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Original investigation

Differentiation underground: Range-wide multilocus genetic structure of the silvery mole-rat does not support current taxonomy based on mitochondrial sequences

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ABSTRACT

The silvery mole-rat (*Heliophobius argenteocinereus*) is a solitary subterranean rodent with its distribution centred mainly in miombo woodlands of eastern Africa. This part of the continent was significantly influenced by the formation of the East African Rift System (EARS) during the last 25 Mya and by pronounced climatic changes in the Plio-Pleistocene that have caused genetic differentiation leading even to speciation events in many organisms. Recently, based on analysis of mitochondrial (mt) DNA sequences, it was suggested that *H. argenteocinereus* is a complex of six to eight species that diverged from the early to middle Miocene. In the present study, we significantly extended the sampling, re-analysed mtDNA datasets and analysed nuclear markers with the aim to assess the evolutionary history of *Heliophobius*. If we do not consider the old museum samples from south-eastern Democratic Republic of Congo (very divergent short mtDNA sequences obtained from ancient DNA, requiring further study from fresh material), the genus *Heliophobius* is composed of three major mtDNA lineages with parapatric distribution. The Rukwa Rift (+Mbeya triple junction) at the Zambia-Tanzania border, Lake Malawi, and the Eastern Arc Mountains form the biogeographical divides among these clades. The relatively shallow differences among the mitochondrial clades, divergence dating based on the use of the fossilised birth-death ratio model and a multi-locus dataset, and a very similar pattern of genetic structure to other rodents inhabiting the same area and habitat, suggest that the evolutionary history of the extant silvery mole-rat was predominantly influenced by the climatic fluctuations in the Plio-Pleistocene. Awaiting further studies employing genomic, ecological, morphological or behavioural data, we advocate for using the single name *H. argenteocinereus* for all evolutionary lineages within this taxon, because (1) comparison of the genetic structure observed in mtDNA and nuclear markers suggest hybridization between at least some mtDNA lineages in the contact zones; and (2) new samples close to the type localities suggest incorrect use of previous names.

Introduction

The immense biological diversity inEastern Africa is caused by an interplay of multiple factors, especially the presence of the Eastern African Rift System (EARS) producing very diverse geomorphology at a relatively small geographical scale and climatic changes in the Plio-Pleistocene (deMenocal, 2004; Trauth et al., 2009). These environmental changes may have had evolutionary consequences for animal taxa leading to genetic divergence, local adaptations, and speciation (Potts, 2013). Multi-species comparison of patterns in the distribution of genetic diversity is a very powerful tool for getting robust signals of historical changes in a biogeographic context. In regions where phylogeographic studies

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are still infrequent (e.g. in many tropical areas), the genetic analysis of co-existing species can bring important information about the evolution of particular ecosystems, distribution of biodiversity, or mechanisms of divergence.

Compared to montane and forested areas, phylogeographic analyses of African taxa living in open habitats are much more scarce. In mammals, most such studies have been carried out on large species such as ungulates (reviewed by Lorenzen et al., 2012) or carnivores (e.g. Dubach et al., 2005). On the contrary, small mammals (e.g. rodents), which may represent more suitable candidates for reconstructing environmental effects on the history of biota at a fine scale owing to their short generation time, limited dispersal ability and strong associations with particular habitats were less frequently studied. Data from the complex savannah mosaics in eastern Africa are especially still limited, but recent investigations revealed that the genetic structure might be very pronounced, with spatially coincident biogeographical divides across different taxa (e.g. Colangelo et al., 2013; Mazoch et al., 2018; McDonough et al., 2015; Mikula et al., 2016).

Outputs from phylogenetic and phylogeographic studies have been often used for systematic revisions by adoption of the Phylogenetic Species Concept, especially its Diagnosability Version (dPSC). Using dPSC, a species is defined as "*the smallest population or group of populations within which there is a parental pattern of ancestry and descent and which is diagnosable by unique combinations of character-states*" (Cracraft, 1997). Diagnosability is based on the lack of overlap in character space, which was originally morphospace. However, with the increasing use of genetic data in taxonomy, DNA sequences are now often used as diagnostic characters for species delimitations and in extreme situations just a few substitutions can lead to a split into several species, because these substitutions are diagnostic for particular "species". The pros and cons of this approach (widely applied e.g. in ungulate taxonomy; Groves and Grubb, 2011) have recently been intensely debated (see references in Zachos, 2016, p. 127). While dPSC might reflect evolutionary processes within a particular taxon (e.g. genus), its strict application often leads to the so-called taxonomic inflation (Zachos et al., 2013).

In African mole-rats (family Bathyergidae), the crucial role of the EARS in shaping the evolution and distribution of recent genera has been repeatedly suggested (Faulkes et al., 2004; Ingram et al., 2004; Van Daele et al., 2007). Two genera (*Heterocephalus* and *Heliophobius*) are distributed mainly eastward of the EARS, while the remaining groups (*Bathyergus*, *Georychus*, *Cryptomys*, *Fukomys*) occur predominantly to the west and south of the rift. Among mole-rats, the genus *Heliophobius* (before 2011 considered by most authors as monotypic with only the species *H. argenteocinereus* Peters, 1846) inhabits the largest area of all bathyergids. Its preferred habitat is miombo woodland in the Zambezian region (*sensu* Linder et al., 2012), so it can serve as a suitable model for testing the role of EARS and past climatic changes on the distribution of genetic diversity of organisms living in this phytochorion. Available data on the mitochondrial genetic diversity of *Heliophobius* (Faulkes et al., 2004, 2011; Ingram et al., 2004) suggest conspicuous genetic structure, with the main clades separated by EARS and the Eastern Arc Mountains (EAM). There are at least two explanations for the observed pattern. First, Faulkes et al. (2011) proposed that the formation of EARS during the Miocene (20-5 Mya) fragmented the ancestral population and created the extant genetic structure. Alternatively, it is also possible that the current genetic pattern is a result of repeated fragmentation of suitable habitats in a geomorphologically heterogeneous area during Plio-Pleistocene climatic cycles (see e.g. Colangelo et al., 2013; Mazoch et al., 2018 for examples of other rodent taxa living often in sympatry with *Heliophobius*).

