University of South Bohemia in České Budějovice
Faculty of Science

The Rufous Sengi is not *Elephantulus* — Multilocus reconstruction of evolutionary history of sengis from the subfamily Macroscelidinae

RNDr. Thesis

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České Budějovice 2022

Krásová, J., 2022: The Rufous Sengi is not *Elephantulus* — Multilocus reconstruction of evolutionary history of sengis from the subfamily Macroscelidinae. RNDr. Thesis, in English. – 15 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation:
We conducted multilocus phylogeny and divergence dating of sengis (also known as elephant shrews) from the subfamily Macroscelidinae. For the first time, we provided genetic evidence that the East African Rufous Sengi (*Elephantulus rufescens*) is closely related to the recently delimited genus *Galegeeska* known from the Horn of Africa and comprising a single species, *G. revolii*. Our findings are in concordance with morphological traits and also biogeographical patterns known from Eastern Africa. Based on the results of divergence dating, the genus *Galegeeska* originated in the Pleistocene era.

Declaration
I hereby declare that I am the author of this thesis and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 1.12. 2022

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Mgr. Jarmila Krásová

Krásová, J., Mikula, O., Šumbera, R., Horáková, S., Robovský, J., Kostin, D. S., Martynov, A. A., Lavrenchenko, L. A., & Bryja, J. (2021). The Rufous Sengi is not *Elephantulus*—Multilocus reconstruction of evolutionary history of sengis from the subfamily Macroscelidinae. *Journal of Zoological Systematics and Evolutionary Research, 59*(4), 918-932. DOI: 10.1111/jzs.12460
(IF=2.424)

Jarmila Krásová was involved in the design of the study and data collection, performed laboratory work, and contributed to the writing of the manuscript. (Contribution=40 %)

Acknowledgments

I feel deeply appreciative of all the support both my supervisors - Radim Šumbera and Pepa Bryja - provided, not just during the writing of this publication, but perhaps more importtnly during the whole course of my studies. It was a great pleasure to learn from them!

I am extremely grateful to Ondra Mikula for beyond-perfect statistical analysis :) and to Honza Robovský for his willingness to collaborate and morphology work. Special thanks goes to Sylva Horáková – I admire her precision working with such small things like rodent's penises. I would also like to thank all other co-authors of this study for their contributions.

Last but not least, I am very grateful to my family.

Content

Received: 25 September 2020 | Revised: 4 January 2021 | Accepted: 9 January 2021

DOI: 10.1111/jzs.12460

ORIGINAL ARTICLE

JOURNAL OF ZOOLOGICAL SYSTEMATICS AND EVOLUTIONARY RESEARCH — WILEY

The Rufous Sengi is not *Elephantulus*—Multilocus reconstruction of evolutionary history of sengis from the subfamily Macroscelidinae

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Funding information
Czech Science Foundation, Grant/Award Number: 20-07091J and 18-17398S; Grant Agency of the University of South Bohemia, Grant/Award Number: 04-048/2019/P and 018/2017/P; Russian Foundation for Basic Research, Grant/Award Number: 19-54-26003

Abstract

The evolutionary history of sengis (Macroscelidea), an order of Afrotheria, has been studied in last decades by molecular phylogenetic approaches. These studies proposed an evolutionary scenario for this group of mammals and, surprisingly, revealed the presence of two new genera, delimited and described in the last five years. However, most research has relied solely upon samples from Southern Africa, while the genetic information from East Africa and the Zambezian region was only fragmentary. Here, we provide the most complete multilocus phylogeny of the subfamily Macroscelidinae, using new material mainly from eastern Africa, Zambia, and Angola. In agreement with previous studies, we confirmed the presence of two major radiations in the group, corresponding to recently defined tribes Macroscelidini and Elephantulini. Contrary to previous studies, however, we provide clear genetic evidence that the widespread East African Rufous Sengi (*Elephantulus rufescens*) belongs to the recently delimited genus *Galegeeska*. This finding is in agreement with morphological traits and with general biogeographical patterns in sub-Saharan Africa. Revised divergence dating using a multispecies coalescent approach revealed much younger splits in Macroscelidea, compared with previous studies, with a majority of recent species appearing in the Plio–Pleistocene. The genus *Galegeeska* thus represents a typical mammalian genus of the Somali-Masai bioregion and its current diversity (at least two recognized species, *G. revoilii* and *G. rufescens*) arose during the Pleistocene climatic oscillations, which is in agreement with other studies of small mammals in this region.

KEYWORDS
elephant shrew, *Elephantulus rufescens*, *Galegeeska*, phylogeny, Somali-Masai

1 | INTRODUCTION

Revolutionary discovery of the supercohort Afrotheria, one of the four main clades of extant placental mammals, in the last decade of the 20th century by molecular phylogenetic approaches, has stimulated intensive research on this ancient group of mammals. Some clades have been disproportionately targeted by researchers, for example, elephants and sirenians, but relatively intensive research has also focused on lesser known smaller taxa, including sengis (order Macroscelidea). Together with members of two other afrotherian families (Tenrecidae, Chrysochloridae), these three taxa represent the most speciose afrotherian groups. Sengis are small insectivorous mammals with a body mass from about 30 to 500 grams. Their biology and phenotype are very different from other mammals of

comparable body size. All sengis are highly cursorial with very long slender limbs. They create stable systems of trails in their territories and never hide in burrows. They live in stable pairs, and the females give births to highly precocial neonates. Many species use foot drumming as a mean of intraspecific communication, and many of them fall into torpor. All species possess a long mobile snout; therefore, they are known also as elephant shrews (Heritage, 2018).

All extant species of sengis are members of a single family Macroscelididae, with two clearly monophyletic subfamilies: Rhynchocyoninae with five species in a single genus *Rhynchocyon* and Macroscelidinae with 15 species in five genera *Macroscelides*, *Petrodromus*, *Petrosaltator*, *Galegeeska*, and *Elephantulus* (Heritage, 2018; Heritage et al., 2020). While the giant sengis from the genus *Rhynchocyon* are distributed in coastal, montane, and lowland forests of tropical Africa, the members of Macroscelidinae live in more open habitats from woodlands (*Petrodromus*) to semi-deserts (*Macroscelides*), with the highest species diversity in Southern African region (Heritage, 2018; see also distributional data at https://www.sengis.org/distribution.php).

Similar to many other groups of small mammals, the use of molecular genetic methods over the last 15 years has led to the recognition and description of (nearly) cryptic species in *Rhynchocyon* (Carlen et al., 2017), *Macroscelides* (Dumbacher et al., 2012, 2014), and *Elephantulus* (Smit et al., 2008). Specifically, the number of recognized species has increased from 15 in 2005 to 20 in 2018 (Burgin et al., 2018). Another important finding was that the North African sengi (formerly *Elephantulus rozeti*; Duvernoy, 1833) does not belong to *Elephantulus*, but instead represents a distinct evolutionary lineage elevated to a new genus *Petrosaltator*, which is more closely related to genera *Petrodromus* and *Macroscelides* (Douady et al., 2003; Dumbacher, 2016). In the latter study, Dumbacher (2016) thus defined two tribes in the subfamily Macroscelidinae: Macroscelidini with genera *Macroscelides*, *Petrodromus*, and *Petrosaltator*; and Elephantulini with species-rich genus *Elephantulus*. Finally, Heritage et al. (2020) recently rediscovered the very poorly known Somali Sengi (*Elephantulus revoilii*; Huet, 1881) in Djibouti. Subsequent genetic analysis revealed that this species belongs to another distinct evolutionary lineage within Macroscelidini, assigned to the generic name *Galegeeska* (Heritage et al., 2020).

Unfortunately, there is still a lack of multilocus phylogenetic studies including all (or most) extant species of sengis, and especially, the genetic data from East Africa are largely missing. The most complete molecular dataset was produced by Smit et al. (2011), but even in this study, several species were only represented by mtDNA sequences from museum specimens and their phylogenetic position remained unresolved. During our research of small mammals in Africa over the last *ca* 15 years, we obtained a new tissue material of several species of sengis. In this study, we sequenced fresh samples at one mitochondrial and six nuclear markers, combined new data with GenBank sequences, and performed multilocus phylogenetic analyses. As a result, we present the most complete and resolved phylogeny of the subfamily Macroscelidinae, where we confirmed monophyly of two recently defined tribes Macroscelidini and Elephantulini. Contrary to previous molecular–phylogenetic studies, we provide clear genetic evidence that the widespread East African Rufous Sengi (*Elephantulus rufescens*; Peters, 1878) belongs to the recently delimited genus *Galegeeska*, which is in agreement with the morphology of the species and with biogeographical patterns known in other mammals of sub-Saharan Africa. We discuss possible reasons why previous genetic studies failed to find this relationship, and based on the revised divergence dating, we also propose an alternative evolutionary scenario for the diversification of sengis in the subfamily Macroscelidinae, which is likely much younger than previously thought.

2 | MATERIALS AND METHODS

2.1 | Sampling

In this study, we analyzed genetic diversity of 48 recently collected elephant shrews. This material includes one *Rhynchocyon cirnei* (Peters, 1847) from Mozambique, one *P. rozeti* from Morocco, six *Petrodromus tetradactylus* (Peters, 1846), and 40 *Elephantulus* specimens belonging at least to five species (Figure 1, Table S1) as identified by external morphology and a preliminary analysis of a mitochondrial marker (see below). Most of the *Petrodromus* and *Elephantulus* specimens originated from eastern Africa (Ethiopia, Kenya, Tanzania, Mozambique, Malawi), Zambia, and Angola, that is, regions significantly under-sampled in previous studies of genetic diversity of elephant shrews. All fieldwork complied with legal regulations in particular African countries, and sampling was in accordance with local legislation (see more details on wildlife authorities that permitted the research in Acknowledgements).

