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Genetic and cytogenetic variation of African root-rats *Tachyoryctes splendens* (Mammalia: Rodentia) from Ethiopia

Leonid A. Lavrenchenko*, Sewnet Mengistu, Nina Sh. Bulatova, Afework Bekele, Sergei G. Potapov, Rena S. Nadjafova & Gurja Belay

ABSTRACT. Genetic and cytogenetic variation of African root-rats *Tachyoryctes splendens* sensu lato from Ethiopia was assessed on 23 complete cytochrome *b* gene sequences (1140 bp) and chromosomal sets from eight individuals. Results of this study suggest that the real diversity of Ethiopian *Tachyoryctes splendens* s.l. has been underestimated due to its apparent cryptic diversification. The molecular genetic analysis reveals genetic subdivision including at least four allopatric and deeply divergent mitochondrial lineages, restricted to the Simien Mountains and the Northern, Southern and Eastern parts of the Ethiopian Plateau. Three of them possess unique karyotypes while chromosomal characteristics of the Eastern lineage remain unknown. These lineages may represent distinct species, however additional analyses of molecular, chromosomal and morphological data should be conducted to confirm our preliminary results and provide a real basis for species delimitation within Ethiopian *Tachyoryctes splendens* s.l.

KEY WORDS: African root-rats, *Tachyoryctes splendens*, Ethiopia, phylogeography, cytochrome *b*, mitochondrial DNA, chromosomes.

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Генетическая и цитогенетическая изменчивость восточноафриканских кротовых крыс *Tachyoryctes splendens* (Mammalia: Rodentia) из Эфиопии

Л.А. Лавренченко, Севнет Менгисту, Н.Ш. Булатова, Афеворк Бекеле, С.Г. Потапов, Р.С. Наджафова, Гурджа Белай

РЕЗЮМЕ. В результате предварительного исследования молекулярно-генетической и цитогенетической изменчивости африканских кротовых крыс *Tachyoryctes splendens* sensu lato из Эфиопии, проведен анализ 23 полных последовательностей гена цитохрома *b* (1140 пн) и хромосомных наборов восьми экземпляров. Результаты данного исследования показали, что реальное генетическое разнообразие эфиопских *Tachyoryctes splendens* s.l. ранее было недооценено из-за очевидной криптической диверсификации в пределах этого надвидового комплекса. Молекулярно-генетический анализ выявил наличие четырех глубоко дивергировавших митохондриальных линий, аллопатрически распространенных, соответственно, в Симиенских горах, а также в северной, южной и восточной частях Эфиопского нагорья. Три из них обладают уникальными кариотипами, хромосомные характеристики Восточной линии остаются неизвестными. Эти четыре диагностируемые линии могут представлять различные виды. Для подтверждения наших предварительных результатов и определения видовых границ в пределах *Tachyoryctes splendens* s.l. необходимы дополнительные анализы молекулярных, хромосомных и морфологических данных.

КЛЮЧЕВЫЕ СЛОВА: африканские кротовые крысы, *Tachyoryctes splendens*, Эфиопия, филогеография, цитохром *b*, митохондриальная ДНК, хромосомы.