Although several species of the genus *Heliophobius* have been described in the past, they have been synonymised with *H. argenteocinereus* (Happold, 2013; Musser and Carleton, 2005). Faulkes et al. (2011) used mitochondrial (mt) sequences of cytochrome *b* (*CYTB*) and suggested that the silvery mole-rat might be a complex of six to eight taxa. Even if mtDNA might be very helpful in the identification of evolutionary significant units (and some of them may have the status of separate species), the use of mtDNA sequences as diagnostic characters in the strict application of dPSC for species delimitation is questionable (Zachos et al., 2013). Unfortunately, the taxonomic view proposed by Faulkes et al. (2011) started to be adopted in the recent literature, e.g. in the compendium Rodents of Sub-Saharan Africa (Monadjem et al., 2015), where the authors wrote "*Recent molecular work . . .. confirms the existence of multiple distinct species . . ..*". However, knowing the properties of mtDNA, the species cannot be delimited using solely this marker.

In our view, additional diagnostic characters (nuclear DNA, morphology, ecology, behaviour, etc.) are required before accepting a multiplication of the species number. In this study, we complemented the genetic data of Faulkes et al. (2011) by the addition of significant numbers of new mtDNA sequences from previously under-studied regions, and for the first time we also assess the genetic structure of *Heliophobius* by using nuclear (n) DNA markers. Using the largest genetic dataset available today for the genus, we had the following aims: (1) to describe the detailed range-wide genetic structure of the genus and to identify which geographic barriers have been the most important for its genetic differentiation; (2) by dating the divergences among genetic lineages, to propose an evolutionary scenario for establishing the recent genetic structure; and (3) to test the hypothesis of several biological species by comparison of mitochondrial and nuclear markers, especially at contact zones between mtDNA lineages.

Material and methods

Sampling

The new material analysed in this study (201 individuals) was collected during field expeditions to Malawi, Tanzania, Kenya, Zambia and Mozambique, performed by the authors in 2000–2016 (Fig. 1, Table S1 in Supplementary material S1). The animals were dissected and pieces of tissue (spleen, kidney, muscle or toe) were stored in 96% ethanol until DNA extraction. An additional seven samples were obtained as pieces of dry skin (vouchers collected in 1971–1972) from the Livingstone Museum in Zambia.

Analysis of genetic structure at mitochondrial DNA

The complete *CYTB* gene was amplified from extracted DNA by polymerase chain reaction (PCR) using primers and protocol from Faulkes et al. (1997). Sanger sequencing of PCR products was conducted from both ends using BigDyeTM chemistry (Applied Biosystems) in a commercial laboratory. The new sequences of *CYTB* used in the phylogenetic analyses were submitted to GenBank under accession numbers MG911035-MG911089 (Table S1 in Supplementary material S1). All sequences were aligned by the MUSCLE algorithm implemented in AliView 1.18 (Larsson, 2014).

We also downloaded all available *CYTB* sequences of *Heliophobius* from GenBank, most of them published by Faulkes et al. (2004, 2011). While their first study used only sequences obtained from fresh material and ethanol- or DMSO-preserved tissue samples, the second one included also sequences obtained from museum specimens. Because DNA in old museum samples is often fragmented, shorter fragments are usually amplified and sequenced, which was also the approach adopted by Faulkes et al. (2011). As a result, *CYTB*

