutus* (a pygmy mouse species with an ancestral-like karyotype, i.e., with no fusions) onto the metaphases of one specimen (male 2) according to the procedure described in Veyrunes et al. (2006).

In addition, a telomeric probe $(TTAGGG)_n$ was synthesized by PCR from total genomic DNA of *M. musculoides* using the primers and protocol described in Gilbert et al. (2008) and then labeled with biotin by nick-translation. Telomeric sequences were revealed by fluorescent in situ hybridization (FISH) following the procedure of Fischer et al. (2000) on metaphase preparations according to Henegariu et al. (2001). Images were captured using a Zeiss photomicroscope equipped with an image analyzer (Genus, Applied Imaging), and chromosomes were identified by inverted DAPI-banding.

Molecular phylogeny

As the African pygmy mice are a complex of morphologically very similar species often occurring in sympatry, the identification of the specimens as *M. minutoides* was confirmed by reconstructing a molecular phylogeny based on the sequences of the cytochrome *b* gene (see Britton-Davidian et al. 2012). Total genomic DNA was extracted from tissues preserved in ethanol using the DNeasy blood and tissue kit (QIAGEN). Complete mitochondrial cytochrome *b* sequences were amplified in all analyzed specimens from Tanzania and South Africa. Primers and PCR conditions are described in Veyrunes et al. (2010a). For one sample, we were not able to amplify the complete cytochrome *b* and we therefore sequenced a shorter fragment as described in Chevret et al. (2014). The new sequences of cytochrome *b* were deposited in GenBank. Additional sequences of African pygmy mice and of other *Mus* sp. used as outgroups were retrieved from GenBank and added to the alignment (accession numbers are indicated in Fig. 1). The molecular phylogeny was reconstructed by maximum likelihood (ML) and Bayesian approaches using, respectively, PhyML (Guindon and Gascuel 2003) and MrBayes v3.2 with three partitions corresponding to each codon position (Ronquist et al. 2012). The best-fit model of sequence evolution (TVM+I+G) was determined based on the corrected AIC score implemented in jmodeltest v2.1 (Darriba et al. 2012) and used for the ML analysis. As this model was not directly available in MrBayes, we set nst = mixed which allows the Markov chain to sample over the space of all possible reversible substitution models, including the TVM model, and rates = invgamma. The robustness of the nodes was estimated by 1,000 ML bootstrap replicates (BP) using PhyML, and by posterior probability (PP) using MrBayes with the following parameters: 10 million generations, 1 tree sampled every 500 generations, the burn-in = 10 % was determined with Tracer v1.6 (Rambaut and Drummond 2009), and we checked that the average SD of split frequencies remained <0.01 after the burn-in threshold.