Our new material was complemented by genetic data from GenBank, collected from 326 elephant shrews from the subfamily Macroscelidinae, most of them from South Africa and Namibia, but we also included recently published sequences of *G. revoilii* from Djibouti (Heritage et al., 2020) (Table S1).

2.2 | Genotyping

DNA was extracted by commercial kits, and all samples were Sanger-sequenced for the mitochondrial marker *cytochrome b* (*CYTB*) and six nuclear markers, that is, exon 1 of *interphotoreceptor retinoid-binding protein* gene (*IRBP*), exon 28 of *von Willebrand factor* gene (*vWF*), gene for *recombination activating protein 1* (*RAG1*), *beta-fibrinogen* intron 7 (*FGB*), *7-dehydrocholesterol reductase* (*DHCR*), and *wnt ligand secretion mediator* (*WLS*). We specifically genotyped *IRBP* and *vWF*, that is, two markers that have been used in previous phylogenetic studies of elephant shrews, to allow the combination of our new and older published datasets. For more details on the molecular markers used (i.e., primer sequences, genotyping protocols, amplicon lengths, and alignment lengths), see Table S2. All new sequences were submitted

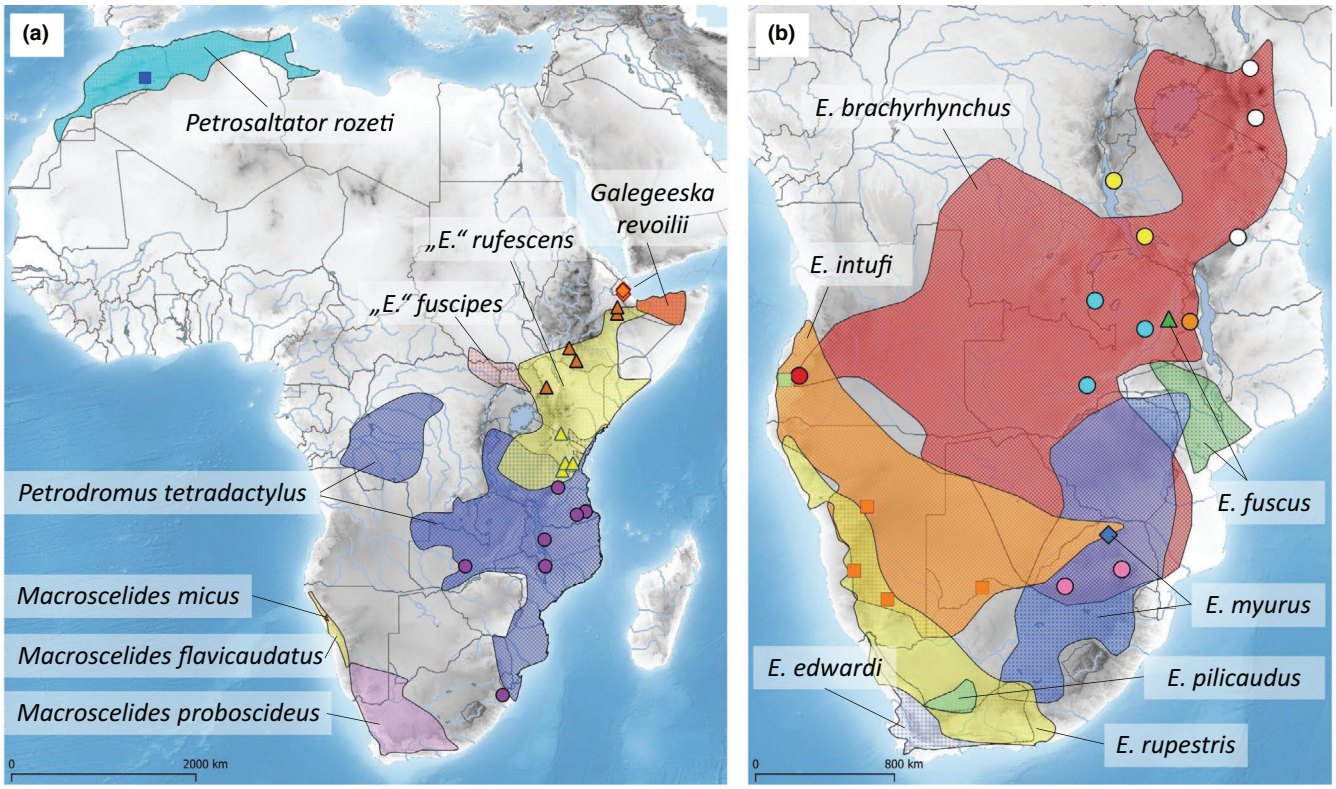

FIGURE 1 Distribution of species of the subfamily Macroscelidinae and localities of samples newly analyzed in this study. (a) Species from the clade 1 (tribe Macroscelidini), as identified in this study. Note that *Elephantulus fuscipes* is included in this map, but its taxonomic position requires further research. (b) Species from the clade 2 (tribe Elephantulini). Different symbol shapes indicate different species, possible intraspecific clades are distinguished by different colors. Only the newly analyzed material is shown on the map, except the georeferenced and genotyped samples of *Petrodromus tetradactylus* (circles), *Galegeeska revoilii* (rhombuses), *Elephantulus intufi* (squares), and *Elephantulus brachyrhynchus* (circles) taken from the previous studies. The predicted distribution of particular species is taken from IUCN maps (https://www.iucnredlist.org/)

to GenBank under accession numbers MW344964-MW345237 (Table S1), and aligned sequences used in particular analyses are available as Data S1.

2.3 | Mitochondrial phylogeny

In the first step, we reconstructed the mitochondrial phylogeny by the Bayesian inference (BI) and maximum-likelihood (ML) approaches. The *CYTB* sequences were edited and aligned in Geneious 9.0.5 (Biomatters, Ltd.). Based on the preliminary analysis, we selected 108 sequences representing unique haplotypes and covering the diversity of the subfamily Macroscelidinae; that is, we removed redundant, short, and low-quality sequences (Table S1). The FindModel web application (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to identify the most appropriate substitution model for the ingroup alignment. The Akaike information criterion (AIC), compared among 12 biologically relevant substitution models, revealed that the model best fitting the data was General Time Reversible plus Gamma (GTR + G). We used four sequences of other species of Afrotheria for outgroups. Close relatives were represented by two species of elephant shrews from the subfamily Rhynchocyoninae, specifically *Rhynchocyon petersi* (GenBank accession no. KU756166) and *R. udzungwensis* (KF742619), while as more distant outgroup, we used *CYTB* sequences of aardvark *Orycteropus afer* (AF107724) and rock hyrax *Procavia capensis* (KM245022). BI analysis was performed in MrBayes v. 3.2.6 (Ronquist & Huelsenbeck, 2003). Three heated and one cold chain were employed with the runs initiating from random trees. Two independent runs were conducted with 10 million generations each; the trees and parameters were sampled every 1000 generations. Convergence was checked using TRACER 1.7 (Rambaut et al., 2018). For each run, the first 20% of sampled trees were discarded as burn-in. Bayesian posterior probabilities were used to assess branch support of the MCMC tree. ML analysis was performed in RAxML 8.2.8 (Stamatakis, 2014), using GTRCAT substitution model as suggested by the authors of the program. The robustness of the nodes was evaluated by the default bootstrap procedure with 1,000 replications.

2.4 | Species tree based on nuclear sequences

The species tree of the subfamily Macroscelidinae was estimated by using the multispecies coalescent model as implemented in STARBEAST 2 (Ogilvie et al., 2017). For this purpose, we used 42 newly collected specimens genotyped at 4–6 nuclear loci,

supplemented by 44 specimens from the GenBank, sequenced at *RAG1* (2 sequences), *IRBP* (31 sequences), and *vWF* (39 sequences) markers (Table S1). Based on the results of mitochondrial phylogeny and morphological identification, the specimens were assigned to 14 species (Table S1), including the split of *E. brachyrhynchus* into one widespread and one Angolan candidate species (see below).

We imported alignments for each marker into BEAUTI 2.4.7 and set the HKY substitution model for each partition (i.e., nuclear locus). The species tree shape was modeled by a birth–death process (Gernhard, 2008) with an uninformative prior for the net diversification rate and a weakly informative prior, Beta(2,2), for the fraction of extinct species. The constant per-branch population sizes were analytically integrated using approach of Hey and Nielsen (2007) as implemented in STARBEAST 2.4.6. Because we do not expect differences in substitution rates among lineages within Macroscelidinae, we assumed a strict molecular clock but partition-specific relative clock rates. We ran two independent runs with 50×10^6 generations with sampling every 5000 generations. The convergence was examined in TRACER 1.7 (Rambaut et al., 2018) with effective sample sizes >200 considered as sufficient for good parameter estimation. Based on TRACER, the first 10% iterations were discarded as a burn-in. The two runs were combined in LOGCOMBINER 2.4.7, the maximum credibility species tree was produced by TREEANNOTATOR 2.4.6. All phylogenetic analyses were run on CIPRES Science Gateway (Miller et al., 2010).