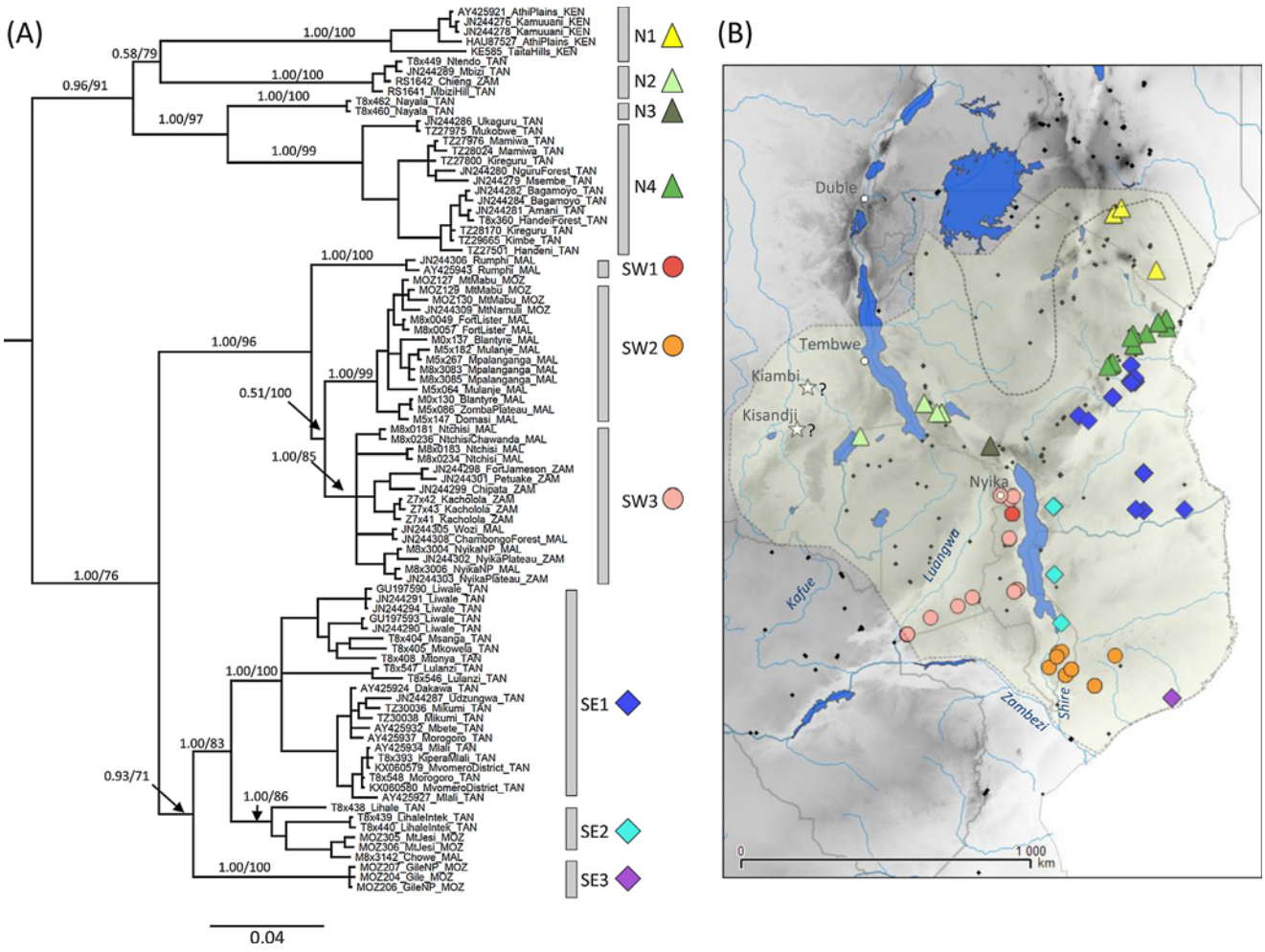

Fig. 1. (A) Bayesian phylogenetic reconstruction of mitochondrial DNA (*CYTB*) of *Heliophobius*. The tree was constructed in MrBayes and the topology was very similar to a maximum likelihood tree calculated in RAxML. The numbers above branches represent posterior probabilities from MrBayes/bootstrap support from RAxML. (B) Distribution of main mitochondrial clades of *Heliophobius*. The symbols correspond to different clades on the phylogenetic tree. Black dots represent localities where our research group has recently collected small mammals (in ca. 10 last years), but no *Heliophobius* was obtained/observed. Background shaded area shows the distribution of *Heliophobius* according to IUCN (its northern part and central Tanzania are considered as "probably extant" by IUCN). Note that previously used sequences by Faulkes et al. (2011) from Dubie, Tembwe, Nyika, Kiambi and Kisandji were not used in the phylogenetic analyses. While those from Kiambi and Kisandji might represent another clade (white stars), those from Dubie, Tembwe and Nyika were clearly contaminations (white circles). See more details in Supplementary materials S1 and S2. Note also that the localities Nguru Forest and Msembe (suggesting distributional overlap between clades N4 and SE1 in Faulkes et al., 2011) were localized incorrectly in Faulkes et al. (2011). Here we used correct GPS coordinates from the database of the Field Museum of Natural History in Chicago. It seems that the two clades have parapatric distribution and do not overlap.

sequences of some individuals were relatively short or split into three fragments, separated by short missing sequences marked as $(N)_n$ in GenBank. We performed detailed preliminary analyses of these short fragments and found that seven of them likely represent contamination or chimeric sequences from different sources (see details in Supplementary material S2). We therefore excluded them from the final analysis. Because of the generally low reliability of sequences from old museum material as described above, we also excluded two additional short sequences from the Democratic Republic of Congo (JN244311 and JN244312), despite the fact that they might represent correct sequences of a very distinct mitochondrial clade (see Discussion and Supplementary material S2). After the selection, we finally included in our dataset 56 georeferenced GenBank sequences of *CYTB* from three previous studies (Faulkes et al., 2004, 2011; Ngalameno et al., 2017) (Table S1 in Supplementary material S1).

The final *CYTB* dataset (1140 bp) was composed of 154 sequences; 98 individuals from 30 localities were newly sequenced within this study, and 56 sequences from 23 localities were downloaded from GenBank (Fig. 1, Table S1 in Supplementary material S1). We reconstructed a mitochondrial phylogeny with a reduced dataset of 89 sequences (Supplementary material S1), representing all main mtDNA lineages identified by the preliminary analyses. The remaining 65 specimens (identical and/or shorter sequences from the same or neighbouring localities) were unambiguously assigned to particular lineages by neighbour-joining analysis (1000 bootstraps, support >90%; not shown) in MEGA 6.06 (Tamura et al., 2013). The number of base substitutions per site of *CYTB*, averaging over all sequence pairs between and within groups (identified by phylogenetic analysis), was calculated as uncorrected *p*-distance in MEGA using 89 sequences.