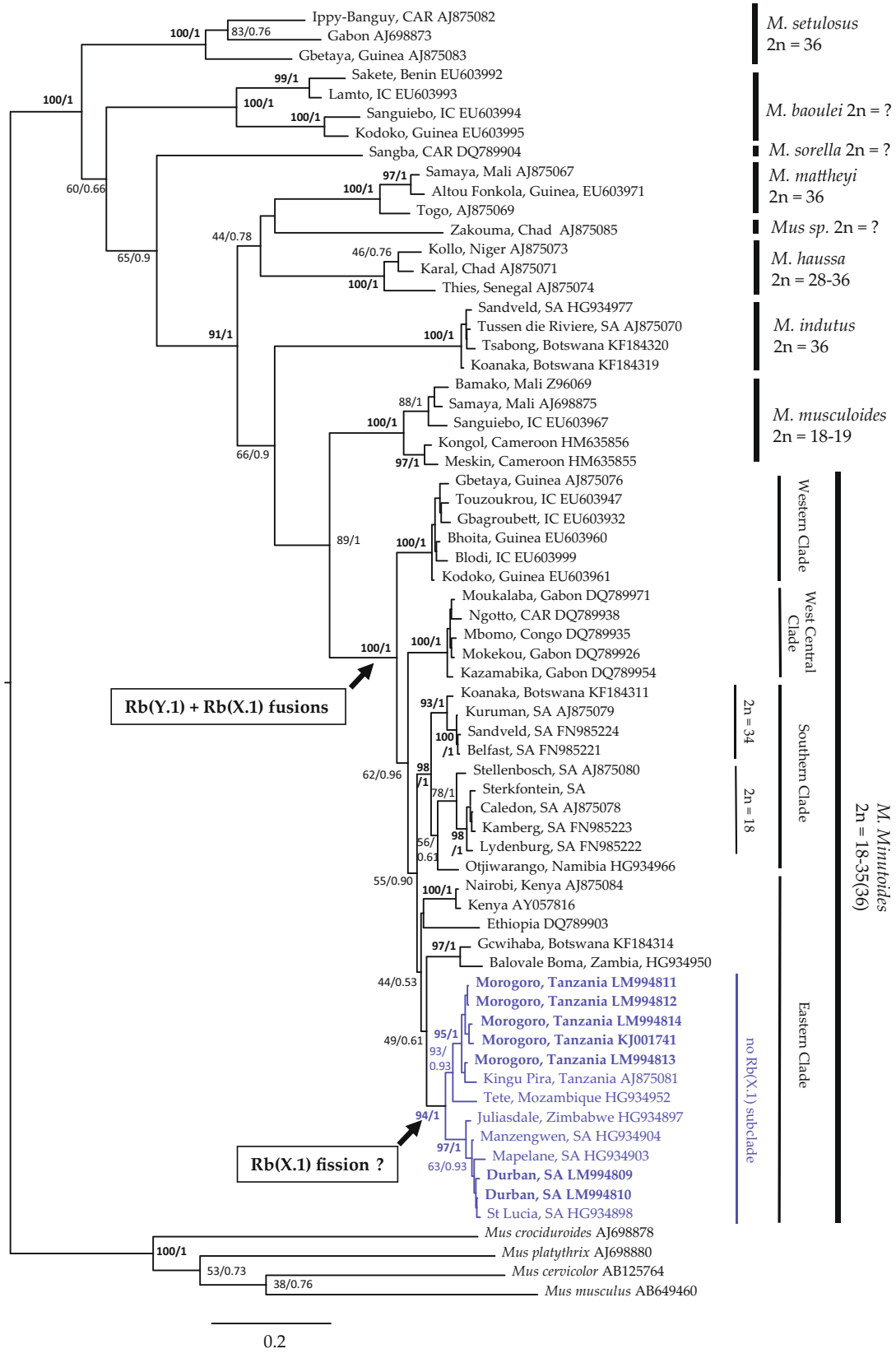
Results

Molecular phylogeny

The phylogenetic trees showed a very similar topology in both ML and Bayesian analyses (Fig. 1). The monophyly of each species is robust, but some relationships between them are unclear. In the same manner, strong support was found for the division of *M. minutoides* into at least four geographic clades but their relationships remain poorly supported. Nevertheless, the animals used in this study can be unambiguously referred to *M. minutoides* as the sequences of the analyzed specimens nest within the Eastern clade, and more precisely within a well-resolved subclade that clusters both specimens from Morogoro (Tanzania) and Durban (South Africa) and other animals from Tanzania, Mozambique, Zimbabwe, and the Eastern part of South Africa (BP= 94/PP=1, Fig. 1).