Using similar methodologies, we also performed alternative analyses. First, because most of specimens retrieved from GenBank and used in the reconstruction of species tree were genotyped at only two nuclear markers (*vWF*, *IRBP*), we performed multispecies coalescent analyses based solely on these two markers. Second, we used the reduced (i.e., two loci) dataset and defined 20 candidate species (instead of 14) based on the structure revealed by mtDNA phylogenetic tree (see Results). Specifically, we split *E. rufescens* into "northern" and "southern" species, *E. intufi* into "South African" and "Angolan" species, and *E. brachyrhynchus* into "Angolan" and five Southeastern African species (see Table S1). The *xml* files used for STARBEAST analyses are provided as Data S2.

2.5 | Divergence dating

Time-calibrated species tree was inferred in STARBEAST for 14 species of Macroscelidinae and two species of *Rhynchocyon* using *vWF* and *IRBP* only, the two nuclear markers genotyped in all these species (*R. cirnei* and *R. petersi* were grouped together as they were indistinguishable at these two nuclear markers; Table S1). The tree was calibrated on its root using *Miorhynchocyon meswae* (Butler, 1984), the oldest known representative of Rhynchocyoninae from the Oligocene–Miocene boundary, 23.5 Ma (Butler, 1984) or 23–22 Ma (Holroyd, 2010). While at least three species of Rhynchocyoninae and two of Macroscelidinae are known from the early Miocene (20–16.8 Ma), only extinct subfamilies are present in early Oligocene and Eocene layers (reviewed by Holroyd, 2010). Accordingly, we calibrated the root with gamma distribution (shape = 2, scale = 2.4, offset = 22), which puts split of Macroscelidinae and Rhynchocyoninae to Oligocene (median = 26 Ma). We used the same model settings as specified above, except relaxed uncorrelated lognormal clock was specified separately for each marker. The *xml* file used for this analysis is provided in Data S2. The convergence of two MCMC runs was checked, and their outputs were summarized by the same way as described above.

2.6 | Karyotypes

For the first time, we described the karyotype of the Rufous Sengi and compared it with other species. Samples of metaphase plate have been obtained from bone marrow of four Ethiopian specimens of *E. rufescens* (LAV2930, LAV2937, LAV3415, and LAV3416) by using a classical procedure (Ford & Hamerton, 1956). Giemsa staining was performed in 4% solution.

2.7 | External morphology

Standard measurements of *E. rufescens* specimens collected during fieldwork were obtained (body mass, length of head + body, tail, ear, and hind feet; Table S3). Males were examined for the presence of nipples (a synapomorphic character of the genera *Petrodromus* and *Petrosaltator*; Olbricht & Stanley, 2009). In addition, we focused on diagnostic traits of *G. revoilii* and *E. rufescens* highlighted in Heritage et al. (2020) and examined them in our material of *E. rufescens*. Those characteristics are as follows: size of the second upper incisor (in *G. revoilli*, it is of the same size as the first and the third incisor, whereas remarkably smaller in *E. rufescens*), presence of hairs on tail (in *G. revoili*, the tail is relatively hairy with a tuft on the distal tip; *E. rufescens* should have short hairs and lacks tuft), haired inferior part of rhinarium (synapomorphy of *G. revoilii* and *E. rufescens*), and pale eye ring with a dark post-ocular spot.

2.8 | Penile morphology

The penis of all sengis is very long, extending from the pelvis up to the sternum. The shape of the tip of the glans penis is highly variable, and it seems to be specific for each genus (Woodall, 1995), providing additional diagnostic criteria for previously proposed traits (e.g., Corbet, 1971; Corbet & Hanks, 1968) and as phylogenetically valuable characters for sengis (Corbet, 1995; Douady et al., 2003). All the sampled males of *E. rufescens* (eight in total) were studied, but only two individuals from the northern and one from the southern clade (see Results), captured in Ethiopia (Jaldessa area), Kenya (Nassalot National Reserve), and Tanzania (Ibuti), were usable for making comparisons of penile morphology in detail due to good tissue preservation. The morphology

of the penis was also compared with four samples of *E. brachyrhynchus* and two of *E. intufi*, both species represented by individuals collected from Angola. The animal bodies were stored and preserved in 70% ethanol until processing in 2019. Dissected penises were later fixed in 10% formaldehyde. Penile characteristics were observed under a binocular microscope (Nikon SMZ 1500), documented by a digital camera, and compared with Woodall (1995).

2.9 | Identification of diagnostic characters of *Galegeeska*

As we identified *E. rufescens* as a closely related species to *Galegeeska revoilii* (see Results), the diagnosis of the recently delimited genus *Galegeeska* requires a revision. Therefore, we compared morphological, reproductive, and chromosomal traits (e.g., Corbet & Hanks, 1968; Heritage et al., 2020; Perrin & Rathbun, 2013) across Macroscelidinae (sensu Heritage et al., 2020) to find apomorphies of the *Galegeeska* clade, using our most complete and updated phylogeny of the subfamily. Definitions of characters followed the original source (e.g., Corbet & Hanks, 1968; Perrin & Rathbun, 2013; Heritage et al., 2020; and references in Matrix S1). Altogether, 68 compiled morphological, reproductive, and chromosomal characters (Matrix S1) were optimized by NONA (ver. 2.0) through the WINCLADA interface (ver. 1.00.08; Nixon, 1999) using the unweighted maximum parsimony approach on a simplified phylogenetic tree (we used the topology of the multilocus species tree). We gave no preference to ACCTRAN nor DELTRAN optimization when alternative reconstructions were of equal cost (for the terminology of optimization, see Agnarsson & Miller, 2008). The taxon sampling composed of 11 taxa is adapted from Corbet and Hanks (1968) to maximize the comparison.

3 | RESULTS

3.1 | Mitochondrial phylogeny

Phylogenetic analysis of *CYTB* sequences provided only a partially resolved mtDNA tree of the subfamily Macroscelidinae (Figure 2a). While all but one (*E. pilicaudus*) species formed strongly supported (PP = 1.00, BS = 99 or 100) monophyletic clades, the supports for their mutual relationships were variable. The genus *Macroscelides* is monophyletic, with *M. flavicaudatus* being sister to *M. proboscideus* (PP = 1.00, BS = 100). The genus *Elephantulus* is composed of three well-supported clades: (1) *E. myurus*, *E. edwardi*, and *E. pilicaudus*, with two latter species being sisters; (2) (*E. rupestris* + *E. intufi*) and their sister lineage (*E. fuscus* + *E. brachyrhynchus*); and (3) *E. rufescens*. While the sister relationship of clades (1) and (2) is the most probable in the Bayesian analysis, albeit with relatively weak support (PP=0.87), *E. rufescens* is the highly supported sister species of *G. revoilii* (PP = 1.00, BS = 100). The phylogenetic position of the latter clade, and of genera *Petrosaltator* and *Petrodromus* within Macroscelidinae is not well resolved by the analysis of mtDNA. On the other hand, mtDNA indicates strong intraspecific phylogeographic structure (or even the presence of cryptic species, as suggested by genetic distances), for example, in *E. rufescens* (with northern and southern lineages), *E. intufi* (with an obvious divergence of the Angolan specimens), and *E. brachyrhynchus* (with clearly distinct Angolan samples and up to five parapatric lineages in Southeastern Africa) (Figure 2a; for the distribution of lineages, see Figure 1).

3.2 | Species tree and divergence dating

Contrary to the mtDNA phylogeny, the nuclear species tree from STARBEAST is almost fully resolved (PP for all but one node is >0.98; Figure 2b). The only exception is the sister relationship between genera *Petrosaltator* and *Petrodromus* with only moderate support (PP = 0.81). The results were very similar regardless of the number of loci used (two or six) and number of candidate "species" (14 or 20) (see Figure S1 for alternative trees). The subfamily Macroscelidinae is divided into two major clades that correspond to recently defined tribes Macroscelidini and Elephantulini (*sensu* Dumbacher, 2016). Most surprisingly, *E. rufescens* clearly belongs to Macroscelidini, as strongly supported sister lineage of *G. revoilii*. The relationships among species of Elephantulini are the same as suggested by mtDNA tree, and this topology is fully resolved by nuclear markers (all PP=1.00; Figure 2b).

The time-calibrated tree of Macroscelidea (Figure 3) has almost the same topology as the species tree of Macroscelidinae based on six loci (Figure 2b). The only exception is the position of *Petrosaltator*, which is here a sister to the recently erected genus *Galegeeska* (including *E. rufescens*). This relationship has only moderate support (PP=0.82) on the time-calibrated tree, and the discrepancy is likely caused by the reduced number of nuclear markers in this analysis (compare with Figure S1, where the trees are also based on *IRBP* and *vWF* loci only). The sister relationship between *G. revoilli* and *E. rufescens* is only moderately supported here (PP=0.89). Otherwise, the tree is well resolved (PPs ≥0.98). The tree shows the most recent common ancestor (MRCA) of Macroscelidinae and Rhynchocyoninae to live in Oligocene, 25.7 Ma ago with 95% highest posterior density (HPD) interval 22.1–30.9 Ma. The split between the tribe Macroscelidini and the genus *Elephantulus* (excluding *E. rufescens*) was dated to the middle Miocene, 11.8 (7.4–16.7) Ma, MRCA of Macroscelidini lived 8.5 (5.1–12.2) Ma, and MRCA of *Elephantulus* lived 9.3 (5.7–13.5) Ma. All other divergences date back to the end of the Miocene up to the Plio-Pleistocene, including the origin of *Petrodromus* at 4.5 (2.0–6.9) Ma and *Petrosaltator/Galegeeska* split at 3.0 (1.1–5.3) Ma. The MRCA of *G. revoilli* and *E. rufescens* was dated to the Pleistocene, 1.7 (0.5–3.1) Ma.

