Chromosomal and molecular characterization of *Aethomys kaiseri* from Zambia and *Aethomys chrysophilus* from Tanzania (Rodentia, Muridae)

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Aethomys is a common and widespread rodent genus in the African savannas and grasslands. However, its systematics and taxonomy are still unclear as no study has covered the entire range. In fact it might not be a monophyletic genus and perhaps should be split into two subgenera, *Micaelamys* and *Aethomys*. In this paper, we present findings based on the cytogenetics and the entire cytochrome b sequence of two species from Zambia (*A. kaiseri*) and Tanzania (*A. chrysophilus*), and we compare them with the sequences of a South African species (*A. namaquensis*) and other allied muroid genera. Comparison of the banded chromosomes revealed complete G-band homology between the autosomes of the two species. However, the X and Y chromosomes clearly differ in size and in C- and G-banding, being much larger in *A. kaiseri*.

Comparison of the cytochrome b sequences places the separation between *A. kaiseri* and *A. chrysophilus* at 4.49 Mya, a period of intense speciation in other African muroids. The resulting phylogeny strongly supports the idea of a paraphyletic group, suggesting the need to elevate the previously described subgenera to the genus rank.

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The genus *Aethomys* Thomas, 1915 (Rodentia, Muridae) is a typical example of an African rodent with a wide distribution but unclear systematics and taxonomy. This is mainly due to the history of European settlement of Africa and the scattered and differential collecting during the first part of the twentieth century. The use of cytogenetics and molecular genetics has brought new insight in the last 15 years and has led to renewed interest in several African rodent species groups (*Arvicanthis*; DUCROZ et al. 1997; CASTIGLIA et al. 2003; VOLOBOUEV et al. 2002).

Aethomys is traditionally divided into two subgenera (DAVIS 1975): *Micaelamys*, including *A. namaquensis* and *A. granti*, and *Aethomys*, with nine recognised species (VISSER and ROBINSON 1986; MUSSER and CARLETON 1993). The two subgenera were originally distinguished on the basis of tail length and the colour of ventral parts (DAVIS 1975), and this subdivision has been confirmed by cytogenetic and molecular analyses (see later).

Chromosomal studies (MATTHEY 1954; VISSER and ROBINSON 1986; BAKER et al. 1988) have shown that the karyotypes of the "*Micaelamys*" species *A. namaquensis* ($2n = 24$) (from southeastern Zambia to South Africa) and *A. granti* ($2n = 32$) (Cape Province) are different from those of the other species studied, all characterized by higher diploid numbers ($2n = 44–50$). The divergence is due to several complex rearrangements including tandem fusions, paracentric inversions and Robertsonian fusions (BAKER et al. 1988).

Recent molecular analyses based on cytochrome b sequences (DUCROZ et al. 2001; RUSSO et al. 2001) confirmed the high genetic divergence between the two subgenera (17–18 % sequence divergence) and provided evidence for paraphyly of the genus within a wider phylogenetic context involving several other African species of Murinae and Otomyinae (DUCROZ et al. 2001).

Furthermore, there is evidence of cryptic species. For example, *A. chrysophilus*, widely distributed from Kenya to South Africa, has been split into two species with different cytotypes (GORDON and RAUTENBACH 1980; VISSER and ROBINSON 1986): the true *A. chrysophilus* ($2n = 50$) and *A. ineptus* ($2n = 44$), both occurring sympatrically. These species also dif-

Table 1. *Species, country, locality with latitude and longitude, the number of specimens examined through karyotyping and through the analysis of the entire cytochrome b sequence, with the EMBL accession number.*

Species	Country	Locality	Latitude and longitude	Karyotype	Cyt- b	EMBL code
A. chrysophilus	Tanzania	Matongolo	05°46′ S–36°28′ E	3♀	–	
	Tanzania	Zoissa	05°40′ S–36°25′ E	1♀	1♀	AJ604525
	Tanzania	Ndaleta	05°14′ S–36°29′ E	1♂, 1♀	–	
	Tanzania	Mombo	04°54′ S–38°13′ E	1♀	–	
	Tanzania	Ngasumet	04°31′ S–37°12′ E	6♀, 5♂	2♀	AJ604523-24
	Tanzania	Kisiwani	04°07′ S–37°57′ E	–	1♂	AJ604521
	Tanzania	Lwami	03°41′ S–37°32′ E	–	1♀	AJ604522
	Tanzania	Idodi	07°46′ S–35°10′ E	1♂	1♂	AJ604516
	Tanzania	Tabora-Inala	05°25′ S–32°49′ E	2♂, 1♀	1♀, 1♂	AJ604517-18
	Tanzania	Chunya	08°36′ S–35°05′E	4♀, 2♂	2♂	AJ604515-26
A. kaiseri	Zambia	Meheba, Solwezi	12°22′ S–26°16′E	2♂	3♂	AJ604514-19-20