Karyotype analyses

Interestingly, the G-banded analysis revealed an identical karyotype between some specimens of South Africa


Ippy-Banguy, CAR AJ875082
Gabon AJ698873
Gbetaya, Guinea AJ875083
Sakete, Benin EU603992
Lamto, IC EU603993
Sanguiebo, IC EU603994
Kodoko, Guinea EU603995
Sangba, CAR DQ789904
Samaya, Mali AJ875067
Altou Fonkola, Guinea, EU603971
Togo, AJ875069
Zakouma, Chad AJ875085
Kollo, Niger AJ875073
Karal, Chad AJ875071
Thies, Senegal AJ875074
Sandveld, SA HG934977
Tussen die Riviere, SA AJ875070
Tsabong, Botswana KF184320
Koanaka, Botswana KF184319
Bamako, Mali Z96069
Samaya, Mali AJ698875
Sanguiebo, IC EU603967
Kongol, Cameroon HM635856
Meskin, Cameroon HM635855
Gbetaya, Guinea AJ875076
Touzoukrou, IC EU603947
Gbagroubett, IC EU603932
Bhoita, Guinea EU603960
Blodi, IC EU603999
Kodoko, Guinea EU603961
Moukalaba, Gabon DQ789971
Ngotto, CAR DQ789938
Mbomo, Congo DQ789935
Mokekou, Gabon DQ789926
Kazamabika, Gabon DQ789954
Koanaka, Botswana KF184311
Kuruman, SA AJ875079
Sandveld, SA FN985224
Belfast, SA FN985221
Stellenbosch, SA AJ875080
Sterkfontein, SA
Caledon, SA AJ875078
Kamberg, SA FN985223
Lydenburg, SA FN985222
Otjiwarango, Namibia HG934966
Nairobi, Kenya AJ875084
Kenya AY057816
Ethiopia DQ789903
Gcwihaba, Botswana KF184314
Balovale Boma, Zambia, HG934950
Morogoro, Tanzania LM994811
Morogoro, Tanzania LM994812
Morogoro, Tanzania LM994814
Morogoro, Tanzania KJ001741
Morogoro, Tanzania LM994813
Kingu Pira, Tanzania AJ875081
Tete, Mozambique HG934952
Juliasdale, Zimbabwe HG934897
Manzengwen, SA HG934904
Mapelane, SA HG934903
Durban, SA LM994809
Durban, SA LM994810
St Lucia, SA HG934898
Mus crociduroides AJ698878
Mus platythrix AJ698880
Mus cervicolor AB125764
Mus musculus AB649460
Rb(Y.1) + Rb(X.1) fusions
Rb(X.1) fission ?
0.2
M. setulosus 2n = 36
M. baoulei 2n = ?
M. sorella 2n = ?
M. mattheyi 2n = 36
Mus sp. 2n = ?
M. haussa 2n = 28-36
M. indutus 2n = 36
M. musculoides 2n = 18-19
Western Clade
West Central Clade
Southern Clade
Eastern Clade
2n = 34
2n = 18
no Rb(X.1) subclade
M. Minutoides 2n = 18-35(36)

◀ **Fig. 1** Molecular phylogeny inferred from Bayesian inference under a TVM+I+G model using cytochrome *b* sequences. Each specimen is designated by its precise locality (when known), its country of origin (*IC* Ivory Coast, *SA* South Africa, *RCA* Republic of Central Africa), and its accession number. Bootstrap percentage and the posterior probability (BP/PP) are indicated for the main nodes, they are in *bold* when BP>90 % and PP=1. The specimens in *bold* refer to the present study. The subclade in *blue* indicates the absence of Rb(X.1). 2n = diploid number

and Tanzania, whereas showed a notable chromosomal diversity within the five specimens from the Tanzanian locality. This diversity is due to autosomal Rb polymorphism and an atypical sex chromosome system, resulting in a diploid number that ranges from 2n=33 to 2n=35.

Male 1 from Tanzania and the two males from South Africa shared the same karyotype with a diploid number of 2n=35, all chromosomes were acrocentric except for Rb(Y.1) (Fig. 2a).

Male 2 from Tanzania had a diploid number of 2n=33, carrying the sex-autosome translocation Rb(Y.1) and the homozygous Rb fusion (7.16). All remaining autosomes and the X chromosome were acrocentric (Fig. 2b).