The most suitable nucleotide substitution model (HKY + G + I) was chosen in MEGA using the Bayesian information criterion (BIC). This model was used for Bayesian inference (BI) of evolutionary relationships in MRBAYES 3.2.6 (Ronquist and Huelsenbeck, 2003). Three heated and one cold chain were employed and runs were initiated from random trees. Two independent runs were conducted with 20 million generations each and trees and parameters were sampled every 1000 generations. Convergence was checked using TRACER 1.6 (Rambaut et al., 2014). For each run, the first 25% of sampled trees were discarded as burn-in. Maximum likelihood (ML) analysis was performed using RAXML 8.0 (Stamatakis, 2014).

Because simpler models are not available in RAXML, the GTR model was selected (option -m GTRCAT as recommended by the author of the program). The robustness of the nodes was evaluated by the default bootstrap procedure with 1000 replications (option -# 1000). As outgroup, we used the sequence of *Bathyergus suillus* (GenBank; AY425913) in all phylogenetic analyses.

Analysis of genetic structure at microsatellite markers

Detailed genetic structure was analysed by eight polymorphic microsatellite markers that were successfully used in the silvery mole-rat in our previous study (Patzenhauerová et al., 2010). They are polymorphic at the population level and have very low frequency of null alleles (see more details in Table S3 in Supplementary material S3 and in Patzenhauerová et al., 2010). In total, we genotyped 182 individuals from 29 localities representing all major mtDNA clades (Supplementary material S1). We applied the individual-based Bayesian clustering approach using the program STRUCTURE 2.3.4 (Pritchard et al., 2000) to reveal the genetic structure without *a priori* geographic information. The program uses the MCMC process to assign individuals to a different number of clusters (K). The program was run with 10 independent simulations (each with 1000,000 iterations after 200,000 iterations of burn-in) for each K-value from 1 to 10. In all simulations, an admixture ancestry model and independent allele frequency model (with $\lambda = 1$) were used. The results of 10 replicate runs for each value of K were combined and evaluated in CLUMPAK (Kopelman et al., 2015). The likelihood of K, i.e. lnPr(X|K), was used to infer the most likely number of real populations in the dataset, using the delta K method of Evanno et al. (2005). Summary outputs for major and minor modes (detected by CLUMPAK) for each K value were displayed graphically using DISTRUCT v. 1.1 (Rosenberg, 2004). Because uneven sampling per population can bias the results of the STRUCTURE analysis (Puechmaille, 2016), we performed an alternative analysis where (1) we lumped together individuals from geographically very close localities, belonging to the same mtDNA haplogroup, and (2) we randomly subsampled 20 individuals from populations with many genotyped individuals. This reduced dataset resulted in 132 individuals from 18 populations.

Nuclear introns, species delimitations and species tree

Based on preliminary phylogenetic analyses of *CYTB*, we selected 32 individuals across the mtDNA diversity (Table S1 in Supplementary material S1) for reconstruction of nuclear phylogeny within the genus using three nuclear introns, i.e. *DHCR*, *TRPV*, and *NADSYN* (primers and genotyping protocols specified in Table S2 in Supplementary material S3). The new sequences are available in GenBank under accession numbers MG911148-MG911176 (*DHCR*), MG911118-MG911147 (*TRPV*), and MG911090-MG911117 (*NADSYN*). To relax prior assumptions about species delimitation, we estimated a species tree using the birth-death-collapse model (Jones et al., 2015) as implemented in the package STACEY (Jones, 2017) for BEAST 2 (Bouckaert et al., 2014). We assumed conspecificity of individuals bearing mtDNA of the same lineage, but there were two exceptions. First, each putative "species" must have at least one sequenced individual for each marker, but we were not able to get sequences of two nuclear introns for specimens from the mitochondrial lineage SE3. Because their *DHCR* sequences were almost identical with sequences of the SE2 lineage, and SE2 and SE3 clustered together also in the analysis of the nuclear microsatellites (see Results), we considered them conspecific. Second, lineage SW1 was represented only by *CYTB* sequences from GenBank, so it was excluded from this analysis. Technically, the candidate species correspond in STACEY to clusters of collapsed tips (ancestral node height = 0) that are not allowed to be split. We used the strict clock model and the best-fit substitution model was assessed by the maximum-likelihood approach in MEGA 6.06 as JC (for *DHCR*), JC + G (*NADSYN*) and HKY (*TRPV*). Two independent runs were carried out for 50×10^6 generations with sampling every 1000 generations in BEAST 2.4.5. The resulting parameter and tree files from the two runs were combined using LOGCOMBINER 2.4.6 (Bouckaert et al., 2014) and examined for convergence and effective sample sizes in TRACER 1.6 (Rambaut et al., 2014). All species trees were visualized in DENSITREE (Bouckaert, 2010) and the number of species was analysed by SpeciesDelimitationAnalyzer (http://www.indriid.com/software.html).

Dating of divergence

The only fossil *Heliophobius* is known from the late Pleistocene at Isenya, Kenya (Brugal and Denys, 1989), so it is not possible to directly apply the primary calibration of the molecular clock within the genus. We therefore used a fossilized birth-death model to estimate phylogenetic relationships and to infer divergence times among bathyergid lineages. This model removes the need for a priori node constraints, infers divergence times by integrating fossil dates into the lineage diversification and extinction model (Heath et al., 2014), and was implemented in BEAST v. 2.4.6 (Bouckaert et al., 2014) using the Sampled Ancestors add-on package (Gavryushkina et al., 2014). We used alignments of one mitochondrial (*CYTB*) and three nuclear (*GHR*, *IRBP*, *VWF*) markers and a wide spectrum of known fossils of African hystricognath rodents. Fossils and associated information (the first fossil occurrence, position on phylogenetic tree, etc.) were acquired from the available palaeontological literature; see Table S4 in Supplementary material S4 for more details). We specified GTR + G (for *CYTB*) or HKY + G (nuclear markers) substitution model with no codon partitioning and a relaxed lognormal molecular clock (Drummond et al., 2006). Clock rates of different markers were allowed to vary around the common mean, specified by the diffuse lognormal prior ($\mu = -5$, $\sigma = 2$). The likelihood stationarity was checked in TRACER, parameter and tree files from the two runs were combined in LOGCOMBINER and the maximum clade credibility tree was calculated in TREEANNOTATOR 2.4.6 (Bouckaert et al., 2014). All phylogenetic analyses were run on CIPRES Science Gateway (Miller et al., 2010).