fer in gross sperm and bacular morphology (VISSER and ROBINSON 1986; BREED et al. 1988), as well as quantitative cranial morphology (CHIMIMBA 1998, 1998; CHIMIMBA et al. 1999).

Any additional data from the northernmost portion of the range of *Aethomys* would help clarify the systematic relationships and taxonomy of its species. Here we present a cytogenetic (C- and G-banding) and molecular (cytochrome b sequence) characterization of *A. kaiseri*, a species from northern Zambia, Malawi, northern Angola and Zaire, and of populations of *A. chrysophilus* from Tanzania, representing the extreme northern distribution of the species. This is also the first description of the banded karyotype of *A. kaiseri*. A brief description of the Giemsa-stained karyotype of the Tanzanian *A. chrysophilus* was reported by FADDA et al. (2001) but the karyotype banding is unknown for the region. Finally, we compare our results with the molecular data for other species and genera of African rodents forming the monophyletic group including *Aethomys* (DUCROZ et al. 2001).

MATERIAL AND METHODS

Specimens and chromosome preparations

Thirty specimens (13 males, 17 females) were collected from one locality in Zambia and seven localities in Tanzania (Table 1 and Appendix). They were live-trapped and transported for karyotyping to the Rodent Research Project, Sokoine University of Agriculture, Morogoro (Tanzania) and to the Mutanda Agricultural Research Station (Zambia). Chromosome preparations were obtained from bone marrow following HSU and PATTON (1969).

Fixed cell suspensions were transported to the University of Rome where slides were prepared according to the standard air-drying technique. G-bands were enhanced with trypsin following the protocol of SEABRIGHT (1971). The heterochromatic portion of the genome was identified by C-banding using 5 % barium hydroxide (SUMNER 1972).

DNA methods

Ten specimens from seven localities in Tanzania and three specimens from one locality in Zambia were used (Table 1). Total genomic DNA was obtained from muscle preserved in 80 % ethanol. DNA extraction was performed using the Qiagen method (DNeasy tissue kit). Mitochondrial sequences containing the 1.140 bp fragment encompassing the entire cytochrome b sequence were isolated by polymerase chain reaction (PCR). Amplified DNA was obtained by combining the primers L14723 (5′-ACCAATGACATGAAAAATCATCGTT-3′) and H15915 (5′-TCTCCATTTCTGGTTTACAAGAC-3′). Amplifications were performed in an MJ MiniCycler machine by the following procedure: a first cycle of an initial denaturation at 94°C for 2 min, then 35 cycles with denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. These steps were followed by a 10 min extension at 72°C. Each PCR reaction used 50–500 ng of template DNA, 200 ng of each primer, 0.2 mM of each dNTP and 2 U of Taq polymerase (Promega) in a final volume of 50 µl. Amplified products were purified using the Qiagen QIAquik purification kit and prepared for automated sequencing with the same primers used for amplification.

To assess the genetic affinities and the phylogenetic position of the Zambian and Tanzanian species of *Aethomys* we used the published sequences by DUCROZ et al. (2001) of *A. chrysophilus* (Berega, Tanzania; EMBL-AF004587) and *A. namaquensis* (Itala, South Africa; EMBL-AF141215). The analysis by DUCROZ et al. (2001) strongly suggested that the genus was paraphyletic. Therefore, we included the sequences of species belonging to different genera of