Males 3 and 4 from Tanzania possessed the same karyotype (2n=34), the only difference to the previous one being the autosomal Rb fusion in a heterozygous state.

Finally, the female from Tanzania also had a diploid number of 2n=35, with a heterozygous Rb(7.16), while both X chromosomes and all other autosomes were acrocentric (Fig. 2c).

The identification of Rb(7.16) was unambiguously confirmed by chromosome painting. The hybridization of the *M. indutus* chromosome 16 paint on metaphases on male 4 (heterozygous for the fusion) revealed signals on the small arm of the autosomal Rb fusion plus one acrocentric, while the paint 17 showed signals on the two smallest acrocentrics (Fig. 3).

Telomeric sequences were detected at the ends of all chromosomes of male 2. Extensive amplification of these sequences was observed in the pericentromeric regions, whereas distal telomeric signals were extremely faint (Fig. 4). In addition, strong interstitial telomeric signals (ITS) were detected in the pericentromeric region of the Rb(7.16), while they were faint in Rb(Y.1). Chromosomes X and 1 showed the same amount of telomeric signals as the other acrocentric autosomes (Fig. 4).

Discussion

The molecular phylogeny (Fig. 1) is consistent with previous phylogenetic analyses (e.g., Britton-Davidian et al. 2012; Chevret et al. 2014), and confirms the identification of the studied specimens as *M. minutoides* that nest within the recently described Eastern clade (Chevret et al. 2014).

M. minutoides is noteworthy for having a highly variable karyotype that ranges between 2n=34 and 2n=18, resulting from different numbers and combinations of autosomal Rb fusions, but all cytotypes share the sex-autosome fusions Rb(X.1) and (Y.1) (Matthey 1970; Veyrunes et al. 2004, 2010a). The specimens studied herein present karyotypes that are at odds; four animals have a 2n=35 that falls outside the *M. minutoides*' diploid number fan (Matthey 1970). In fact, the cytogenetic analyses revealed two notable traits in this new cytotype.

First is the presence of a polymorphic autosomal fusion within the population of Morogoro, Tanzania: Rb(7.16) is homozygous in male 2, heterozygous in males 3, 4, and the female, and absent in male 1. Karyotypic variability at the species level is not a rare feature in mammals and is well studied in two species models, the house mouse *M. musculus* and the common shrew *Sorex araneus* (Searle and Wojcik 1998; Pialek et al. 2005). However, karyotypic polymorphism at the population level is much rarer, and is often associated with chromosomal hybrid zones (Searle 1993). The mouse, the shrew, and the gerbil *Gerbillus nigeriae* provide some of the rare cases of true polymorphism (i.e., not related to a hybrid zone; Banaszek et al. 2009; Hima et al. 2011; Medarde et al. 2012). This scarcity can easily be explained by the meiotic defects and lowered fertility often associated with chromosomal heterozygosity (e.g., Gropp et al. 1982; King 1993; Chatti et al. 2005). Hence, the polymorphism of Rb(7.16) is noteworthy. Other cases of locally restricted Rb polymorphisms have already been reported in several species of African pygmy mice, *Mus haussa* in Mali; *M. minutoides* in Ivory Coast, Republic of Central Africa, and Zambia; and *M. triton* in Burundi (see Table 2 in Veyrunes et al. 2004). Finally, this is the first time that Rb(7.16) is identified in *M. minutoides*.