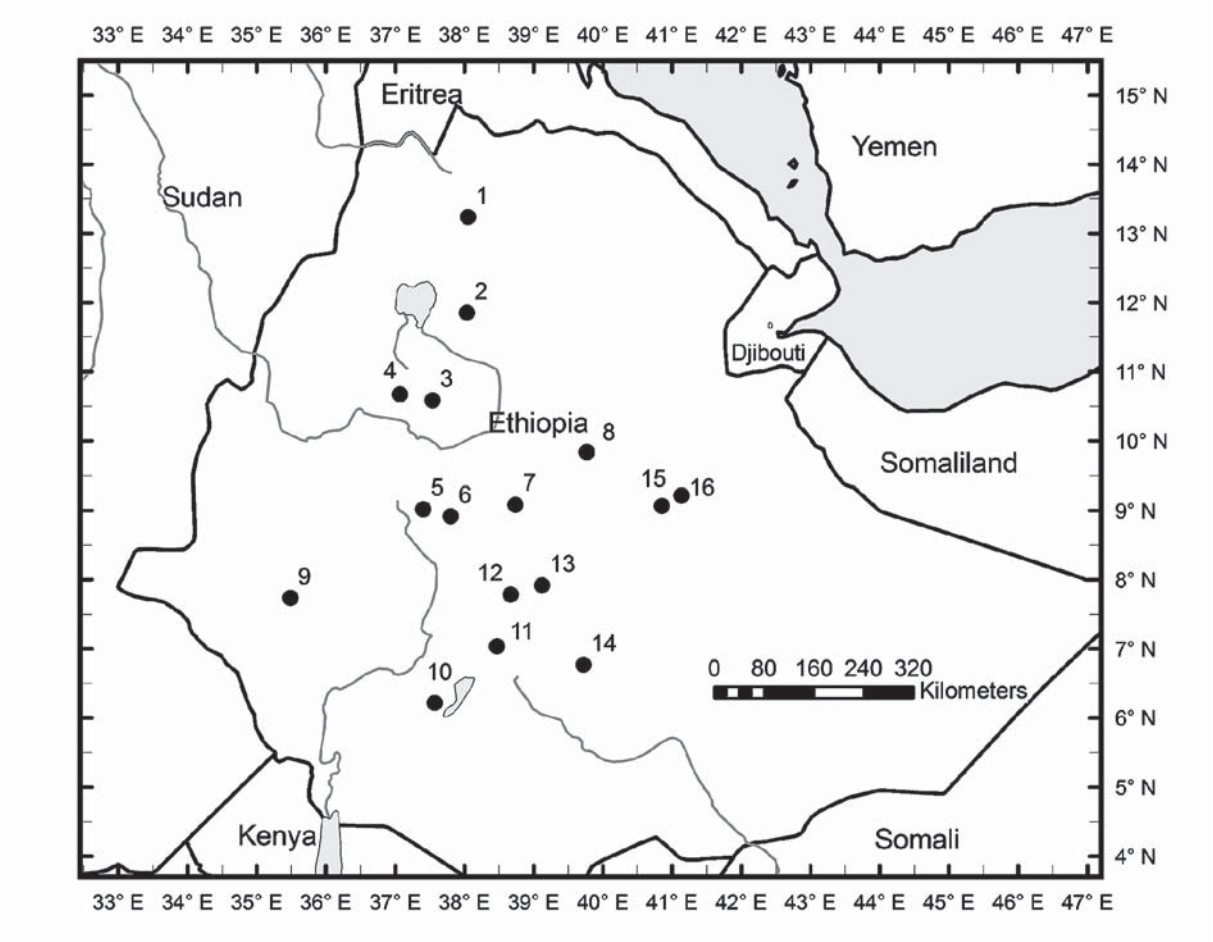

Figure 1. Map of sampling localities for Ethiopian *Tachyoryctes splendens* s.l. For the designations of the localities see the text.

Introduction

The root-rats of the African endemic genus *Tachyoryctes* Rüppell, 1835 are solitary subterranean rodents, living underground in semi-permanent burrow-systems. They are exclusively herbivores feeding on a wide range of vegetation including grasses, herbs and underground storage organs (Jarvis, 2013). The genus is distributed in East Africa and eastern parts of Central Africa. It ranges from the eastern Democratic Republic of the Congo into Rwanda and Burundi, northern Tanzania, Kenya, Uganda and throughout much of Ethiopia to northwestern Somalia at altitudes ranging from 1200 m to over 4000 m (Yalden *et al.*, 1976; Musser & Carleton, 2005; Jarvis, 2013). The Northern (Ethiopian) part of the range of this genus is separated from the rest by dry lowland uninhabitable for *Tachyoryctes*. Their isolated position attributes particular interest to Ethiopian root-rats, which presumably evolved in this area under isolation. The taxonomy of the Ethiopian *Tachyoryctes* has a turbulent history. Seven taxa were described from Ethiopia and northwestern Somalia, based on the variation in pelage coloration and external and cranial measurements: *T. splendens* (Rüppell, 1835); *T. s. somalicus* Osgood, 1910; *T. s. omensis* Neumann and Rümmler, 1928; *T. pontifex* Neumann and Rümmler, 1928; *T. cheesmani* Thomas, 1928; *T. s. canicaudus* Osgood, 1936; and *T. c. gallarum* Osgood, 1936. Craniometric analyses based on rather limited samples have revealed significant differences between Ethiopian populations attributed to *T. splendens* s.str. and *T. cheesmani* (Afework Bekele, 1986; Sewnet Mengistu & Afework Bekele, 2003). Nevertheless, until recently all these taxa were lumped under *T. splendens* (Musser & Carleton, 2005). Furthermore, large *T. macrocephalus* (Rüppell, 1842) is known only from a restricted range in the Bale Mountains (South-Eastern Ethiopia) (Yalden *et al.*, 1976). This specialised Afroalpine species is morphologically well differentiated from any other representative of the genus. Eleven additional species were recognized from the remaining part of the distribution range of the genus outside Ethiopia: *T. ankoliae* Thomas, 1909; *T. annectens* (Thomas, 1891); *T. audax* Thomas, 1910; *T. daemon* Thomas, 1909; *T. ibeanus* Thomas, 1900; *T. naivashae* Thomas, 1909; *T.*

rex Heller, 1910; *T. ruandae* Lönnberg and Gyldenstolpe, 1925; *T. ruddi* Thomas, 1909; *T. spalacinus* Thomas, 1909; and *T. storeyi* Thomas, 1909. An extended study of cranial morphology based on geometric morphometric analyses confirmed only the specific status of *T. macrocephalus* and suggested the lumping of all other taxa into a single species, *T. splendens* (Beolchini & Corti, 2004). In particular, only slight differences were observed between *T. cheesmani* and *T. splendens* s.str. Therefore, only two *Tachyoryctes* species have been recognised in Ethiopia: *T. splendens*, widespread throughout most of East Africa, and *T. macrocephalus*, endemic to the Bale Mountains. Cytogenetic analyses revealed complex karyotypic differences between these two taxa studied in the Bale Mountains, regarding diploid number and chromosome morphology (fundamental, or chromosome arm number): 2n=48, FNa=65–86 for *T. splendens*, and 2n=50, FNa=62 for *T. macrocephalus* (Aniskin *et al.*, 1997). It was uncertain whether FN differences reported for *T. splendens* from a few samples in- and outside Ethiopia had any taxonomic value or reflected specific adaptations of populations to a limited ecological niche under isolation conditions. To further test the hypotheses extended analyses based on chromosomal studies and DNA sequencing are needed. In this paper we examine, for the first time, the cytogenetic and mitochondrial DNA (mtDNA) variation of representatives of *T. splendens* s.l. from Ethiopia.