Results

Distribution of mitochondrial DNA diversity

ML and BI phylogenetic analyses produced a similar topology of the tree and revealed three major clades with a parapatric distribution (Fig. 1). We will call them "Northern" (with four subclades N1–N4), "South-eastern" (subclades SE1-SE3), and "South-western" (subclades SW1-SW3). The Northern clade is distributed in south-eastern Kenya (N1), on both sides of the southern part of Lake Tanganyika (N2), in the Poroto Mts. in south-western Tanzania (N3), and along the central and northern part of the Tanzanian Eastern Arc Mountains (e.g. Nguru, Ukaguru, Usambara; N4). The South-eastern clade is composed of three subclades: SE1 is largely distributed in south-eastern Tanzania, including the Uluguru Mts and eastern foothills of the Udzungwa Mts., SE2 on the eastern side of Lake Malawi, and SE3 in the Gile National Park in coastal central Mozambique. The South-western clade has the following distribution: SW1 in a single locality (Rumphi) in northern Malawi, SW2 in the highlands of southern Malawi and central Mozambique, east of the Shire River, and SW3 in central and northern Malawi and south-eastern Zambia (Fig. 1). Note that the localities Nguru Forest and Msembe (suggesting distributional overlap between clades N4 and SE1 in Faulkes et al., 2011) were

positioned incorrectly by Faulkes et al. (2011). Here we used the correct GPS coordinates from the database of FMNH in Chicago, and it seems that the clades N4 and SE1 have a parapatric distribution with no evidence of overlap.

Genetic distances among lineages belonging to three different major clades ranged from 7.8% to 13.7% (Table 1). Within major clades, the differentiation among lineages was lowest in SW (*p*-distances smaller than 5%), intermediate in SE, and highest in N (with the maximum *p*-distance = 11.2% between N1 and N2).

Population structure at microsatellites

The Bayesian analysis in STRUCTURE suggests highly structured populations, because lnPr(X|K) increased with the number of inferred clusters in analyses of the complete dataset (182 individuals; not shown) as well as of the reduced dataset (132 individuals; Fig. S3 in Supplementary material S3). The best model for the complete dataset according to Evanno's method is for K = 2, but models with K = 3, 5, and 8 have also good support (not shown). We therefore show more details on the genetic structure for major modes of K = 5 (major mode was revealed in 9 of 10 iterations) and K = 8 (major mode in 7 of 10 iterations) in Fig. S2 in Supplementary material S3. Analysis of a reduced dataset revealed a very similar structure with the highest delta K for K = 3, K = 6 and K = 8 (Fig. S3 in Supplementary material S3). Barplots showing individual assignments for all models are shown in Fig. S5 in Supplementary material S3, while the geographic distribution of genetic variability is shown only for the most likely models K = 6 (Fig. 2) and K = 8 (Fig. S4 in Supplementary material S3). The assignment of populations to genetic clusters is only partially consistent with the results of the mitochondrial phylogeny (compare Figs. 1 and 2). Populations from N̈orthernm̈itochondrial lineages are highly structured. While Pop1 (=N1 at mtDNA; southern Kenya) is similar to Pop3 (=N3; Poroto Mts.), Pop2 (=N4; Usambara Mts.) and Pop4 (=N2; Mbizi Mts.) are distinct at the microsatellites already at K = 5 (Fig. S5 in Supplementary material S3). The only individual from the Mbizi Mts (Pop4) was assigned to the cluster of "South-eastern" populations (dark blue at Fig. 2), but this can be an artefact of the low sample size. However, there are other signatures of microsatellite introgression (or ancestral polymorphism) between populations bearing "Northern" and "South-eastern" mitochondrial DNA. For example, 35% (57% in minor mode) of the genotypes in Pop7 in the Eastern Arc Mountains (mtDNA SE1) were assigned to the cluster represented by Pop2 from the Usambara Mts (mtDNA N4) (=green colour in Fig. 2). Similarly, the populations in the Poroto Mts. (Pop3) and Lihale (Pop8) in southernmost Tanzania may represent a partial admixture between the "Northern" and "South-eastern" genetic clades (Fig. 2). On the other hand, there seems to be a genetic structure present southwards of Lake Malawi, where all individuals possess mtDNA from the SW2 lineage. The possible contact between mitochondrial "South-eastern" and "South-western" lineages in southern Malawi is reflected by the admixed character of Pop9 located in the possible contact zone between these two mitochondrial lineages (Fig. 2).

Species delimitation based on nuclear sequences

The posterior sample of species trees obtained by STACEY indicates two main groups of taxa, with all "Northern" candidate species clustered together, while the two "South-eastern" sub-clades were clustered with the two "South-western" sub-clades (Fig. 3A). Given the moderate number and generally low variation of the used nuclear markers, we took a conservative stance and assumed a pair of candidate species collapsed if there was not strong evidence (posterior probability >0.95) to the contrary. Using this criterion and prior collapse height 0.0001, only candidate species SW2 and SW3 collapsed, suggesting a strong genetic structure also at the nuclear markers (Fig. 3B). More candidate species collapsed with increasing posterior collapse height, e.g. for 0.002 all northern taxa formed a separate group distinct from all southern taxa.