African Murinae found by DUCROZ et al. (2001) to fall in the same monophyletic clade as *A. namaquensis* and *A. chrysophilus*: *Arvicanthis niloticus* (EMBL-AF004572), *Arvicanthis somalicus* (EMBL-AF004574), *Grammomys dolichurus* (EMBL-AF141218), *Hybomys univittatus* (EMBL-AF141219), *Otomys irroratus* (EMBL-AF141222), *Otomys sloggetti* (EMBL-AF141223), *Parotomys brantsi* (EMBL-AF141224), *Parotomys littledalei* (EMBL-AF141225), *Dasymys rufulus* (EMBL-AF141216), *Dasymys incomtus* (EMBL-AF141217), *Mylomys dybowskii* (EMBL-AF141212), *Pelomys campanae* (EMBL-AF141213), *Rhabdomys pumilio* (EMBL-AF141214), *Desmomys harringtoni* (EMBL-141206), *Lemniscomys striatus* (EMBL-AF141211), *Lemniscomys macculus* (EMBL-AF141208). *Rattus norvegicus* (EMBL-MIRNXX) and *Mus musculus* (EMBL-MITOMM) were used as outgroup.

Genetic divergence levels were evaluated by estimating the genetic distances with the Kimura two-parameter model (KIMURA 1980). Saturation analysis was performed by plotting the uncorrected distances versus the absolute number of pairwise differences for transitions (Ti) and transversions (Tv) at all three codon positions.

Maximum parsimony (MP), maximum likelihood (ML) and neighbor-joining (NJ) methods were used to construct phylogenetic relationships. MP trees were obtained with Paup 4.0b10 using a heuristic search and tree-bisection-reconnection (TBR) and random addition of sequences (10 replicates). A weighting scheme was adopted to correct for possible substitution saturation, according to the results of saturation analysis.

NJ trees were obtained using Kimura distances (K2P) in the MEGA program (version 2.1; KUMAR et al. 1993).

ML trees were reconstructed using the GTR + G + I model in Paup 4.0b10. The appropriate model of substitution was chosen using the program Model Test 3.04 (POSADA and CRANDALL 1998).

The robustness of the nodes was assessed by bootstrap with 500 replicates for MP and NJ and 100 replicates for ML.

Divergence times between *A. chrysophilus* and *A. kaiseri* and between the subgenus *Aethomys* and other African Murinae were estimated. The molecular clock hypothesis was tested with a relative rate test for transversions at the 3rd codon position using the program Phyltest 2.0 (KUMAR 1996). The dichotomy between the Mus and Rattus lineages (12 Mya) was used to calibrate the divergence time (JAEGER et al. 1986).

RESULTS

Cytogenetics

Aethomys kaiseri. 2n = 50, NFa = 60, X submetacentric, Y submetacentric.

The karyotype consists of nineteen pairs of telocentric chromosomes of decreasing size and five pairs of small meta/submetacentrics (Fig. 1). The sex chromosomes are very large and biarmed, the Y being slightly larger than the X. The length of each sex chromosome is approximately double the length of autosome number 1 (Fig. 2).

The C-banding pattern revealed a weak presence of heterochromatin in the autosomes (not shown). The short arm of the X chromosome is entirely heterochromatic but in some plates a more intensely stained band is visible at the centromere (Fig. 2). The Y chromosome is entirely heterochromatic but in some plates the distal regions of both the short and long arms and the centromere are more intensely stained. The different intensity of C-bands suggests heterogeneous heterochromatin, also shown by the occurrence of discrete G-bands at the heterochromatic region of the sex chromosomes (Fig. 2).

Aethomys chrysophilus. 2n = 50, NFa = 60, X telocentric, Y submetacentric.

The autosomal complement is identical to that of *A. kaiseri* for all specimens analysed. The X chromosome is a large telocentric, but smaller than autosome number 1. The Y chromosome is a medium-sized submetacentric (Fig. 2 and 3).

The C-banding pattern revealed conspicuous blocks of heterochromatin at the centromere of all autosomes (not shown). The X chromosome cannot be identified after C-banding. The Y chromosome is

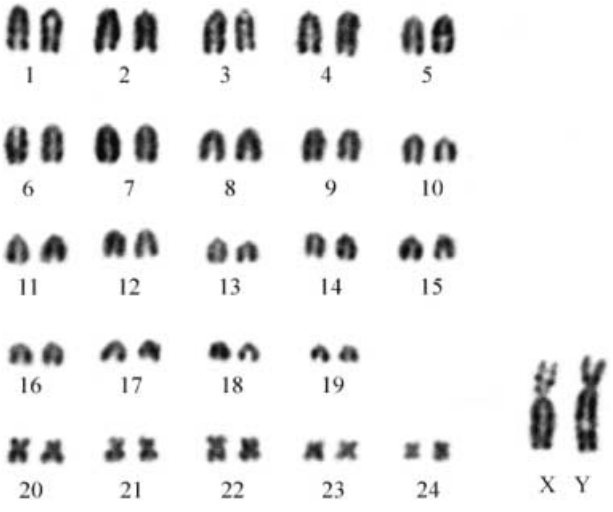

Fig. 1. Giemsa-stained karyotype of *A. kaiseri* (male).