3.3 | Karyotype of *E. rufescens*

The diploid number of the examined specimens is 26. The karyotype consists of 12 pairs of biarmed (metacentrics and submetacentrics) autosomal chromosomes continually decreasing in size; thus, the autosomal fundamental number is 48. (Figure 4). In one specimen from Borena NP (LAV2930) we found one heteromorphic pair (no. 5 at Figure 4a)

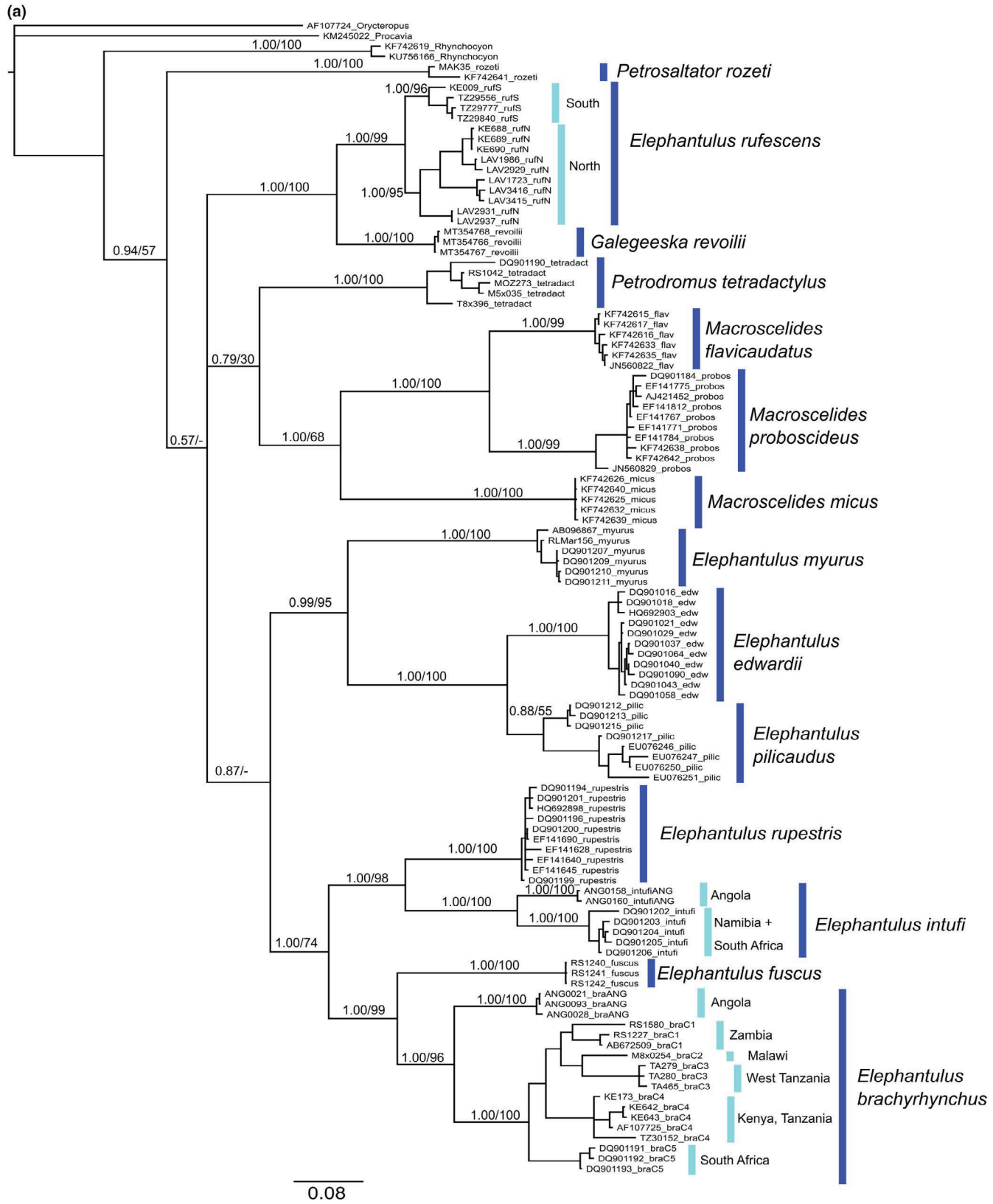

FIGURE 2 (a) Mitochondrial phylogeny of sengis. Bayesian tree based on 108 unique *CYTB* sequences (+ four outgroups) is shown. Numbers above branches show posterior probability from MrBayes/bootstrap support from RAXML for major nodes. Light blue bars indicate intraspecific mitochondrial lineages. (b) Species tree of the subfamily Macroscelidinae inferred from six nuclear loci using a multispecies coalescent approach in STARBEAST2. The numbers above branches indicate posterior probability

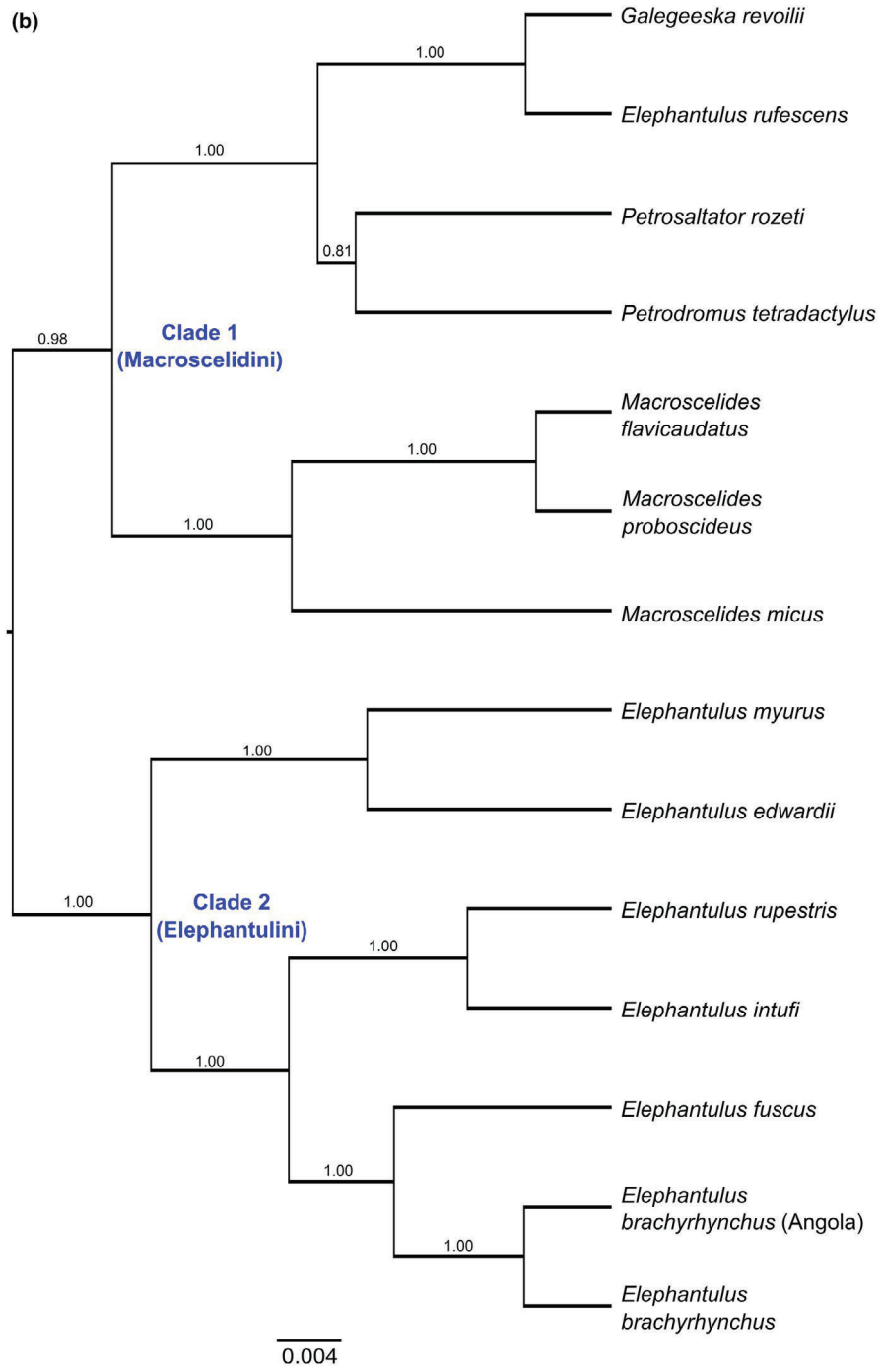

FIGURE 2. (Continued).

represented by one submetacentric and one large acrocentric chromosome. X chromosome is small metacentric, and Y chromosome is the smallest submetacentric (Figure 4).

3.4 | External morphology of *E. rufescens*

The mean body mass of adult specimens of *E. rufescens* is 62.4 grams, and the relative tail length is 104.5% of the head+body length (Table S3). All animals possess a relatively wide white ring around the eye, which is broken by brown post-ocular spot and a rather wide stripe extending posteriorly. The inferior part of rhinarium is covered with dense short fur. The fur on the feet is mainly uniformly white. Examined males do not possess nipples. Apart from animals with worn teeth, it was clear that the second incisors are smaller compared with the first and the third one. The tail had remarkable homogeneous hair cover without a tuft at the distal tip.