Second, this new cytotype is characterized by the sex-autosome translocation Rb(Y.1), while the X chromosome and the remaining chromosome 1 are acrocentric (Fig. 2). This configuration leads to a particular sex

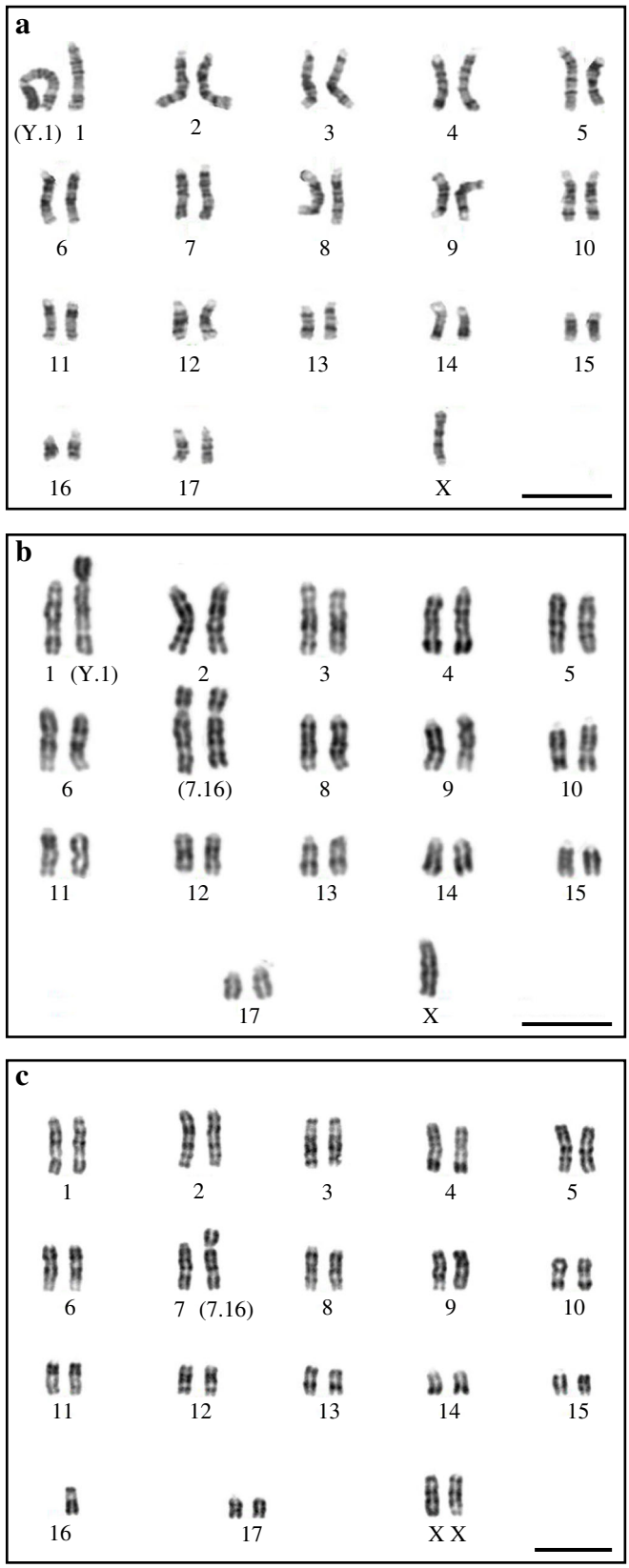


Fig. 2 G-banded karyotype of one male from South Africa with 2n=35 (**a**), inverted DAPI-banded karyotype (equivalent to a G-banding) of male 2 from Tanzania with 2n=33 (**b**), and of the female from Tanzania with 2n=35 (**c**). The *numbers* in parentheses indicate the chromosomes involved in the Rb fusions. *Scale bars*=10 μm