Material and methods

The specimens examined were collected during trapping sessions in the course of the Joint Ethiopian-Russian Biological Expedition (JERBE) between 2005 and 2011. All voucher specimens are housed in the Natural History Museum of the Addis Ababa University, Ethiopia, and the Zoological Museum of the Moscow State University (ZMMU), Russia. *Tachyoryctes splendens* s.l. were collected at the following localities in Ethiopia (Fig. 1): (1) Simien Mountains National Park, Sankaber area (13°14′N 38°03′E, 3250 m a.s.l.) — 2 specimens, Chennek area (13°15′N 38°13′E, 3800 m a.s.l.) — 2 specimens; (2) Debre-Tabor (11°51′N 38°02′E, 2645 m a.s.l.) — 10 specimens; (3) Dembecha (10°35′N 37°32′E, 2258 m a.s.l.) — 8 specimens; (4) Bure (10°40′N 37°04′E, 2060 m a.s.l.) — 10 specimens; (5) Gedo (09°01′N 37°24′E, 2058 m a.s.l.) — 10 specimens; (6) Ambo (08°55′N 37°48′E, 2300 m a.s.l.) — 12 specimens; (7) Addis Ababa, Mt. Entoto (09°05′N 38°44′E, 2982 m a.s.l.) — 10 specimens; (8) Debre Sina (09°50′N 39°44′–39°46′E, 2570–3240 m a.s.l.) — 20 specimens; (9) Masha (07°44′N 35°29′E, 2250 m a.s.l.) — 8 specimens; (10) Chencha, Doko Mesho (06°13′N 37°34′E, 2550 m a.s.l.) — 12 specimens; (11) Hawassa (07°02′N 38°28′E, 1704 m a.s.l.) — 10 specimens; (12) Adami Tullu (07°47′N 38°40′E, 1654 m a.s.l.) — 12 specimens; (13) Asela, Chilalo Mt. (07°55′N 39°07′E, 2525 m a.s.l.) — 10 specimens; (14) Bale Mountains National Park, Rira (06°46'N 39°43'E, 2940 m a.s.l.) — 10 specimens; (15) Asbe Teferi (09° 04′N 40°51′E, 1880 m a.s.l.) — 10 specimens; (16) Hirna, Chercher Mts. (09°13′N 41°08′E, 2400 m a.s.l.) — 10 specimens.

We sequenced a total of 23 entire (1140 bp) sequences of the mitochondrial gene cytochrome *b*. DNA was extracted from 96% alcohol preserved liver, kidney and muscle tissue by the standard phenol-chloroform method (Mathew, 1984). Complete cytochrome *b* gene was amplified by PCR with the combination of the forward L14727-SP (5'-GACAGGAAAAATCATCGTTG-3') (Fink *et al.*, 2004) and reverse H15915-SP (5'-TTCATTACTGGTTTACAAGAC-3') (Fink *et al.*, 2004) primers.

Template DNA was amplified by PCR in 25 μL reaction volume containing the following: 1X reaction buffer, 10 mM $(NH_4)_2SO_4$, 0.1% TWEEN 20, 2 mM $MgCl_2$, 1 mM of each dNTP, 0.1 mM of each primer, 1 unit of Taq polymerase and 25–100 ng of template DNA. PCRs were performed under the following temperature profile: hot start at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 1 min, and a final elongation at 72°C for 10 min. Sequencing was performed on an ABI PRISM 310 DNA automatic analyser (Applied Biosystems, Forster City, CA, USA) using the same primers mentioned above and BigDye Terminators chemistry (Applied Biosystems). The sequences were checked for quality and aligned by eye. To avoid erroneous inclusion of Numts (nuclear sequences of mitochondrial origin) in the data sets, the codons of the complete cytochrome *b* gene were translated into aminoacids to check for non-functional mutations. The sequences were submitted to the GenBank under accession numbers KM523600–KM523622.

We also used sequence data on relevant rodents from GenBank: *Myospalax psilurus* Milne-Edwards, 1874 (AF326271), *Eospalax fontanierii* Milne-Edwards, 1867 (AF326266), *E. rothschildi* Thomas, 1911 (AF326268) and *Rhizomys sinensis* Gray, 1831 (AF326274) (Zhou *et al.*, 2004). These four Spalacidae taxa were selected as outgroups. We also included *cytb* sequence data on the only available non-Ethiopian representative of *Tachyoryctes* (AF160602) from Kenya (precise geographic locality unknown) (Jansa *et al.*, 1999).

Phylogenetic relationships among entire sequences of the cytochrome *b* gene were assessed using neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) criteria implemented in PAUP* version 4.0b10 (Swofford, 2000), complemented with PAUPUP, version 1.0.3.1 (Calendini & Martin, 2005). The NJ tree was reconstructed using the uncorrected *p*-distance. Unweighted MP analysis was performed using heuristic search starting with stepwise addition trees and employing tree bisection–reconnection branch-swapping algorithm. ModelTest, version 3.04 (Posada & Crandall, 1998) was employed for the choice of the

best model of sequence evolution for the ML analyses, using the corrected Akaike information criterion (AICc) value. The supported substitution model was Hasegawa–Kishino–Yano 1985 (HKY+G+I, alpha=1.543, pin=0.511). Maximum likelihood was conducted using the heuristic search option and the parameters obtained from ModelTest. Clade stability of the NJ-MP and ML trees was assessed by bootstrapping with 1000 and 500 replicates, respectively. Genetic divergence between and within clades were computed as uncorrected *p*-distances with the software Mega version 3.1 (Kumar *et al.*, 2004).

The chromosomal analysis was performed on *Tachyoryctes splendens* s.l. from localities 1, 8 and 12. Somatic metaphases of individuals were prepared from bone marrow by the usual air-drying technique according to Ford & Hamerton (1956). Slide preparations were stained with 4% Giemsa in phosphate buffer with pH = 7.0 for routine karyotyping and C-stained for heterochromatin location according to Sumner (1972).