3.5 | Penile morphology

The collar-like penis was detected in the southern clade of *E. rufescens* (Figure 5d–f), but the disk-like penis in the northern clade *of E. rufescens* (Figure 5a–c). Specifically, the glans penis of the southern clade

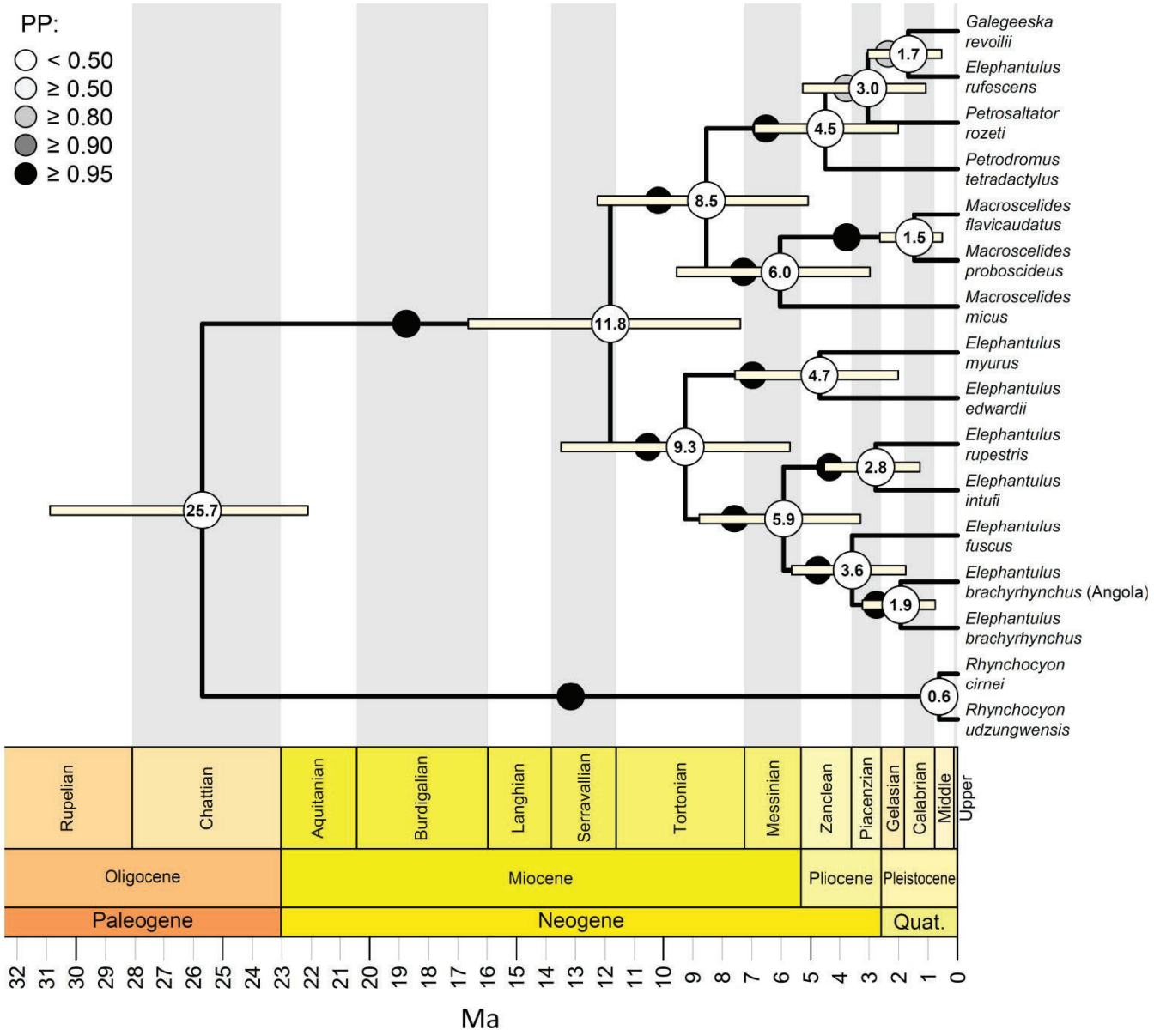

FIGURE 3 Time-calibrated species tree of the extant Macroscelidea. The numbers in white circles are node ages in Ma before present, and the associated yellowish bars indicate their 95% HPD intervals. Posterior probabilities of the nodes are shown in shades of gray on the branches supporting them

exhibited a high degree of similarity to *M. proboscideus* (Woodall, 1995—Plate IV, f), but with much deeper lateral lobes forming the collar. The penis of the northern clade almost matched to the sketch of the tip of glans penis in *E. rufescens* presented by Woodall (1995, Figure 2b), and showed also the similarity with *E. edwardii* (plate V-d, in Woodall, 1995), but the lateral lobes were ventrally much closer and the crater with the urethra was more recessed.

3.6 | Identification of diagnostic characters of *Galegeeska*

The parsimony analysis (Figure 6) recognized three apomorphies of the *Galegeeska* clade, specifically hairy rhinarium below (trait no. 2, non-homoplasious apomorphy), fully developed pectoral gland (no. 8, non-homoplasious apomorphy), and y-shaped sulci of the dorsal openings for the stapedial artery (no. 56, homoplasious apomorphy) (for more details on used traits, see Matrix S1). Two more characters might be diagnostic for *Galegeeska*, because they are shared within Macroscelidini only by *revoilii* and *rufescens*, but the reconstruction of ancestral conditions of some clades remains uncertain due to heterogeneous character states in related species. Both *Galegeeska* species exhibit a slightly developed double root in C1 and an absence of bicolored tail (*revoilii* with a white tail and *rufescens* with dark brown) (Corbet & Hanks, 1968; Perrin & Rathbun, 2013). The results of an alternative analysis (with *Galegeeska* being sister of *Petrosaltator* as suggested by the species tree based on two nuclear loci only) were very similar (for details, see Figure S2).

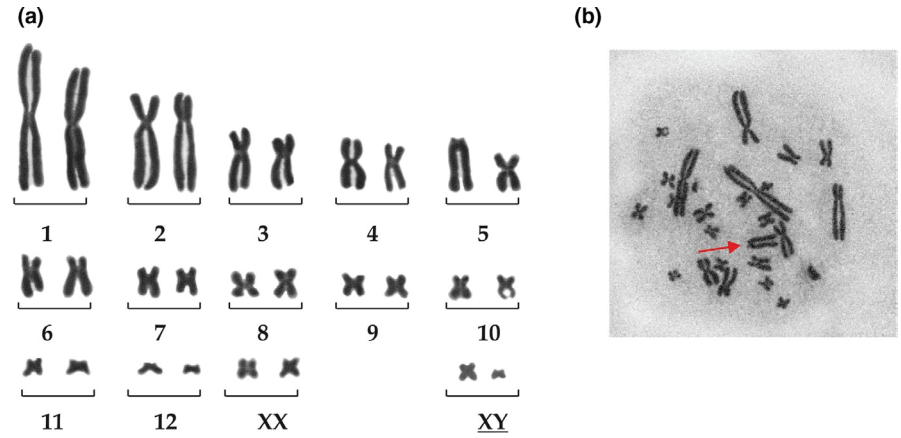

FIGURE 4 Karyotype of *Elephantulus rufescens* from Ethiopia. (a) Giemsa-stained karyotype based on female specimen LAV2930. XY pair is shown for a male specimen LAV2937. (b) Metaphase plate obtained from the specimen LAV2930. Arrow shows large acrocentric (for details, see text)