Dating of divergences

The fossilized birth-death model based on four genetic markers and a wide spectrum of fossil taxa provided well supported estimates (i.e. ESS for all parameters were much higher than 200 in TRACER) of divergence times among bathyergid lineages (Fig. 4). The split of *Heterocephalus* from other genera occurred very soon after the bathyergid radiation in the early Miocene (21.7 Mya). The *Heliophobius* clade split off in the middle Miocene (11.3 Mya), while the lineages leading to the remaining extant genera diversified in the late Miocene. All divergences within current genera, including those in *Heliophobius*, were dated to the Plio-Pleistocene (i.e. younger than 5 Mya).

Discussion

Genetic structure of the silvery mole rat and major biogeographical divides

The silvery mole rat (if considered as a single species) is the African mole-rat species with the largest distribution. Comparing our data with the IUCN map (Fig. 1), however, it seems that its distribution is probably more restricted. Its absence in some areas might be caused by the lack of sampling (e.g. northern Mozambique), but in others could be caused by inhospitable conditions (the Massai steppe in part of central-northern Tanzania). The species is very likely not present in western Zambia and in the northern part of the Albertine rift, where previous records should be considered dubious (see below). On the other hand, the genus is definitely present in southeastern DRC (Katanga province; Faulkes et al., 2011).

An increasing number of recent studies shows that phylogeographic divides in *Heliophobius* correspond with those of other small mammal taxa living in non-forested habitats, whose genetic lineages are separated especially by the Eastern Arc Mountains (EAM), Lake Malawi and its outlet the Shire River and in some cases also by the Rukwa rift and Mbeya triple junction (Bryja et al., 2014; Colangelo et al., 2013; Mazoch et al., 2018; Petružela et al., 2018). Contrary to murid rodents, the silvery mole-rats seem to prefer the relatively moist miombo woodlands in foothills, especially along EAM. Humid habitats were repeatedly very fragmented in the northern part of the Zambezian region (e.g. Lawson, 2010), which likely resulted into allopatric differentiation of isolated populations during dry periods of the Pleistocene; see e.g. large differences between the geographically close Mbizi Mts. (mtDNA lineage N2) and Poroto Mts. (N3) in southern Tanzania. On the contrary, the distribution of the South-western and South-eastern lineages is more continuous, which suggests more stable (mesic) environmental conditions in the southern part of the Zambezian region.

Calibration of molecular clock has important implications for reconstructions of evolutionary history

We agree with Faulkes et al. (2011) that EARS plays an important role in genetic differentiation, but we see its role mainly as a secondary barrier to gene flow rather than as a primary driver of diversification. The evolutionary scenario strongly depends on the dating of historical events, i.e. the calibration of the molecular clock. We used the fossilized birth-death model and multilocus genetic data to estimate the times to the most recent common ancestors (MRCAs) within bathyergids. In contrast to Faulkes et al. (2011), we

Table 1
Mean genetic *p*-distances at *CYTB* between (below the diagonal) and within (white numbers on the diagonal) genetic lineages of *Heliophobius* calculated in MEGA 6.06 (Tamura et al., 2013). Standard errors of inter-lineage distances (calculated by 1000 bootstraps) are above the diagonal. The distances between lineages within the three major mitochondrial clades (as specified in Fig. 1) are shaded grey. Abbreviations of genetic lineages correspond to Fig. 1.

Clade	N1	N2	N3	N4	SE1	SE2	SE3	SW1	SW2	SW3
N1	0.016	0.011	0.008	0.009	0.009	0.009	0.009	0.010	0.009	0.009
N2	0.112	0.003	0.009	0.009	0.009	0.009	0.010	0.010	0.010	0.009
N3	0.099	0.100	0.001	0.007	0.008	0.008	0.009	0.009	0.009	0.009
N4	0.105	0.101	0.071	0.021	0.008	0.008	0.008	0.009	0.009	0.009
SE1	0.126	0.119	0.117	0.122	0.028	0.005	0.006	0.008	0.007	0.007
SE2	0.125	0.116	0.109	0.116	0.054	0.025	0.006	0.008	0.007	0.007
SE3	0.123	0.114	0.111	0.111	0.072	0.067	0.001	0.008	0.008	0.008
SW1	0.137	0.135	0.125	0.130	0.091	0.086	0.091	0.005	0.005	0.006
SW2	0.136	0.123	0.120	0.126	0.088	0.085	0.090	0.046	0.011	0.005
SW3	0.126	0.121	0.111	0.123	0.080	0.078	0.083	0.045	0.036	0.018