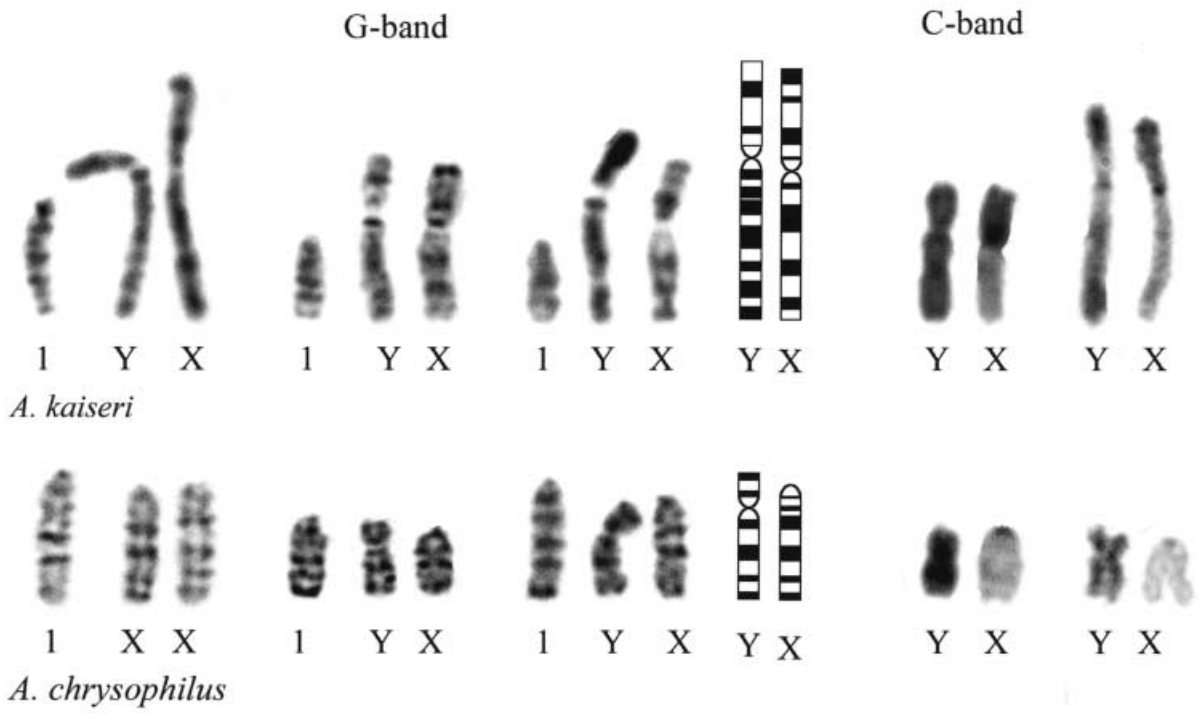

Fig. 2. Sex chromosomes and autosome number 1 from three G-banded metaphases and two C-banded metaphases of *A. chrysophilus* and *A. kaiseri*. A schematic representation of the G-banded sex chromosomes of the two species is also shown.

entirely heterochromatic with heterogeneous heterochromatin, as shown by the presence of a discrete G-banding pattern along the heterochromatic arm (Fig. 2).

G-banding comparison

Comparison of the G-banding pattern revealed complete homosequential G-band homology between the autosomes of the two species (Fig. 3). However, the X and Y chromosomes clearly differ in size and in G-banding (Fig. 2 and 3); in fact no homologous areas were observed. The small euchromatic X chromosome of *A. chrysophilus* can be considered as ancestral because the banding pattern is similar to the one observed in the euchromatic portion of the X chromosome in other genera of African Murinae, such as *Arvicanthis dembeensis* and *Lemniscomys rosalia* (selected X chromosomes of these two species are shown in the insert in Fig. 3).