Results

Analysis of mtDNA

The complete cytochrome *b* gene sequences among ingroup 23 haplotypes show 307 variable sites of which 240 are informative under parsimony. Analysis of the nucleotide composition within the ingroup revealed a deficiency in guanine (mean cytochrome *b*: *A*=0.254, *C*=0.290, *G*=0.127, *T*=0.329), which was typical of the cytochrome *b* of rodents (Jaarola *et al.*, 2004), as well as of mammals in general (Irwin *et al.*, 1991). Mean pairwise transition/transversion ratio for cytochrome *b* was 4.15 (as estimated by ML using the HKY substitution model with gamma distribution and alpha estimated from the data).

Phylogenetic trees constructed by use of the three main methods (NJ, MP, and ML) had very similar topology (Fig. 2). Uncorrected *p*-distances between distinct haplotypes are given in Tab. 1. The phylogenetic analyses of complete cytochrome *b* gene sequences retrieved three highly divergent lineages: Southern, Northern and Eastern. The results suggest the basal position of the Eastern lineage within the ingroup (bootstrap support (BI) = 54 to 87%). The mean levels of the genetic divergence (*p*-distance) between major phylogroups based on complete cytochrome *b* gene sequencing are: Eastern–Northern — 11.82 ± 0.87% of nucleotide substitutions, Eastern–Southern — 11.27 ± 0.91%, and Northern–Southern — 9.62 ± 0.58%. Besides the distinctness of clades, the relatively high distance values suggest that the clades may represent species and/or even species-groups.

The Southern phylogroup appeared to be a sister group to the single available specimen from Kenya (BI = 59 to 81%). Average *p*-distance between the Kenyan specimen and representatives of the Southern lineage was 9.03 ± 0.71%. The Southern phylogroup was further subdivided into three distinct subclades ((1) samples from south-eastern mountains (loc. 13, 14), (2) locality 7, (3) resting southern samples (loc. 10, 11, 12)) supported in NJ, MP and ML-analyses by sufficiently high bootstrap indices (BI = 96 to 100%). The mean levels of the genetic divergence (*p*-distance) between these three distinct subclades are: 1–2 — 6.11 ± 0.73%, 1–3 — 5.97 ± 0.69%, 2–3 — 6.45 ± 0.64%. According to the results of NJ, MP and ML-analyses, within the Northern phylogroup the most basal lineage was represented by the haplotype from the Simien Mountains (loc. 1) (BI = 62–76%). Average *p*-distance between the Simien specimen and other representatives of the Northern lineage is 8.70 ± 0.62%. The rest of the Northern phylogroup was further subdivided into four distinct subclades ((1) northern localities 2 and 4, (2) locality 3, (3) locality 8, (4) samples from central (loc. 5, 6) and western (loc. 9) parts of the country). Three subclades were supported in NJ, MP and ML-analyses by sufficiently high bootstrap indices (BI = 88 to 100%), whereas one (loc. 2, 4) obtained only poor bootstrap support (BI = 50 to 73%). The level of genetic divergence (*p*-distance) among these four subclades was in the range 6.02–8.61% (mean 7.13%). The mean levels of the genetic differentiation (*p*-distance) within major phylogroups based on complete cytochrome *b* gene sequences are: Northern — 6.68 ± 0.44%, Southern — 4.03 ± 0.40%, and Eastern — 1.65 ± 0.40%.

Cytogenetic analysis

Cytogenetic analyses detected two different 48-chromosome complements and a 50-chromosome variant new for Ethiopian *T. splendens* s.l. (Tab. 2). The karyotypes of specimens from localities 12 and 8, both having 2n=48, resemble structurally the two formerly known karyotypes with minimal (3 pairs) and maximal (5 pairs) numbers of autosomal metacentrics, or, correspondingly, the 3MA and 5MA karyotypes (Fig. 3). They all have both large metacentric sex chromosomes, X and Y, with a characteristic C-banding pattern. The Y is entirely heterochromatic and looks dense even in routine stained preparations, and there is a large portion

Figure 2. Neighbour-joining (NJ) tree illustrating the phylogenetic relationships among the complete cytochrome *b* gene haplotypes in the selected 23 specimens of the Ethiopian common root-rat (*Tachyoryctes splendens*). The tree is based on the uncorrected *p*-distances. The values of bootstrap index (>50%) are shown by the upper figures for NJ analysis, and by lower figures (slash-separated), for maximum parsimony (MP) and maximum likelihood (ML) analyses, respectively. *Myospalax psilurus*, *Eospalax fontanierii*, *E. rothschildi* and *Rhizomys sinensis* are used as outgroups. Collection localities are given before each Ethiopian specimen name with numbers that refer to those used in text and Fig. 1.