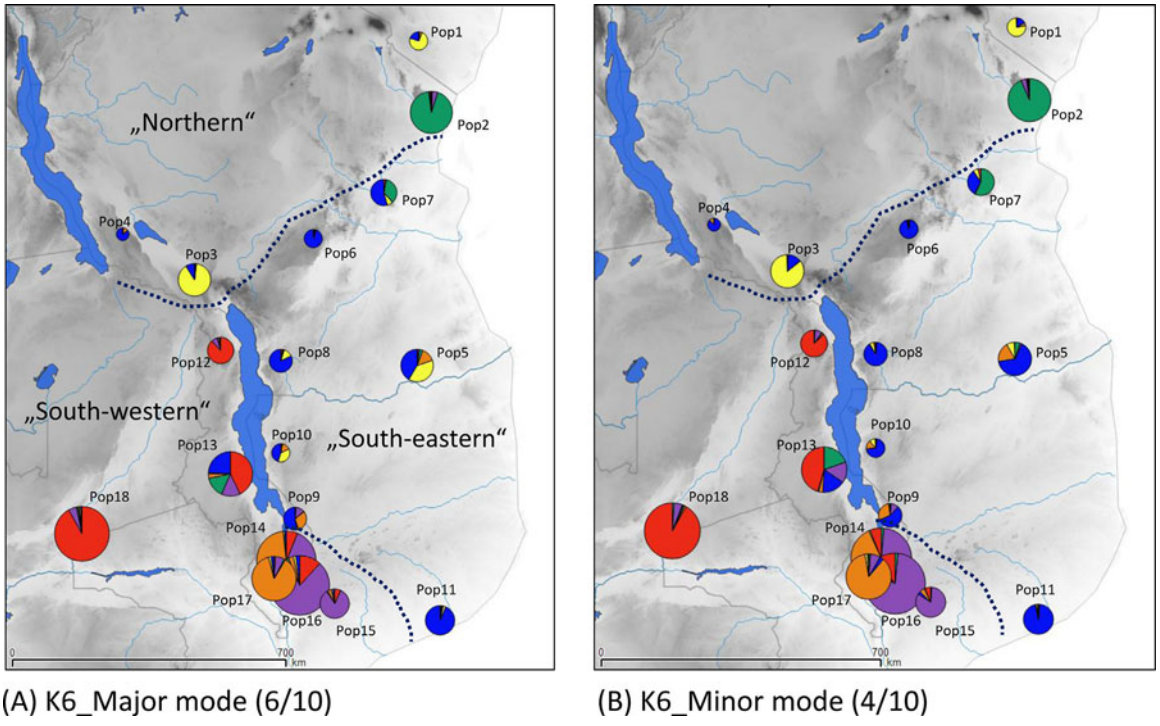

Fig. 2. Population structure estimated in STRUCTURE from 132 individual genotypes (eight loci) from 18 population samples. The colours in pie charts represent the proportional membership of individuals to microsatellite based clusters inferred from STRUCTURE for K = 6 for major (A) and minor (B) modes; the size of circles corresponds to the number of genotyped individuals per locality (Min = 1, Max = 20). Dotted lines separate regions inhabited by three major mitochondrial lineages. Major and minor modes were identified by CLUMPAK (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.).

found that the splits within *Heliophobius* are remarkably younger (Plio-Pleistocene). To get realistic estimates of divergence dates it is necessary to have reliable fossil data correctly placed on phylogenetic trees. Faulkes et al. (2011) placed *Proheliophobius* at the most basal split of contemporary *Heliophobius*, which is incorrect. *Proheliophobius* is one of the extinct genera of bathyergids, being part of the intensive early Miocene radiation of the mole-rats cca 20 Mya (Mein and Pickford, 2003). However, there is no firm evidence that these fossil genera represent direct ancestors of extant genera (e.g. Winkler et al., 2010). Even if *Proheliophobius* belongs to the lineage including *Heliophobius*, it would be its stem member possibly pre-dating MRCA of all extant genera apart from *Heterocephalus* (as hypothesised by Lavocat, 1973). The whole story about the role of the EARS on early Miocene adaptive radiation of the genus (Faulkes et al., 2011) is therefore unlikely and should be reconsidered in the light of new analyses.

There are also indirect indications that the very old (early Miocene) divergence dates within *Heliophobius* estimated by Faulkes et al. (2011) are artefacts. Using various calibrations of the molecular clock based on fossil evidence, even the oldest divergences within other rodent groups living in sympatry with *Heliophobius* (and similarly divergent if *CYTB* distances are used) were estimated to occur in the Plio-Pleistocene (e.g. Bryja et al., 2014; Colangelo et al., 2013; Mazoch et al., 2018; McDonough et al., 2015; Mikula et al., 2016; Petružela et al., 2018; Verheyen et al., 2011). It thus seems very unlikely that the genetic structure of *Heliophobius* would be conserved during the last 15 My in the same shape that evolved 10 My later in numerous other sympatric taxa as a result of allopatric diversification caused primarily by climatic fluctuations.

Our estimates of divergence dating in bathyergids are very similar to recently published palaeontological reconstructions (Barbière and Marivaux, 2015), but significantly younger than in previous studies employing a molecular clock. For example, the oldest divergence (split of *Heterocephalus*) was 21.7 Mya in our study, while it was 33–35 Mya in Ingram et al. (2004) and 40–48 Mya in