Cytochrome b sequence divergence and phylogenetic relationships

Of the 1140 base pairs, 512 are variable (44.91 %) and 400 are parsimony informative (35.09 %); of these, 77 are at the 1st codon position, 24 at the 2nd codon position and 299 at the 3rd codon position.

Mean genetic distances among the three species range from 14.4 % to 19.1 %. The genetic divergence within *A. chrysophilus* ranges from 0.3 % to 4.5 %, with the highest divergence between the specimens from Chunya and Tabora. Among the three specimens of *A. kaiseri*, all from the same locality (Mutanda research station, Zambia), the genetic divergence ranges from 0.2 % to 1 %.

Saturation analysis showed slight saturation for transitional changes at the 3rd base position. Thus we adopted a weighting scheme in the MP analysis, downweighting transitions at the 3rd codon position (Ti:Tv = 1:10) to correct for the observed substitution saturation.

The MP and ML trees have similar topologies, with *A. kaiseri* and *A. chrysophilus* forming a monophyletic clade. For this reason, we show only the MP tree in Fig. 4a. *Grammomys dolichurus* is closest to this clade and all the arvicanthine rodents are in a monophyletic clade. The only difference is the position of *A. namaquensis*: more basal and close to *Hybomys univittatus* in the MP tree, closer to the Otomyinae clade in the ML tree. The NJ tree differs from the ML and MP trees (Fig. 4b) since the arvicanthine rodents (*Lemniscomys*, *Arvicanthis*, *Mylomys*, *Pelomys*, *Desmomys* and *Rhabdomys*) are in a paraphyletic unit, with *A. namaquensis* close to *Lemniscomys*; moreover, *D. rufulus* and *D. incomtus* cluster together with *H. univittatus* in the more basal clade.

The bootstrap values supporting relationships among the different Murinae genera are very low in

all the phylogenies. This makes it difficult to identify the topology that best explains the phylogenetic relationships among the *Aethomys* species included in this analysis. Nonetheless, all the phylogenetic analyses clearly show that the genus *Aethomys* is not monophyletic, confirming the previous findings by DUCROZ et al. (2001). *A. chrysophilus* and *A. kaiseri* always represent a monophyletic group, with *G. dolichurus* forming the sister genus. The unstable position of *A. namaquensis* could be due to long-branch attraction; this could probably be avoided by including more samples of the same species in the analysis, allowing better resolution for this species.

The monophyly of *A. chrysophilus* is highly supported by bootstrap values (> 99 %) and the clustering of the specimens is geographically related. There are two clades supported by high bootstrap values, one including the samples from southern Tanzania (Chunya, near the border with Zambia) and another including the specimens from the western and northern regions of Tanzania.

The relative rate test did not reject the hypothesis of a constant substitution rate among the different lineages. Therefore, we attempted to estimate the time of divergence between *A. kaiseri* and *A. chrysophilus* and to date the origin of the subgenus *Aethomys*. *M. musculus* and *R. norvegicus* differ for 71 transversions at the 3rd codon position; assuming a divergence of 12 Myr between the two lineages (JAEGER et al. 1986), we estimated a rate of divergence of 1.56 % per Myr, very close to the rates reported by other authors (DUCROZ et al. 1998; MARTIN et al. 2000; LECOMPTE et al. 2002). The estimated divergence between *A. kaiseri* and *A. chrysophilus* is 4.49 ± 0.77 Myr while the estimated divergence between the subgenus *Aethomys* and *G. dolichurus* is 8.08 ± 0.96 Myr.