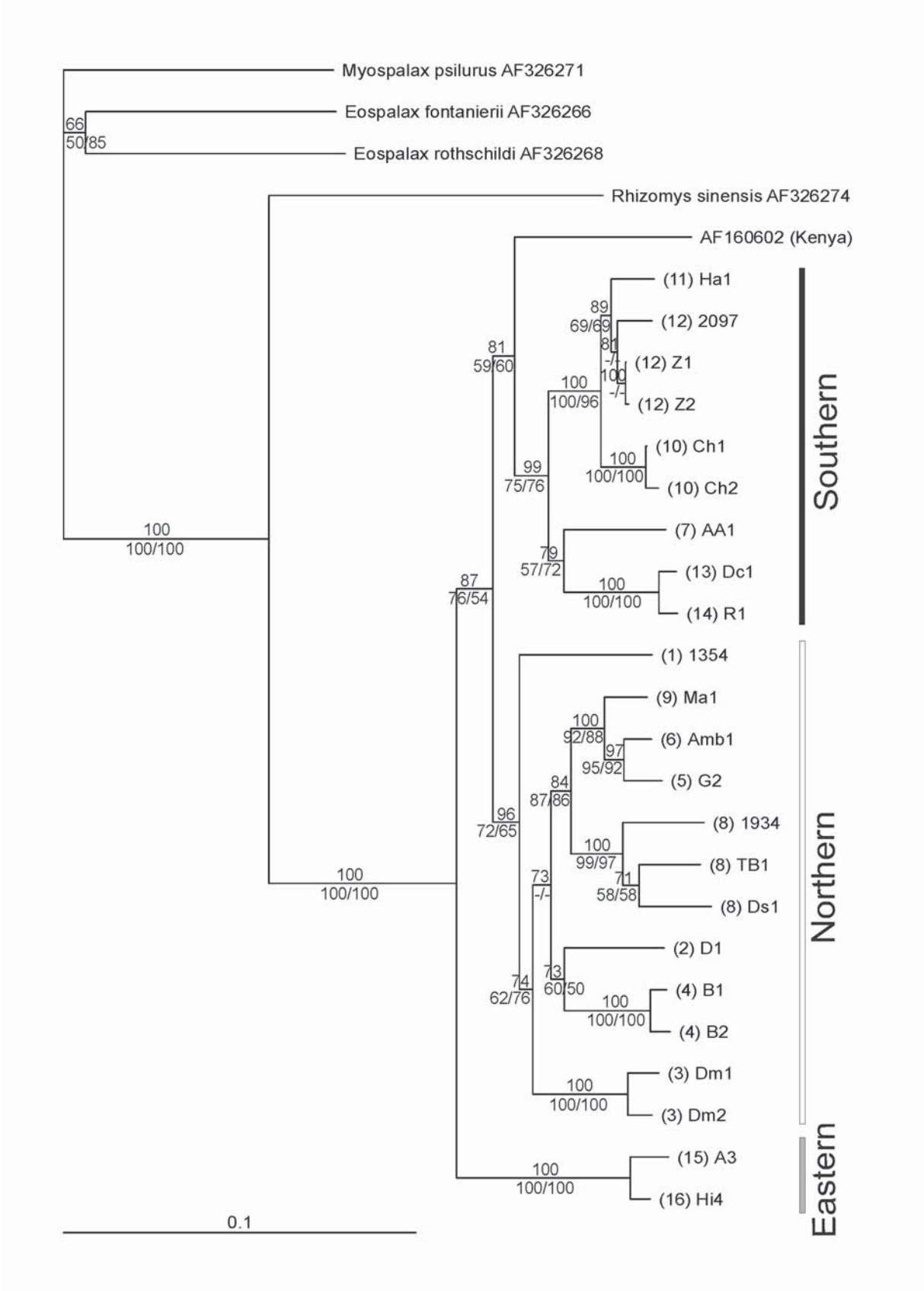
Myospalax psilurus AF326271
Eospalax fontanierii AF326266
Eospalax rothschildi AF326268
Rhizomys sinensis AF326274
AF160602 (Kenya)
(11) Ha1
(12) 2097
(12) Z1
(12) Z2
(10) Ch1
(10) Ch2
(7) AA1
(13) Dc1
(14) R1
(1) 1354
(9) Ma1
(6) Amb1
(5) G2
(8) 1934
(8) TB1
(8) Ds1
(2) D1
(4) B1
(4) B2
(3) Dm1
(3) Dm2
(15) A3
(16) Hi4
Southern
Northern
Eastern
0.1