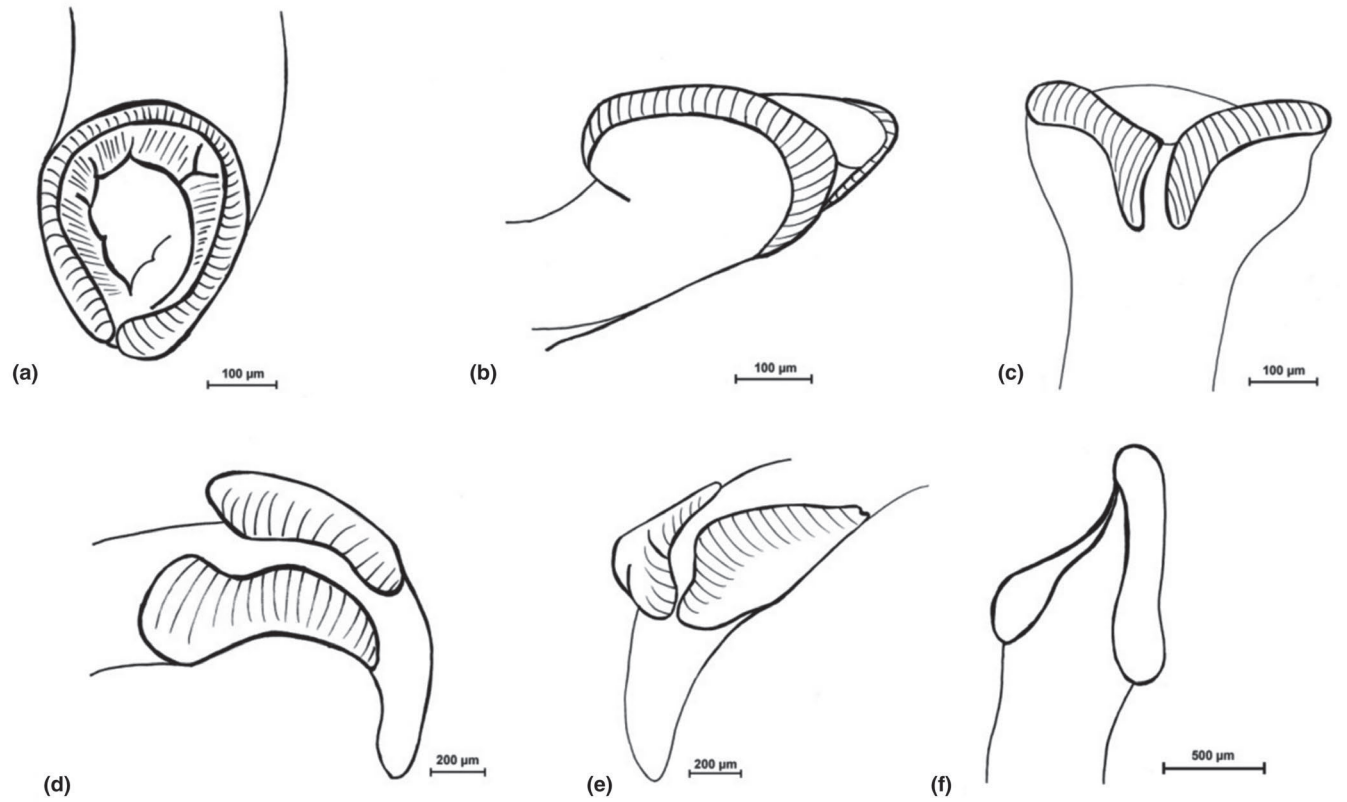

FIGURE 5 Schematic drawing of the tip of the glans penis in the northern (a–c) and the southern (d–f) clades of *Elephantulus rufescens*, with the particular scales. Dorsal view (a); lateral view (b, d, e); ventral view (f); and mid-ventral view (c)

4 | DISCUSSION

The most important result of our study, which represents the most complete multilocus phylogenetic analysis of the subfamily Macroscelidinae, is unambiguous evidence for the sister relationship of the Rufous Sengi (*E. rufescens*) with the Somali Sengi (*G. revoilii*). Both species share multiple morphological apomorphies and are related to the genera *Petrodromus* and *Petrosaltator*. We therefore propose to include the Rufous Sengi into the recently erected genus *Galegeeska* (Heritage et al., 2020), and hereafter, we call this species *Galegeeska rufescens*. Below we summarize additional supporting facts for this taxonomic change and discuss the revised evolutionary scenario for the subfamily Macroscelidinae and the reasons why the phylogenetic position of *G. rufescens* remained obscure for such a long time.

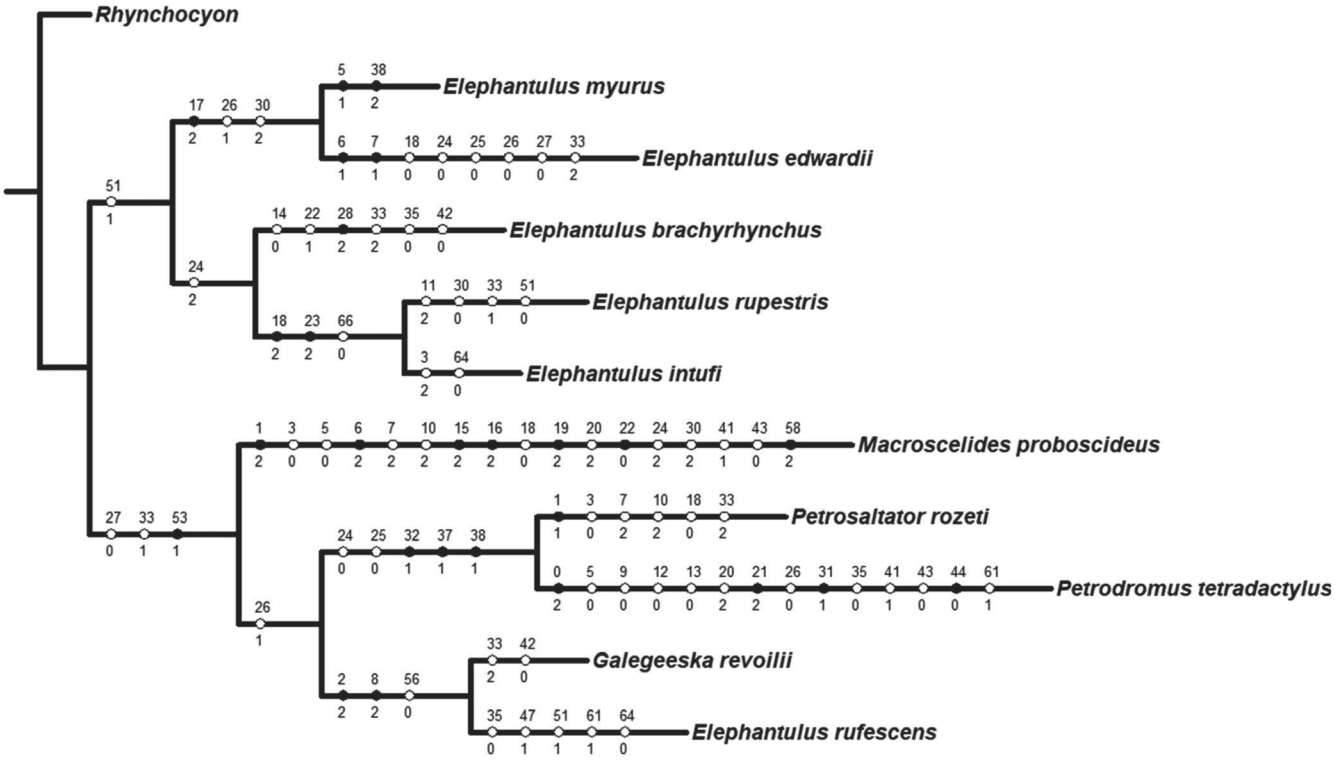

FIGURE 6 Overview of non-homoplasious (black) and homoplasious (white) apomorphies reconstructed by the maximum parsimony approach and the topology of the species tree, where *Petrosaltator* and *Petrodromus* are sister taxa (i.e., based on six nuclear markers). Numbers above circles indicate character numbers and numbers below character states (for more details, see Matrix S1). Ancestral states for Macroscelidinae presented as a list of character number (with character state for the particular character in bracket): 0(0); 1(0); 2(0); 3(1); 4(0); 5(2); 6(0); 7(0); 8(0); 9(2); 10(0); 11(?); 12(2); 13(2); 14(2); 15(0); 16(0); 17(0); 18(1); 19(1); **20(1)**; 21(0); 22(2); 23(0); 24(1); **25(2)**; 26(2); 27(2); 28(0); 29(2); 30(?); 31(2); 32(0); 33(0); 34(?); 35(1); 36(0); 37(?); 38(0); 39(?); 40(0); 41(0); 42(1); 43(1); 44(1); 45(1); 46(1); 47(0); 48(1); 49(?); 50(0); 51(0); 52(1); 53(0); 54(1); 55(1); 56(1); 57(0); 58(?); 59(0); 60(1); 61(0); 62(0); 63(?); 64(1); 65(0); 66(1); 67(0). Ancestral states that vary according to different relationships between *Petrodromus* and *Petrosaltator* are in bold

4.1 | Multiple evidence for the Rufous Sengi belonging to the genus *Galegeeska*

Even if the newly revealed position of the Rufous Sengi might seem surprising (because it was not recovered in previous molecular phylogenetic studies; see below), there have been previously supported indicators of its taxonomic status. First of all, the close relationship between *G. revoilli* and *G. rufescens* is reasonable from a biogeographical point of view. Contrary to other sengis (with a single exception of *E. brachyrhynchus*, whose distribution in the northernmost part overlaps with that of *G. rufescens*), both *Galegeeska* species live in the Somali-Masai biogeographical region (sensu Linder et al., 2012). The genus *Galegeeska* can thus be considered a typical element of Somali-Masai fauna, similar to the deeply divergent clades characteristic for this region, revealed by recent studies in rodents (e.g., Aghová et al., 2017, 2019; Mikula et al., 2016).

External morphology of *G. rufescens* is very similar to *G. revoilii*. The spectacled eye pattern and hairy lower rhinarium are two traits uniquely shared by these two species suggesting their sister relationship (see Figure 3 in Heritage et al., 2020). A distinguishable white circle around the eyes and a dark spot behind can also be found in the genus *Petrodromus*, and it is traditionally mentioned as their synapomorphy (Corbet & Hanks, 1968). Nevertheless, the white ring in *Galegeeska* is remarkably wider, continuing posteriorly together with the dark spot, which differs from *Petrodromus* (see Heritage et al., 2020). The detailed inspection of our material confirmed that *G. rufescens* possess a rhinarium that is hairy below, which is thus a character occurring only in *G. rufescens* and *G. revoilii* (Corbet & Hanks, 1968). Both species nevertheless significantly differ in relative tail lengths to head + body lengths, which are 104% in *G. rufescens* and 117% in *G. revoilii* and mean body mass (62.4 g and 49.9 g, respectively) (Table S3; Heritage et al., 2020). Considering male nipples, Olbricht and Stanley (2009) mentioned that this character is present only in *Petrodromus* and *Petrosaltator*. Heritage et al. (2020) mentioned that *G. revoilii* lacks male nipples, this character therefore seems to be unique for the genera *Petrodromus* and *Petrosaltator* and provides additional support for their sister relationship, in agreement with our 6-loci species tree (Figure 2b). We did not find any hair tuft on the tail in *G. rufescens*, which is also similar to *G. revoilii* (Heritage et al., 2020). Although less bushy than in *G. revoilii* from the Djibouti population, we found substantial hair cover across the whole tail in all specimens of *G. rufescens*. This does not agree with Figure 3 in Heritage et al. (2020). Lack of fur on the tail of the voucher specimen inspected by Heritage et al. (2020) can potentially be explained by the preparation method or by its old age (it was collected in 1912).