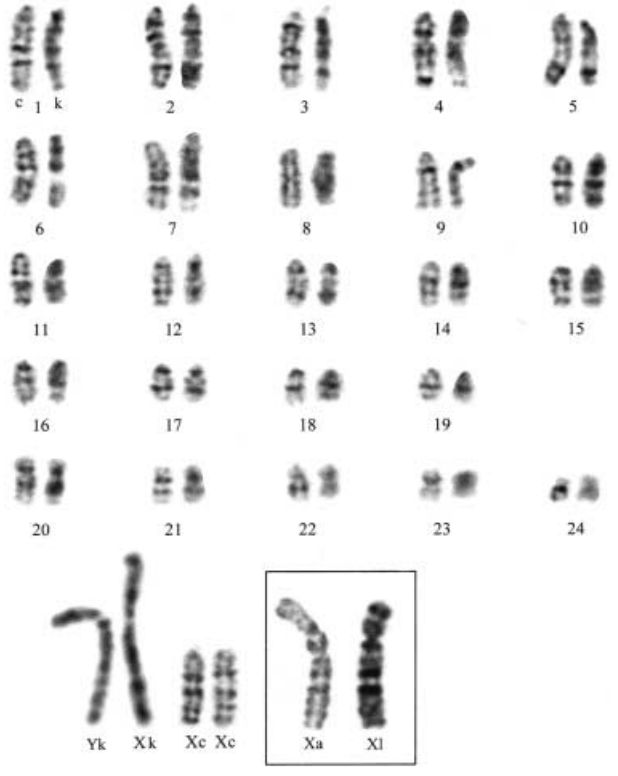

Fig. 3. Side by side comparison of the complete G-banded karyotypes of an *A. chrysophilus* female (c-left) and *A. kaiseri* male (k-right). In the insert, the G-banded X chromosomes of *Arvicanthis dembeensis* and *Lemniscomys rosalia* are shown for comparison.

DISCUSSION

There are two main points of discussion: the constancy of the autosomal set and the homologous G-banding sequence vs the striking heterochromosomal differences; the systematic definition of the taxa.

The diploid number ($2n = 50$) of *A. kaiseri* and *A. chrysophilus* is also shared by *A. bocagei* (MATTHEY 1954; VISSER and ROBINSON 1986), suggesting that this is the ancestral chromosomal number for the subgenus *Aethomys*. Furthermore, the G- and C-banding patterns of *A. chrysophilus* from Tanzania are identical to those described by VISSER and ROBINSON (1986) from South Africa, confirming the specific attribution of the Tanzanian specimens to the true *A. chrysophilus*, while the "cryptic species" *A. ignitus* should be restricted to South Africa.

The complete autosomal homology between *A. kaiseri* and *A. chrysophilus* strongly contrasts with the significant differences in size and constitution of the sex chromosomes, which are much larger in the former (Fig. 1 and 2). This magnification has two components: one is the occurrence of large heterochromatin blocks in both sex chromosomes of *A. kaiseri*; the other is unidentified rearrangements in the euchromatic portion of the X. The X chromosome of *A. chrysophilus* is similar in size and G-banding to the euchromatic portion of the X of many other Muridae, such as *Arvicanthis* (CORTI et al. 1996), *Lemniscomys* (CASTIGLIA et al. 2002) and *Dasymys* (VOLOBOUEV et al. 2000). In contrast, it appears to be different in *A. kaiseri* and consequently can be considered a derived condition. Such differences in sex chromosome size have also been observed among the South African species *A. ineptus*, *A. namaquensis* and *A. granti* (VISSER and ROBINSON 1986) but a precise comparative description of the rearrangements is not yet possible.

Variation of heterochromatin content and distribution in sex chromosomes is widespread in Muridae, representing a component of intra- and interspecific