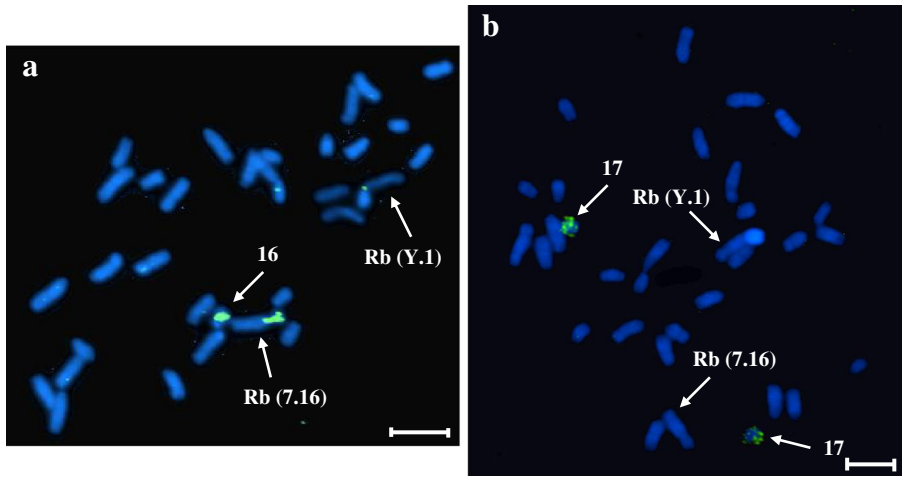


Fig. 3 Fluorescence in situ hybridization using a *Mus indutus* chromosome painting probe on a metaphase spread of male 4 (heterozygous for the autosomal Rb fusion): **a** with chromosome 16, **b** with chromosome 17. *Scale bar*=10 μm

chromosome system which is described by convention as $X_1X_1X_2X_2$ / X_1X_2Y, where X_1 is the original X chromosome and X_2 is the free homologue of the autosome involved in the Y-autosome translocation. Such a system is relatively rare and has been observed in a few groups of primates (e.g., *Alouatta* and *Aotus*), in mongooses (*Herpestes* and *Atilax*), bovids (*Tragelaphus* and *Taurotragus*), bats (*Mesophylla macconnelli*), the two-toed sloth (*Choloepus hoffmanni*), one marsupial (*Lagorchestes conspicillatus*), and very few species of rodents (*Deltamys kempi* and *Cryptomys hottentotus*) (Fredga 1970; White 1973; Deuve et al. 2008; Rubes et al. 2008; Yoshida and Kitano 2012). In fact, this may not be the first time that such a system is described in *M. minutoides*. In 1965, Matthey reported specimens from Harare, Zimbabwe with 2n=35 in males and 2n=36 in females, but curiously these data were not mentioned in subsequent publications (e.g., Matthey 1970). Although the figures shown in Matthey's publication are convincing, confirmation by unequivocal direct evidence, such as G-banding, is clearly required. More recently, Denys et al. (2011) described a male and a female *M. minutoides* from Kingu Pira, Tanzania with 2n=35 and 34, respectively, but no G-banded karyotypes were published. Vladimir Aniskine (pers. comm.) confirmed that the male had the sex-autosome translocation Rb(Y.1), but no (X.1), and that the reduction in diploid number of the female was due to autosomal Rb fusions, while the X chromosomes were acrocentric. These two reports and the data presented here suggest the widespread occurrence in Eastern Africa of a cytotype of *M. minutoides* characterized by Rb(Y.1), and an acrocentric X chromosome, i.e., not translocated to autosome 1. The cytochrome *b*-based phylogeny that we obtained (Fig. 1) strongly supports the chromosomal data. In effect, within the Eastern mitochondrial clade, the analyzed specimens from Tanzania and South Africa form a distinct subclade with the animals from Kingu Pira (Tanzania) and from Zimbabwe which present the