Four basic types of glans penis were described in elephant shrews: (i) spatulate-like with a distal row of spines (*Rhynchocyon*), (ii) spear-like with two lateral lobes (*Petrodromus*), (iii) collar-like with distal expanded tip (*Macroscelides*), and (iv) disk-like with two lateral lobes (*Elephantulus*) (Woodall, 1995). Surprisingly, the collar-like penis was detected in the southern clade of *G. rufescens*, but the disk-like penis in its northern clade. It seems that the genus *Galegeeska* could share glans penis features of distant clades (*Elephantulus* and *Macroscelides*), but it remains unclear whether this is a result of convergent evolution or the presence of polymorphisms in its ancestors. A detailed survey of penile morphology of *Galegeeska* and the whole Macroscelidini is required for the proper inspection of this issue, in addition to quantifying inter- and intraspecific variability.

Chromosomal variability within Macroscelidinae is apparently low. Thus, in the most comprehensive overview of sengis cytogenetics, Smit et al. (2011) have shown identical diploid number (26) for all representatives of the subfamily with three exceptions: 2*n* = 30 for *E. myurus* and 2*n* = 28 for *Petrosaltator rozeti* and *P. tetradactylus*. The karyotype of *G. rufescens*, presented for the first time in our study, revealed high similarity (at least by the routine staining) with those of *E. intufi* and *M. proboscideus* (McDonough & Sotero-Caio, 2019; Smit et al., 2011). Taken into account the large phylogenetic distances among these taxa (Figure 2), one can assume a karyotype with 2*n* = 26 as a plesiomorphic character state for the Macroscelidinae subfamily. This hypothesis is supported also by our parsimony analysis (Figure 6).

4.2 | Identification of diagnostic characters of *Galegeeska*

The sister relationship between *G. rufescens* and *G. revoilii* was suggested already by Corbet (1995) based on his comprehensive overview of morphological and reproductive characters (Corbet & Hanks, 1968). Our analysis of the same dataset with additional characters identified several definitive and some possible apomorphies of the *Galegeeska* clade. They are related to external (hairy rhinarium, pectoral gland, tail coloration), dental (roots in C1 and cusp in P1), and anatomical (stapedial artery) features. The non-homoplasious nature of the fully developed pectoral gland can be modified by future analyses, because it is possessed also by *E. fuscipes* (Corbet & Hanks, 1968), whose DNA sequences are not available. Heritage et al. (2020) mentioned the following characters as diagnostic for *Galegeeska*: pale eye ring with a dark post-ocular mark, hair on the lower portion of the rhinarium, a tufted tail, and second upper incisors that are subequal in size to adjacent upper incisors. However, because *G. rufescens* was not recognized as a member of the genus, some of these diagnostic features were not genus-specific, but species-specific to *G. revoilii* (Heritage et al., 2020). Our analysis supported only the hairy rhinarium and possibly the pale eye ring with a dark post-ocular mark with a thick white strip above it (i.e., the modified definition of this character) as genus-specific characters of *Galegeeska*.

TABLE 1 Overview of sequences of the Rufous Sengi used in previous phylogenetic studies

Locus	GenBank	Origin	Sengi phylogeny[a]
12S+16S rRNA	U97339	Springer et al., 1997	(1), (2), (3)
CYTB	AF107725	Springer et al., 1999	(1), (3), (4)
IRBP	U48584	Stanhope et al., 1996	(1), (2), (3), (4)
vWF	U31612	Porter et al., 1996	(1), (2), (3), (4)
GHR	AF392876	Malia et al., 2002	(1), (4)

[a]References: (1) Kuntner et al., 2010; (2) Smit et al., 2011; (3) Heritage et al., 2020; (4) Upham et al., 2019. The study of Douady et al., 2003 is not included, because they do not provide detailed data ("... sequences were extracted from GenBank."). The study of Upham et al., 2019 used additional 20 DNA sequences from GenBank, a majority of them obtained within the phylogenetic studies of the order Mammalia, so likely originating from the same (misidentified) voucher specimen as in the studies mentioned in this Table.

4.3 | Why the correct phylogenetic position of the Rufous Sengi was not discovered earlier?

To our knowledge, there are five previous studies that included the Rufous Sengi in phylogenetic reconstructions and they all consistently reported that it belongs to the genus *Elephantulus* as a sister species to *E. brachyrhynchus* (Douady et al., 2003; Heritage et al., 2020; Kuntner et al., 2010; Smit et al., 2011; Upham et al., 2019). This result is in contrast to our results, and one must ask for the reasons for such a discrepancy. All of these previous studies were based on the limited number of sequences of *G. rufescens* from GenBank (Table 1). These sequences were produced in the frame of several influential studies dealing with the phylogenetic relationships of mammalian orders in the 1990s, leading (among others) to the discovery of the taxon Afrotheria (Malia et al., 2002; Porter et al., 1996; Springer et al., 1997, 1999; Stanhope et al., 1998). Although none of the studies describes an actual voucher specimen linked to the associated sequences, the most parsimonious explanation of the phylogenetic discrepancy is species misidentification. All but one (GHR gene; Malia et al., 2002) of these studies were published by M. J. Stanhope as senior author, and it is very likely that all sequences originated from the same specimen of *E. brachyrhynchus* and not *G. rufescens*, probably from Kenya as suggested by our genetic analysis (see Figure 2a—analysis of *CYTB* placed the sequence AF107725 into the clade of *E. brachyrhynchus* together with other Kenyan samples). Because the distribution of *G. rufescens* and *E. brachyrhynchus* overlaps in Kenya and Tanzania (and the aim of the above-mentioned studies from the 1990s was not to resolve relationships among sengis; the sequence was always used just as a representative of Macroscelidea), it is possible that the sequences were uploaded

to GenBank erroneously as "*Elephantulus*" *rufescens*, even if they in fact represent *E. brachyrhynchus*. On the other hand, the GenBank sequence of the *RAG1* gene (AY011877; Murphy et al., 2001) seems to originate from the correctly identified Rufous Sengi as it unambiguously clustered with our new sequences of this species (but the voucher specimen is also not detailed in Murphy et al., 2001). However, this gene has never been used in previous phylogenetic analyses of sengis (Table 1), with the exception of Upham et al. (2019), where its effect on phylogenetic placement was overwritten by 23 additional DNA fragments downloaded from GenBank. Another factor causing the misplacement of the Rufous Sengi in previous phylogenetic trees is the generally low amount of available genetic data from sengis from eastern Africa as most previous research focused on the southern part of the continent (see references in Introduction). These facts again strengthen the necessity of a collection of fresh samples from understudied areas directly in the field (together with as much additional data as possible), as well as the need for linking physical voucher specimens to GenBank sequences, especially if they should be used for genomic analyses of biodiversity and its evolution (reviewed by Ferguson, 2020; see Heritage et al., 2020, for a recent example in sengis).

4.4 | Revised evolutionary scenario for Macroscelidinae

The most detailed evolutionary biogeographical scenario of Macroscelidea was recently proposed by Heritage et al. (2020). Compared with their study, all estimates of divergence times presented here are significantly younger. In particular, our dating of the split between *G. revoilii* and *G. rufescens* (1.7 Ma) is in striking contrast to 5.4 Ma estimated by Heritage et al. (2020) as the intraspecific divergence time of Djiboutian and Somalian *G. revoilii*. This is even more striking given that both studies put similar calibration densities on the MRCA of extant Macroscelidea. The likely reason for this difference is methodological. The concatenated analysis of Heritage et al. (2020) (and to some extent also Douady et al., 2003) cannot account for coalescences that are deep due to demographic stochasticity rather than to species divergence, which is particularly important when both mitochondrial and nuclear loci are included. Furthermore, conspecific sengis were analyzed together with representatives of deeply divergent Afrotherian species and such unbalanced sampling could also contribute to overestimation of their divergence times. While definitely younger than in Heritage et al. (2020), our estimates of divergence times are burdened with large uncertainty, which precludes associating them precisely with particular geoclimatic events. Clearly, there is a need for a phylogeny based on many more loci (e.g., flanking regions of ultraconserved elements; McCormack et al., 2012) and calibrated by more fossil data (e.g., via fossilized birth–death model; Heath et al., 2014).