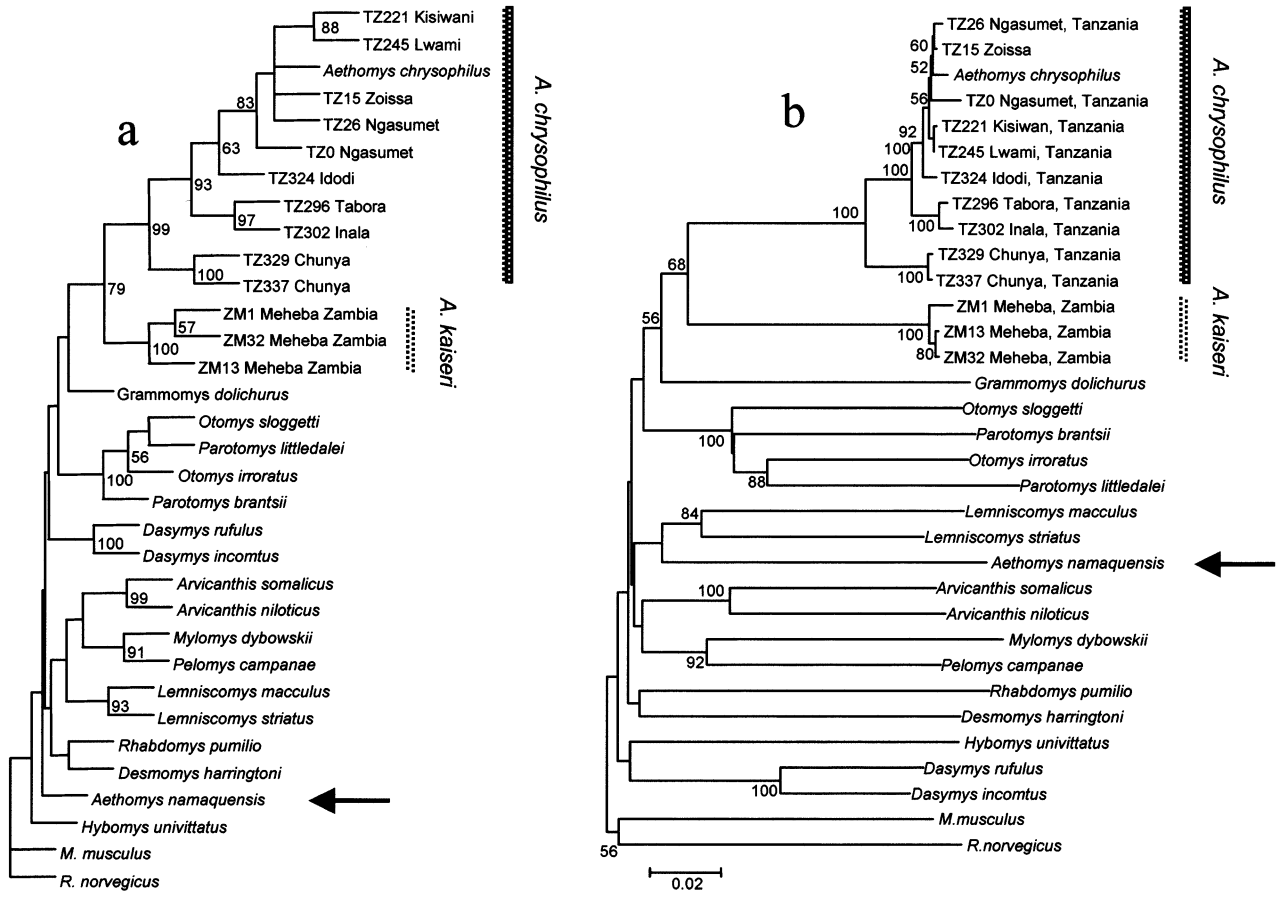

Fig. 4. Maximum parsimony (a) and Neighbor-joining (b) trees showing the phylogenetic relationships among *Aethomys kaiseri*, *Aethomys chrysophilus* and *A. namaquensis* (arrows). The sequences of several species belonging to various genera of African Murinae published by DUCROZ et al. (2001) were included in the analysis. See text for explanation.

variation for many African species (CIVITELLI et al. 1995; CORTI et al. 1996; VOLOBOUEV et al. 2000; CASTIGLIA et al. 2002). Nevertheless, differences in sex chromosome size between congeneric species seldom reach the magnitude reported here: the X and Y of *A. kaiseri* are twice the size of the X and Y of *A. chrysophilus*. Similar sex chromosome differentiation has been found in *Microtus agrestis*, which has accumulated large amounts of constitutive heterochromatin with respect to congeneric species in a short evolutionary period (less than 1 Myr) (KALSCHEUER et al. 1996). Although the nature of heterochromatin and the molecular mechanism of its addition are known in some cases (SINGH et al. 2000), the functional role of constitutive heterochromatin in shaping or regulating gene expression remains unclear (IVANOV and MODI 1996).

The euchromatic part of the X chromosome is conservative among mammalian species as a consequence of sex chromosome dosage compensation (OHNO 1967). NESTEROVA et al. (1998) found rearrangements in the euchromatic portion of the X in a comparative analysis of five species of *Microtus*, but they also identified certain homologous blocks with a constant G-banding pattern in all species studied.

One of the most conservative regions is the *Xic*, marked by the *Xist* gene, the regulator of X inactivation (PENNY et al. 1996). We are not able to accurately describe the changes in the euchromatic part of the X chromosome of *A. kaiseri*, but a simple paracentric inversion is not sufficient to explain the increase of the euchromatic portion. Therefore, other unidentified rearrangements must have occurred. This is an interesting case, given the high conservativism of the euchromatic portion of the X in most rodents.