Table 1. Estimates of divergence (below diagonal) and standard errors (above diagonal) among the complete cytochrome *b* gene haplotypes from the Ethiopian common root-rats (*Tachyoryctes splendens*), using the uncorrected *p*-distances. See Figs. 1 and 2 for collection locality of each specimen.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	Ha1		0.004	0.003	0.003	0.005	0.005	0.008	0.008	0.008	0.008	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.008	0.009	0.009	0.008	0.008	0.009	0.009
2	2097	0.017		0.003	0.003	0.005	0.005	0.008	0.008	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.008	0.009	0.009
3	Z1	0.012	0.008		0.001	0.004	0.004	0.008	0.008	0.007	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.008	0.009	0.009	0.008	0.008	0.009	0.009
4	Z2	0.013	0.009	0.001		0.004	0.004	0.008	0.008	0.007	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.008	0.009	0.009	0.008	0.008	0.009	0.009
5	Ch1	0.026	0.024	0.015	0.016		0.002	0.008	0.008	0.008	0.009	0.009	0.009	0.009	0.010	0.009	0.009	0.009	0.009	0.009	0.010	0.009	0.009	0.009	0.009
6	Ch2	0.030	0.028	0.020	0.021	0.004		0.008	0.008	0.008	0.009	0.009	0.009	0.009	0.010	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009
7	AA1	0.061	0.062	0.056	0.057	0.055	0.058		0.007	0.008	0.009	0.009	0.010	0.010	0.010	0.010	0.010	0.009	0.010	0.009	0.009	0.009	0.009	0.009	0.009
8	Dc1	0.064	0.064	0.056	0.057	0.062	0.065	0.058		0.003	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.010	0.010
9	R1	0.061	0.061	0.055	0.056	0.063	0.066	0.063	0.011		0.009	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.008	0.008	0.010	0.010
10	1354	0.090	0.087	0.085	0.086	0.088	0.090	0.091	0.095	0.100		0.007	0.008	0.008	0.008	0.008	0.008	0.007	0.009	0.008	0.007	0.007	0.009	0.009
11	Ma1	0.084	0.091	0.084	0.083	0.090	0.091	0.094	0.098	0.097	0.071		0.005	0.005	0.007	0.007	0.007	0.007	0.007	0.008	0.008	0.008	0.010	0.009
12	Amb1	0.093	0.095	0.090	0.091	0.093	0.096	0.098	0.097	0.097	0.078	0.026		0.004	0.008	0.007	0.007	0.007	0.007	0.008	0.008	0.008	0.010	0.010
13	G2	0.090	0.094	0.087	0.086	0.091	0.095	0.102	0.101	0.099	0.086	0.027	0.017		0.007	0.007	0.007	0.008	0.007	0.008	0.008	0.009	0.010	0.010
14	1934	0.097	0.094	0.093	0.092	0.100	0.104	0.110	0.107	0.106	0.087	0.062	0.063	0.064		0.006	0.007	0.008	0.009	0.009	0.008	0.009	0.010	0.010
15	Tb1	0.106	0.106	0.102	0.103	0.103	0.105	0.116	0.109	0.110	0.091	0.061	0.056	0.061	0.038		0.006	0.008	0.008	0.009	0.008	0.009	0.010	0.010
16	Ds1	0.106	0.105	0.101	0.100	0.102	0.105	0.111	0.114	0.117	0.082	0.058	0.059	0.059	0.052	0.039		0.008	0.008	0.008	0.008	0.008	0.009	0.010
17	D1	0.094	0.095	0.088	0.087	0.096	0.095	0.095	0.104	0.107	0.078	0.056	0.064	0.069	0.080	0.080	0.085		0.007	0.007	0.008	0.007	0.010	0.010
18	B1	0.095	0.094	0.090	0.091	0.095	0.096	0.099	0.103	0.105	0.088	0.053	0.053	0.061	0.077	0.080	0.079	0.059		0.004	0.009	0.009	0.009	0.010
19	B2	0.100	0.097	0.095	0.096	0.098	0.099	0.100	0.102	0.105	0.082	0.057	0.056	0.065	0.075	0.080	0.079	0.056	0.011		0.009	0.009	0.010	0.010
20	Dm1	0.092	0.095	0.090	0.088	0.095	0.095	0.100	0.100	0.097	0.076	0.068	0.072	0.073	0.086	0.085	0.082	0.069	0.076	0.073		0.004	0.010	0.010
21	Dm2	0.086	0.090	0.084	0.083	0.092	0.093	0.097	0.101	0.098	0.073	0.068	0.075	0.076	0.088	0.088	0.086	0.065	0.076	0.078	0.015		0.010	0.009
22	A3	0.115	0.115	0.111	0.110	0.116	0.119	0.104	0.116	0.117	0.120	0.118	0.119	0.119	0.121	0.130	0.129	0.123	0.111	0.115	0.117	0.110		0.004
23	Hi4	0.109	0.111	0.105	0.104	0.110	0.113	0.104	0.111	0.112	0.116	0.114	0.115	0.115	0.115	0.123	0.122	0.118	0.105	0.109	0.111	0.104	0.016	

Table 2. Karyotypic data on *Tachyoryctes* collected from Ethiopia.

Species	Location	Number/ Sex	2n	DMA (n)	FN**	Reference
T. macrocephalus	Bale Mts.	3♀♀	50	2	54	Aniskin *et al.*, 1997
*T. splendens**	Bale Mts.	2♂♂, 7♀♀	48	3	54	Aniskin *et al.*, 1997
*T. splendens**	Koka Lake (Rift Valley)	2♂♂, 2♀♀	48	3	54	Baskevich *et al.*, 1993
T. cf. *splendens**	Vanzaye	1♀	48	5	58	Bulatova & Lavrenchenko, 2005
T. splendens s.l.	Debre-Sina	2♂♂	48	5	58	This study
T. splendens s.l.	Adami-Tulu	2♂♂	48	3	54	This study
T. splendens s.l.	Simien Mts. (Sankaber)	1♂, 1♀	50	4	58	This study
T. splendens s.l.	Simien Mts. (Chennek)	2♀♀	50	4	58	This study

* As named in corresponding publications. DMA(n) — haploid number of diagnostic metacentrics, which only were regarded as bi-armed elements against the rest "all-acrocentric" content in simplified FN** counts.

of C-heterochromatin in the long arm of the X chromosome not well distinct from its euchromatic part. Among autosomes, 3MA and 5MA diagnostic pairs look C-negative.

C-banding pattern in the remaining autosomes is, however, different. In the 3MA karyotype from locality 12, C-blocks are stained in pericentromeric area of many, if not all the acrocentric chromosomes (Fig. 3A), as in *T. splendens* from the Bale Mountains or near the north-eastern shore of Koka Lake (Tab. 2) where some of the largest elements looked subtelocentric due to an increased amount of heterochromatic material in a short arm. By contrast, in the 5MA karyogram (loc. 8) C-staining is restricted to short arms of four subacrocentric pairs (Fig. 3B).

The new karyotype with 2n=50 and four small metacentric pairs was detected in all four specimens obtained from two sites investigated in the Simien Mountains (loc. 1). It is structured similar to the above mentioned karyotypes and comprises large bi-armed sex chromosomes, four pairs of small metacentrics and 20 pairs of acro- or subacrocentrics. In the absence of C-staining, it may be extrapolated, however, that short arms of larger subacrocentrics should be heterochromatic, as in the case of 5MA karyotype. The Y-chromosome looks compact and very likely should be C-positive as well (Fig. 3C).