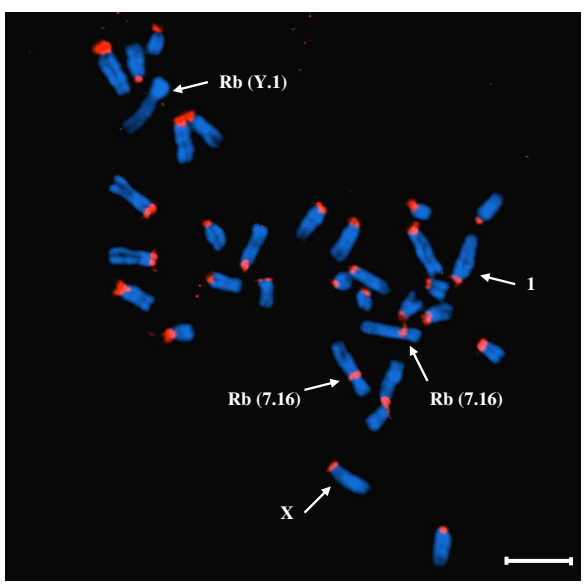


Fig. 4 Fluorescence in situ hybridization using a telomeric probe on a metaphase spread of male 2. Distal telomeric signals were extremely faint, and not even visible here because of the mode of capture of the image due to the bright intensity of the pericentromeric signals. *Scale bar*=10 μm

same $X_1X_1X_2X_2$ / X_1X_2Y sex chromosome system according to Denys et al. (2011) and Matthey (1965); this subclade excludes the animals from Kenya and Zambia that are geographically close but carry Rb(Y.1) and Rb(X.1) (Castiglia et al. 2002, 2006). We thus propose that *M. minutoides* is characterized by Rb(Y.1) only, in a vast distribution that extends from Tanzania to the Eastern shores of South Africa, including Mozambique and Zimbabwe. The females of this group do not have a sex-autosome translocation, and may even present an ancestral-like karyotype with only acrocentric chromosomes (2n=36). These results challenge our previous observations that suggested that the sex-autosome translocations Rb(X.1) and Rb(Y.1) are reliable characters for discriminating *M. minutoides* from other pygmy mouse species (Veyrunes et al. 2004, 2005). In fact, they remain diagnostic when one or both are present, but conversely, their absence does not rule out *M. minutoides*. This subclade is robustly supported and genetically well-differentiated and it is tempting to suggest that the absence of Rb(X.1) may have been crucial in accelerating this genetic divergence.

Sex-autosome fusions are excellent models for investigating the early steps of sex chromosome evolution. In effect, contrary to most of the X and Y chromosomes that are ancient and have lost most of the information on the processes that initiated their evolution (e.g., as in mammals, *Drosophila*, or ZW in birds), the autosome pairs recently involved in sex-autosome fusion are expected to progressively show neo-sex chromosome characteristics but still maintain the early traces of its differentiation (e.g., Charlesworth et al. 2005; Zhou and Bachtrog 2012). The presence of Rb(Y.1) and absence of Rb(X.1) in Eastern Africa offers a unique opportunity to investigate the sequential evolution of the neo-sex chromosomes in *M. minutoides*. The non-ubiquitous distribution of Rb(X.1) questions the order of appearance of the sex-autosome translocations; in other words, which of the X or the Y chromosome first fused to autosome 1? The observed pattern may be explained by two competing hypotheses: (i) Rb(Y.1) appeared first, followed by the subsequent fixation of Rb(X.1) in all populations except in part of Eastern Africa, (ii) both sex-autosome fusions were fixed, but a fission of Rb(X.1) occurred in this lineage. Both evolutionary scenarios can be investigated using a robust phylogenetic framework. The molecular tree suggests that the subclade in which Rb(X.1) is absent is not basal within *M. minutoides*, and thus tentatively supports the second scenario, i.e., fixation of both Rb(Y.1) and Rb(X.1) at the onset of the diversification of *M. minutoides* followed by an Rb(X.1) fission event in the Eastern subclade (Fig. 1). Unfortunately, the interclade relationships remain too poorly resolved to provide a conclusive argument for this hypothesis (see also Chevret et al. 2014). New genetic markers (nuclear sequences) are thus required to provide an accurate fine-scale phylogeny. In order to confirm the second hypothesis, i.e., fission of Rb(X.1), an alternative approach was performed using the distribution of telomeric repeats, since interstitial telomeric signal (ITS) often retain traces of past chromosomal rearrangements, particularly in the case of fission/fusion events (e.g., Ruiz-Herrera et al. 2008). First, the fluorescence in situ hybridization of a telomeric probe onto the metaphases of male 2 showed an extreme amplification of the telomere repeats in the pericentromeric regions of all chromosomes (Fig. 4). Such strong pericentromeric signals have already been observed in some mammal species where they often co-localize with constitutive heterochromatin or ribosomal DNA repeats and may be related to a component of satellite DNA (Garagna et al. 1997; Pagnozzi et al. 2000; Ventura et al. 2006; Zhdanova et al. 2007; Rovatsos et al. 2011). More importantly, strong ITS were present in the pericentromeric region of the Rb(7.16) while they were not detected on Rb(Y.1). Castiglia et al. (2002, 2006) previously reported ITS on autosomal Rb fusions, but also on Rb(X.1) and Rb(Y.1) in *M. minutoides* from Zambia and Kenya. The presence of ITS suggests that the mechanism of Rb fusion occurring within African pygmy mice is different from the one of the house mouse *Mus musculus domesticus* which involves the complete loss of the proximal telomeric sequences (Garagna et al. 1995; Nanda et al. 1995). As a result, Rb fissions are highly unlikely in the house mouse, as they would require the de novo formation of telomeres. In *M. minutoides*, however, the persistence of ITS in the pericentromeric region of metacentrics would increase the probability of fission and thus provide support for the Rb(X.1) fission scenario. Furthermore, several authors have proposed that ITS erode with time: most recent Rb fusions would retain their ITS, while the oldest would have lost them (Fagundes and Yonenaga-Yassuda 1998; Zhdanova et al. 2005). In agreement with this hypothesis, Rb(Y.1), which is one of the first chromosomal rearrangements to be fixed in the *M. minutoides* lineage (Veyrunes et al. 2010a), is depleted of ITS, whereas Rb(7.16), which is specific to this population, has strong ITS (Fig. 4). Therefore, if Rb(X.1) appeared around the same time as