However, our dating, together with the corrected phylogenetic position of *G. rufescens*, invokes a significantly modified evolutionary scenario for the subfamily Macroscelidinae. Heritage et al. (2020) proposed that the common ancestor of Macroscelidini lived in Central Africa during the late Oligocene (25.5 Ma), from where it colonized the entire continent of Africa from the Maghreb region (*Petrosaltator*), through the Horn of Africa (*Galegeeska*), central and eastern Africa (*Petrodromus*) to Africa´s southern cape (*Macroscelides*). Our data agree with this biogeographical pattern, but the timescale is strikingly different. The most recent common ancestor of Macroscelidini was estimated to 8.5 Ma (Figure 3), so the continental diversification was three times faster than previously thought. This is not surprising (even if all sengis have low dispersal ability as pointed out by Heritage et al. (2020)), taking into consideration the cases of colonization of whole sub-Saharan Africa by smaller mammals with presumed low dispersal capacity during Pleistocene (e.g., African Pygmy Mouse, *Mus minutoides*; Bryja et al., 2014). The split of South African *Macroscelides* and three remaining genera of the tribe (their ancestor probably occurred in East Africa) in late Miocene is in concordance with other non-forest small mammals, for example, the split of the East African clade of gerbils of the genus *Gerbilliscus* (Aghová et al., 2017, 2018) or the split of South African spiny mice (*Acomys subspinosus/spinosissimus* clade) from the rest of the genus (Aghová et al., 2018, 2019; Petružela et al., 2018). All of these divergences are estimated between 6 and 9 Ma, when the formation of the Rift Valley and the decline in global temperatures resulted in greater rainfall seasonality, and the African Miocene "coast-to-coast" forest started to be fragmented into the current Guineo-Congolese forests and coastal and mountain forests of East Africa (Bobe, 2006; Plana, 2004). It is likely that the repeated closing of savanna corridors between eastern and southern Africa by equatorial forest intensified diversification processes in organisms adapted to open habitats and worked as "a speciation pump" for such groups. The climate in the Pliocene and Pleistocene was highly variable, and multiple hypervariable periods were identified (e.g., Potts, 2013). A reversal of the general cooling and aridification trend occurred in the Early Pliocene, which represents the warmest period over the last 5 Myr (Feakins & deMenocal, 2010). The differentiation of fauna in three bioregions with predominance of open habitats (Somali-Masai, Zambezian and South African; *sensu* Linder et al., 2012) was often dated to the Miocene/Pliocene boundary (e.g., pouched mice; Mikula et al., 2016), when the East African forests may have expanded (see review in Couvreur et al. 2020). It is also in agreement with our divergence dating of the split between the genera *Galegeeska* (Somali-Masai) and *Petrodromus* (Zambezian), and similarly between Zambezian *E.brachyrhynchus/fuscus* and South African *E. rupestris/intufi* clades. These hypotheses still remain to be tested by fossil data. Unfortunately, fossil sengis from the late Miocene and early Pliocene are scarce—they were found at just two Kenyan fossils sites: Lukeino (6.1–5.8 Ma, Mein & Pickford, 2006) and Kanapoi (4.1 Ma, Manthi & Winkler, 2020), but in both cases, the remains were too fragmentary to be classified more precisely.

Subsequently, the aridification trend from the Early Pliocene led to the spread of savanna-like habitats and their flora and fauna (Bobe, 2006). Current overlap of the distributions of sengi species, which

originated in different bioregions, is clearly secondary. This is best exemplified by the finding of both *E. intufi* and *E.* cf. *brachyrhynchus* in Angola, less than 100 km from each other. Even if geographically relatively close, there is a big ecological divide between their sampling sites. While Angolan *E.* cf. *brachyrhynchus* was captured on the top of the Angolan Escarpment, whose small mammal fauna is derived from that of Zambezian savanna in the eastern Africa, *E. intufi* was found below the Escarpment, in a semidesert habitat on its western side, whose fauna has much in common with Southern Africa (Krásová et al., unpubl. data). Similarly, we can hypothesize relatively recent (definitely Pleistocene) spread of originally Zambezian *E. brachyrhynchus* to the north (where it currently overlaps with Somali-Masai species *G. rufescens*), or southward colonization of *G. rufescens* to Tanzania, where it overlaps with Zambezian *Petrodromus*.

The recently delimited genus *Galegeeska* is a typical Somali-Masai biogeographical element and its intrageneric diversification was most likely affected by the same evolutionary mechanisms as in other taxa of this region, especially rodents and reptiles. For example, phylogenetic and phylogeographic studies of murid rodents from the genera *Acomys* (Aghová et al., 2019), *Arvicanthis* (Bryja et al., 2019), or *Gerbilliscus* (Aghová et al., 2017) suggest that the most important diversification factor creating extant species diversity was Pleistocene climate oscillations. The estimated Pleistocene split between sister taxa *G. revoilii* and *G. rufescens* (ca. 1.7 Ma; Figure 3) is well concordant with other small mammals, sympatric with *Galegeeska* in the Somali-Masai region, for example, *Arvicanthis somalicus* versus *A. neumanni* (Bryja et al., 2019), two clades of *Acomys louisae* (Frynta et al., 2020), *Gerbilliscus robustus* versus *G.* sp. n. "Babile" (Aghová et al., 2017), or *Acomys mullah* versus *A.* sp. "A" (Aghová et al., 2019); all of them being dated to 1.5–2 Ma. These splits in savanna mammals can be a result of a vicariance event caused by the African rift lakes or forested mountain ranges (e.g., in Ethiopia or Somaliland) forming important barriers to gene flow during humid periods of the Pleistocene (Trauth et al., 2010; see also discussion in Aghová et al., 2017).

Distribution of divergence times also calls into question the taxonomic status of three genera in the *Petrodromus/Petrosaltator/Galegeeska* clade, because their mutual divergences are younger than those observed within the remaining two genera. One extreme solution could be the split of *Elephantulus* into several genera (i.e., *E. myurus/edwardi* clade should be a different genus). The opposite extreme was proposed by Smit et al. (2011), who suggested subsuming of *Petrodromus* and *Macroscelides* (i.e., all recognized genera of the subfamily Macroscelidinae at that time) in *Elephantulus*. Based on our most comprehensive phylogenetic study, we prefer to keep the *status quo*, but thorough morphological (or possibly ecological and behavioral) study should assess whether the currently delimited genera *Petrodromus/Petrosaltator/Galegeeska* are substantially more distinctive compared with differentiation within *Macroscelides* and *Elephantulus*.

ACKNOWLEDGEMENTS

This study was supported by the Czech Science Foundation (projects no. 20-07091J and 18-17398S to JB and RS), the Grant Agency of the University of South Bohemia (projects nos. 04-048/2019/P and 018/2017/P to JK), and the Russian Foundation for Basic Research (project no. 19-54-26003 to LAL, AAM, and DSK). Samples utilized in the study have been lawfully acquired and were collected prior to the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from Their Utilization to the Convention on Biological Diversity has been in effect. We are indebted to many local authorities for providing permits to carry out the research, especially Angolan Ministry of Environment, and Instituto Superior de Ciências da Educação da Huíla (ISCED-Huíla) in Angola, Ethiopian Wildlife Conservation Authority (EWCA), and the Oromia Forest and Wildlife Enterprise (OFWE) in Ethiopia, the Sokoine University of Agriculture in Morogoro (Tanzania), the Kenyan Forest Service and the Kenyan Wildlife Service (Kenya), Zambia Wildlife Authority (ZAWA), and National Directorate for Protected Areas (DINAC—Mozambique). We are grateful to A. Darkov (Joint Ethio-Russian Biological Expedition, Fourth Phase—JERBE IV) and S. Keskes (Ethiopian Ministry of Innovation and Technology) for managing the JERBE expedition. For help during the fieldwork and providing additional samples, we acknowledge S. Gryssels, J. Šklíba, S. Šafarčíková, M. Lövy, A. Konečný, A. Hánová, L. Cuypers, V. Mazoch, H. Konvičková, J. Zima jr., F. Sedláček, F. Vejmělka, J. Mbau, A. S. Katakweba, A. Halajian, Y. F. Ivlev, and all local collaborators. We also thank two anonymous reviewers for constructive comments on the previous version of the manuscript. We declare no conflicts of interests.

AUTHORS' CONTRIBUTIONS

JK, RS, LAL, and JB conceived and designed the study; JK, OM, RS, DSK, AAM, LAL, and JB collected the material; JK performed genotyping; JB and OM analyzed genetic data; LAL, AAM, and DSK produced karyotypes; RS, SH, and JR performed morphological analysis; JB wrote the first draft of the manuscript that was complemented by all authors. All authors also approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1. List of used specimens with details on their DNA sequences and localities.

Table S2. Details to the genotyping protocol.

Table S3. External measurements of specimens of *Elephantulus rufescens*.

Data S1. Alignments used in this study in FASTA format.

Data S2. Input data files for the STARBEAST2 (*xml* format prepared in BEAUti) analyses of species trees of the subfamily Macroscelidinae (2 or 6 loci, 14 or 20 candidate species) and for divergence dating of the family Macroscelididae.

Matrix S1. Matrix with a list of characters, definitions, and associated references, for a cladistic analysis.

Figure S1. Species tree of the subfamily Macroscelidinae inferred from two nuclear loci (*IRBP* and *vWF*) using a multi-species coalescentapproach in STARBEAST2.

Figure S2. Alternative cladistic analysis of ancestral traits.

How to cite this article: Krásová J, Mikula O, Šumbera R, et al. The Rufous Sengi is not *Elephantulus*—Multilocus reconstruction of evolutionary history of sengis from the subfamily Macroscelidinae. *J Zool Syst Evol Res*. 2021;59:918–932. https://doi.org/10.1111/jzs.12460