Our results suggest a corrected count of chromosome number in heterochromatically variable karyotypes. It is worth to note that in former descriptions the elements with C-positive short arms were treated usually as subtelocentrics, and their proportion influenced the FN counts which were reported in a wide range from 62–65 to 86 due to the authors' views (Baskevich *et al.*, 1993; Aniskin *et al.*, 1997). One can see, however, that variation in C-negative small metacentrics and subtelocentrics or subacrocentrics (in our current terminology) with heterochromatic additional short arm should be essentially independent (Fig. 3). To exclude the uncertain variety of heterochromatin, we compare FN counts only for "true", i.e. euchromatic metacentrics against originally one-armed groups, which comprised acrocentrics together with subacrocentrics or subtelocentrics. In view of this, the three karyotypes are to be presented in the following figures in our data: 2n=48, FN=56, FNA=52 for 3MA karyotype; 2n=48, FN=62, FNA=58 for 5MA karyotype; 2n=50, FN=62, FNA=58 for 4MA karyotype (Tab. 2). It follows from such a consideration that correspondence of 2N/FN figures might show the participation of a Robertsonian-like rearrangement only between karyotypes 3MA/4MA, but remains unsolved in the case of 5MA karyotype relations with each of those two karyotypes.

Regarding the FN comparisons, one more correction has to be made. A minute bi-armed pair was reported as a peculiarity of the of 5MA karyotype in its first presentation for Vanzaye (Bulatova & Lavrenchenko, 2005). The quality of field chromosome preparations from a new site, Debre-Sina (loc. 8), was not good enough to recognise the arms in members of the smallest pair looking like the tiny chromatin bodies (Fig. 3B, Tab. 2). The same is true for the 4MA karyotype, though some heteromorphisms between two minimal chromosomes can be registered in metaphase spreads on the same preparation. It is likely that unsteady chromosome size in this case is probably due to variation of active/inactive condition of the nucleolar organizing region, or NOR, thus varied in size and influenced on the size of a short arm where probably NOR is located. Therefore, data on differential NOR staining or specific DNA marks should add to morphological details of *Tachyoryctes* karyotypes. AgNOR localization was first reported for two large pairs of subtelocentrics in the 3MA karyotype (Koka, by Baskevich *et al.*, 1993) and doesn't specify something more for small chromosomes. The smallest chromosomal pair in all these cases is

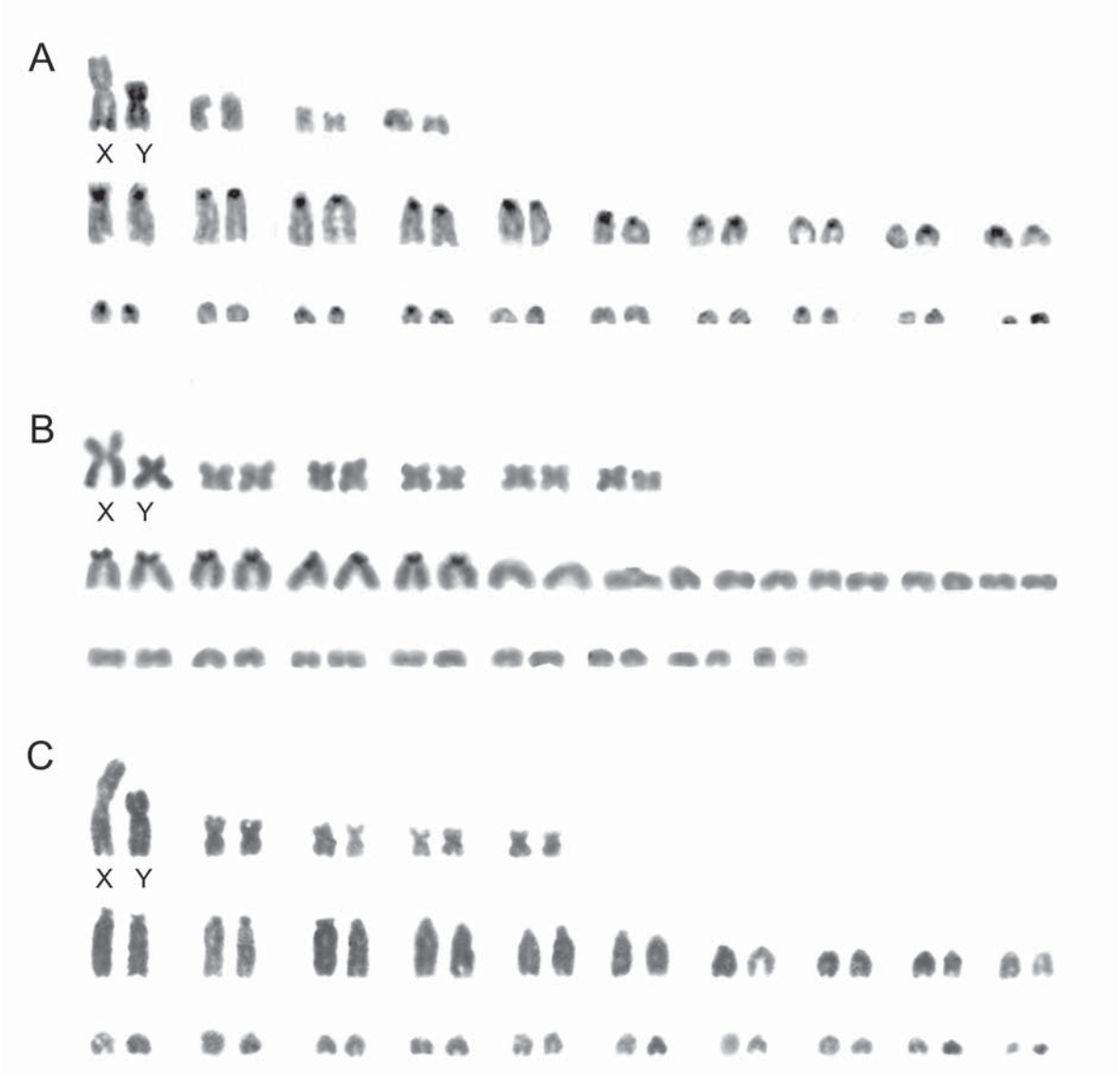

Figure 3. Karyograms of Ethiopian *Tachyoryctes splendens* differing in the number of small autosomal metacentrics (MA) and C-heterochromatin. A — Adami-Tulu, 3MA, autosomal C-blocks in a majority of acrocentric pairs; B — Debre-Sina, 5MA, C-positive short arms in 4 large subacrocentric pairs; C — Simien (Sankaber), 4MA, short arms in largest subacrocentrics are very likely heterochromatic. X and Y — the male sex chromosomes.

interpreted therefore as acrocentric for FN counts, before more detailed data become available.