Rb(Y.1) and subsequently underwent a fission in this lineage, we would expect a weak telomeric signal in the pericentromeric region of the acrocentric X and pair 1. This is, however, not the case, as these chromosomes showed the same strong signals as the other autosomes (Fig. 4), thus weakening the hypothesis of a past fission event. Still, we could not exclude the possibility that subsequent nonhomologous recombination occurred in the pericentromeric region of the acrocentrics leading to sequence homogenization and a secondary large amplification of ITS (e.g., Brannan et al. 2001; Linardopoulou et al. 2005; Cazaux et al. 2011).

In summary therefore, this study highlights a new cytotype of interest for further research, and more specifically for investigating the role of the selection in the formation of neo-sex chromosomes (Charlesworth and Charlesworth 1980; Yoshida and Kitano 2012), and conversely the role of the neo-sex chromosomes in speciation processes (Veltsos et al. 2008; Kitano et al. 2009). For such studies, an accurate stepwise model for neo-sex chromosome evolution is required, and our results support a scenario involving a reversal event, i.e., fusion then fission of Rb(X.1), an hypothesis that needs further corroboration. Furthermore, we reaffirm that African pygmy mice are remarkable for their diversity of neo-sex chromosomes and sex chromosome systems (Veyrunes et al. 2007, 2010b). For instance, besides the conventional XX / XY system, X-autosome and Y-autosome translocations have given rise, respectively, to XX / XY_1Y_2 in *M. musculoides* (Veyrunes et al. 2004) and the $X_1X_1X_2X_2$ / X_1X_2Y system described herein in *M. minutoides*. Novel sex determining mechanisms have emerged as well, with the fascinating XO / XO in *M. triton* where sex determination occurs in the absence of a Y chromosome (Jotterand-Bellomo 1988), and the recently described XX, XX*, X*Y / XY system in *M. minutoides* in which a large proportion of females are sex-reversed due to an X-linked mutation (Veyrunes et al. 2010b).

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