The phylogenetic relationships (Fig. 4) confirm the inclusion of *A. kaiseri* and *A. chrysophilus* in the subgenus *Aethomys* and support the attribution of *A. namaquensis* to the subgenus *Micaelamys* (RUSSO et al. 2001; DUCROZ et al. 2001).

The genetic divergence between *A. kaiseri* and *A. chrysophilus* is in the upper part of the range of values for other pairs of congeneric rodent species (BRADLEY and BAKER 2001; DUCROZ et al. 2001).

The estimated value of 4.49 Myr is similar to those found between other congeneric species of African muroids, e.g. in *Arvicanthis* (DUCROZ et al. 2001) and *Mastomys* (LECOMPTE et al. 2002). These data suggest that the split between *A. kaiseri* and *A. chrysophilus* occurred during the Late Miocene and Early-Middle Pliocene. Indeed, paleontological data show that several new species appeared in various Murinae lineages in this period (DENIS 1999). The appearance of new taxa in the eastern Africa fauna was influenced by the emergence of the Rift Valley during the Late Miocene and Pliocene, resulting in geographic barriers and habitat fragmentation (DENIS 1999). The origin of the subgenus *Aethomys* can be estimated at about 8 Myr, in agreement with DUCROZ et al. (2001) who placed the origin of several other genera of African Murinae in this period (*Grammomys*, *Dasymys*, *Hybomys* and *Aethomys*).

The distributions of the two clades found in *A. chrysophilus* correspond to two areas with different vegetation and ecological conditions (temperature, rainfall, etc.): the Zambesian area, from southern Tanzania to southern Africa, and the Somali-Masai area, from Ethiopia to central Tanzania (WHITE 1983).

In conclusion, our cytogenetic and genetic analyses confirm the high divergence between the two subgenera and the attribution of *Aethomys kaiseri* to the nominate subgenus. The species have a contrasting pattern of chromosomal diversity, with conspicuous autosomal diversification between "cryptic species" in South Africa and between representatives of the two subgenera. Notwithstanding the high genetic divergence between *A. kaiseri* and *A. chrysophilus*, the autosomes appear to be conservative, in contrast to the strong sex chromosome differentiation.

Autosomal rearrangement is believed to act as a post-mating barrier leading to speciation in many cases (KING 1993; RIESEBERG 2001). However, the role of sex chromosome diversification in the establishment of reproductive isolation is less clear and needs further investigation. A few possible examples are: in *Microtus savii*, rearrangements in the euchromatic portion of the X are thought to cause sterility in F1 male hybrids (GALLENI et al. 1998); in *Microtus agrestis*, Y chromosome differences are thought to be involved in genomic incompatibility in a hybrid zone between genetically differentiated populations (JAROOLA et al. 1997).

Parallel cytogenetic and phylogenetic analyses of other *Aethomys* species will definitively identify the chromosomal pattern of evolution of the genus. More attention should be given to the study of geographical variation in sex chromosome differences within and among species by high resolution banding and comparative gene mapping.

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APPENDIX

Following are the museum codes of the specimen examined, together with the locality and the country. The C and B letters denote the specimens for which the karyotype and the cytochrome b sequence are available, respectively.

Musee Royal de l'Afrique Centrale of Tervuren:
Tanzania: Matongolo, T50008 (C), T50007 (C), T50005 (C). Ngasumet, T50181 (C), T50191 (C), T50196 (C), T50169 (C), T50193 (C), T50163 (B, C), T50175 (C), T50184 (C), T50182 (C), T50152 (B), T50156 (C), T50173 (C). Zoissa, T50120 (B, C). Ndaleta, T50250 (C), T50251 (C). Mombo, T50224 (C). Kisiwani, T50430 (B). Lwami, T50500 (B). Idodi, T50641 (B, C). Tabora-Inala, T50618 (B), T50576 (B, C), T50621 (C), T50611 (C). Chunya, T50679 M (B, C), T50670 (B), T50703 F (C), T50662 F (C), T50657 M (C), T50703 F (C), T50668 F (C).

Museo di Anatomia Comparata dell'Università di Roma "La Sapienza":
Zambia: Meheba, Solwezi ZM13 (B, C), ZM32 (B, C), ZM1 (B).

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