Discussion

The results of our molecular genetic study indicate that the Ethiopian *T. splendens* s.l. is composed of at least three geographically separated and well-supported mitochondrial lineages (Southern, Northern and Eastern) that represent potential phylogenetic species. All three lineages are deeply divergent and separated from one another by uncorrected *cytb* distances (~11%) usually corresponding to species-level divergence in mammals (Bradley & Baker, 2001). The Southern lineage possesses a unique karyotype (2n=48, 3MA), while the chromosomal characteristics of the Eastern lineage remain unknown. Within the Northern lineage two distinct karyotypes were found: 2n=48, 5MA and 2n=50, 4MA. The latter chromosomal set was revealed only in specimens from the Simien Mountains, representing one of the northernmost localities of *Tachyoryctes* distribution range. The root-rats from this locality represent the most basal subclade of the Northern lineage and are separated from the remaining representatives of the lineage by an uncorrected *cytb* distance (~9%) generally considered to indicate cryptic taxonomic diversity (Baker & Bradley, 2006). Therefore, the results of

combined cytogenetic and molecular genetic analyses indicate that the Ethiopian *T. splendens* s.l. is a species complex containing at least four putative species, corresponding to independently evolved lineages: 1) Southern lineage, 2) Simien Mountains, 3) remaining subclades of Northern lineage, 4) Eastern lineage.

One critical remark on the use of the divergence criterion for defining species limits is that species, mainly those recent or incipient, are not necessarily exclusive in their gene pool due to retention of ancestral polymorphisms or introgression (Geurgas & Rodrigues, 2010). As mitochondrial haplotypes are related by a strictly bifurcating genealogy due to their non-recombining and matrilineal mode of inheritance, deeply divergent mitochondrial lineages may be maintained in contiguous populations even in the absence of any reproductive barrier, resulting in a phylogeographic structure derived merely from the stochastic lineage sorting of ancestral polymorphisms. The population structure is boosted in species with limited vagility in fragmented habitats, in which populations can be partially isolated not only by distance alone but also by biotic and abiotic factors that can act as barriers to dispersal on a fine geographic scale (Irwin, 2002; Kuo & Avise, 2005; Avise, 2009). In particular, subterranean rodents fit these criteria and represent a real challenge to species delimitation via molecular data. In general, they occupy fragmented habitats and present limited dispersal abilities in relation to the spatial scale of the habitat discontinuities (Steinberg & Patton, 2000). Subterranean rodents occupy small population units with low genetic variation and high inter-population divergence (Lacey, 2000). Moreover, they exhibit in general the most spectacular chromosomal variability yet known in mammals (Reig *et al.*, 1990). Most of their chromosomal diversity can be centered at both intraspecific (genera *Thomomys* Wied-Neuwied, 1839, *Spalax* Guldenstaedt, 1770 and *Ellobius* Fischer, 1814) and interspecific (genus *Ctenomys* Blainville, 1826) level (Bidau *et al.*, 2003). Therefore, in the case of subterranean rodents (including *Tachyoryctes*), cytogenetic and mtDNA-based approaches might overestimate the number of potential species, and additional complementary data sources, such as sequencing of nuclear genes and multivariate analysis of cranial morphology (including type specimens) should be combined to help in species delimitation. Whether these mitochondrial and cytogenetic differentiations represent species boundaries remains to be investigated further, but what is clear from the results is that the diversity of the Ethiopian *T. splendens* has been underestimated. Although more data are needed prior to formal recognition of four lineages described above as taxonomic species, this study provides a framework for future studies in this complex.

All known fossil remains of the genus *Tachyoryctes* are restricted to Ethiopia. Three fossil species were described from the country: the Late Miocene *T. makooka* Wesselman, Black and Asnake, 2009, the Pliocene *T. pliocaenicus* Sabatier, 1978 and the Pleistocene *T. konjiti* Sabatier, 1982. The former species represents a link between *Protachyoryctes tatroti* Hinton, 1933 and *Eicooryctes kaulialensis* Flynn, 1982 from the Late Miocene of Pakistan and the derived African Tachyorictini (López-Antoñanzas & Wesselman, 2013). Therefore, paleontological data suggest that the Ethiopian Plateau might be a primary center of diversification for modern representatives of the genus. The limited evidence available today supports this hypothesis. Thus, sole available non-Ethiopian *Tachyoryctes* from Kenya used in our phylogenetic analysis appears as a sister of the Southern lineage (Fig. 2). Furthermore, the sole described karyotype of non-Ethiopian *Tachyoryctes*, *T. ruandae* from East Congo (Matthey, 1967), is very similar to that of Ethiopian Southern lineage. Obviously, further study involving more samples (including *T. macrocephalus* and non-Ethiopian taxa) and based on the complex molecular, karyological and morphological investigation is required to clarify phylogenetic relationships among modern *Tachyoryctes* and analyse the taxonomic composition of the genus.

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