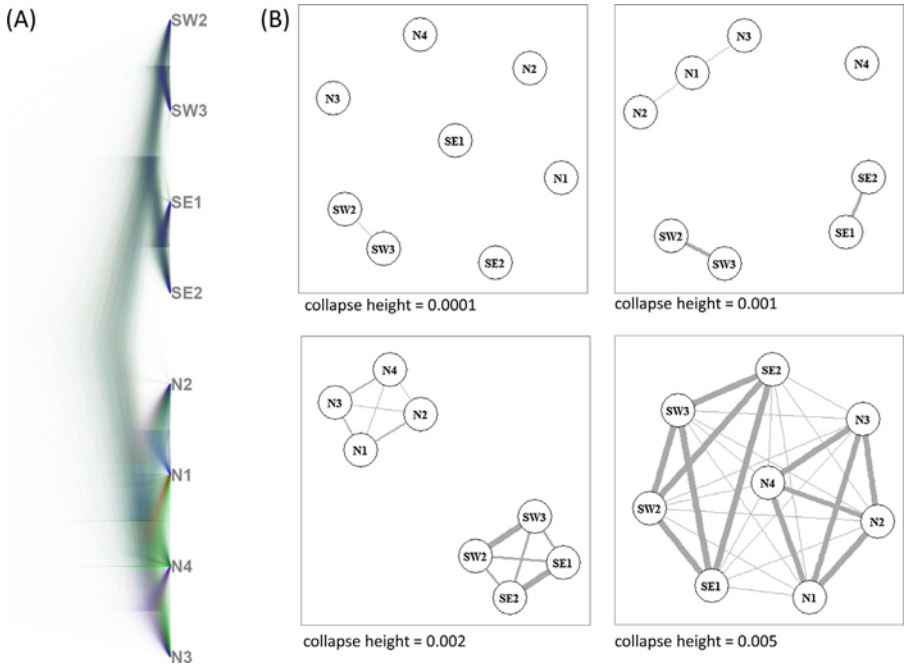

Fig. 3. Species trees and species delimitation from STACEY based on three nuclear introns. (A) Visualisation of all 90,000 sampled trees in DensiTree. (B) Clustering of candidate species (abbreviations correspond to 8 candidate species defined on the basis of mtDNA genotype) in the posterior sample from STACEY. The linked pairs are collapsed with posterior probability >0.05, the width of the link corresponds to collapsing probability. The prior collapse height used in STACEY was 0.0001, but collapsing with higher arbitrary values is also shown to indicate the hierarchical structure of *Heliophobius*.

Faulkes et al. (2004). Similarly, the *Heliophobius* split from other South African genera was estimated at 11.3 Mya in our study, but much earlier in previous studies (19–20 Mya in Ingram et al., 2004; 34–40 Mya in Faulkes et al., 2004). The main reason for these different estimates lies in the calibration of the molecular clock. Ingram et al. (2004) used a 20 Mya split of *Heliophobius* and other South African taxa ("*based on the available fossil evidence . . .. a date of 20–19 Mya was used as a calibration point for the divergence of the Heliophobius lineage (A. Winkler pers. comm.)*"). However, there is no evidence that early Miocene bathyergids (many fossil bathyergid genera appeared at 20–23 Mya) were direct ancestors of extant genera (Winkler et al., 2010). The approach of Ingram et al. (2004) resulted in a 10–12 Mya split estimate for *Fukomys*/*Cryptomys* that was subsequently used as a secondary calibration constraint also in other studies (e.g. Van Daele et al., 2007). Faulkes et al. (2004) used the secondary calibration in their dating analysis and constrained the basal divergence of bathyergids to 40–48 Mya. These dates are based on the single-locus (exon 28 of the nuclear von Willebrand factor gene; vWF) study of Huchon and Douzery (2001), who used a single calibration point of 31 Myr as the time of radiation of caviomorph rodents in South America. As a by-product of their study, the oldest divergence in one of the outgroups, i.e. among *Heterocephalus* and other bathyergids, was estimated at either 40 Mya (based on positions 1 and 2 of vWF) or 48 Mya (based on aminoacid sequences of wWF). Besides the fact that secondary calibration itself can produce significant biases (e.g. Schenk, 2016), single-gene studies are also crucially influenced by the selection of the marker (see e.g. huge differences in divergence dates estimates using two loci in Ingram et al., 2004).

Taxonomic implications

The six species proposed by Faulkes et al. (2011) and elevated to full species status on the basis of *CYTB* sequences by Monadjem et al. (2015) were thought to occur in allopatry, i.e. easily diagnosed by sequencing mtDNA marker at particular localities (see Fig. 5). However, our recent sampling shows that mtDNA lineages may occur in parapatry. Further, microsatellite data suggest gene flow among some of them, which means that if the most widely used Biological Species Concept is applied, they should not be considered separate species. We also discovered new mitochondrial lineages not found by Faulkes et al. (2011) (i.e. N3, SE2, SE3). If we would use the approach adopted by Faulkes et al. (2011) and Monadjem et al. (2015), i.e. dPSC using mtDNA as diagnostic characters, they must be elevated to new species as well.

Contrary to Faulkes et al. (2011) and Monadjem et al. (2015), we prefer a more conservative approach for species delimitation. We think that the null hypothesis should be conspecificity of closely related populations until multiple biologically relevant data are collected (Zachos, 2016, p. 160, and references therein). It now becomes clear that single-locus molecular phylogenies or genetic distances (especially if based on mtDNA only) cannot be considered as sufficient diagnostic criteria for species delimitations. Further, our new sampling close to the type localities of the species delimited by Faulkes et al. (2011) showed that some names used by Monadjem et al. (2015) were incorrectly assigned (see below). There are nine *Heliophobius* holotypes described in the literature of the late 19th and early 20th centuries. Except for *H. albifrons* (Gray, 1864), whose provenance is questionable ("East Africa", see Swynnerton, 1945), we discuss below the use of the remaining eight names and suggest possible taxonomic implications and directions for future work. We organized this part following the list of species used by Monadjem et al. (2015) and discuss why at least some of these names are problematic (see Fig. 5 for graphical summary).

(1) ***H. argenteocinereus*** Peters, 1852 (type locality Tete, Lower Zambezi River, Mozambique):

Monadjem et al. (2015) follows the recommendation of Faulkes et al. (2011) that this species is represented by mtDNA lineage 4 (sensu Faulkes et al., 2011) = "Southwestern" lineage in our study. Faulkes et al. (2011) further discussed the use of "*angonicus* Thomas, 1917" for some subclades (either SW1

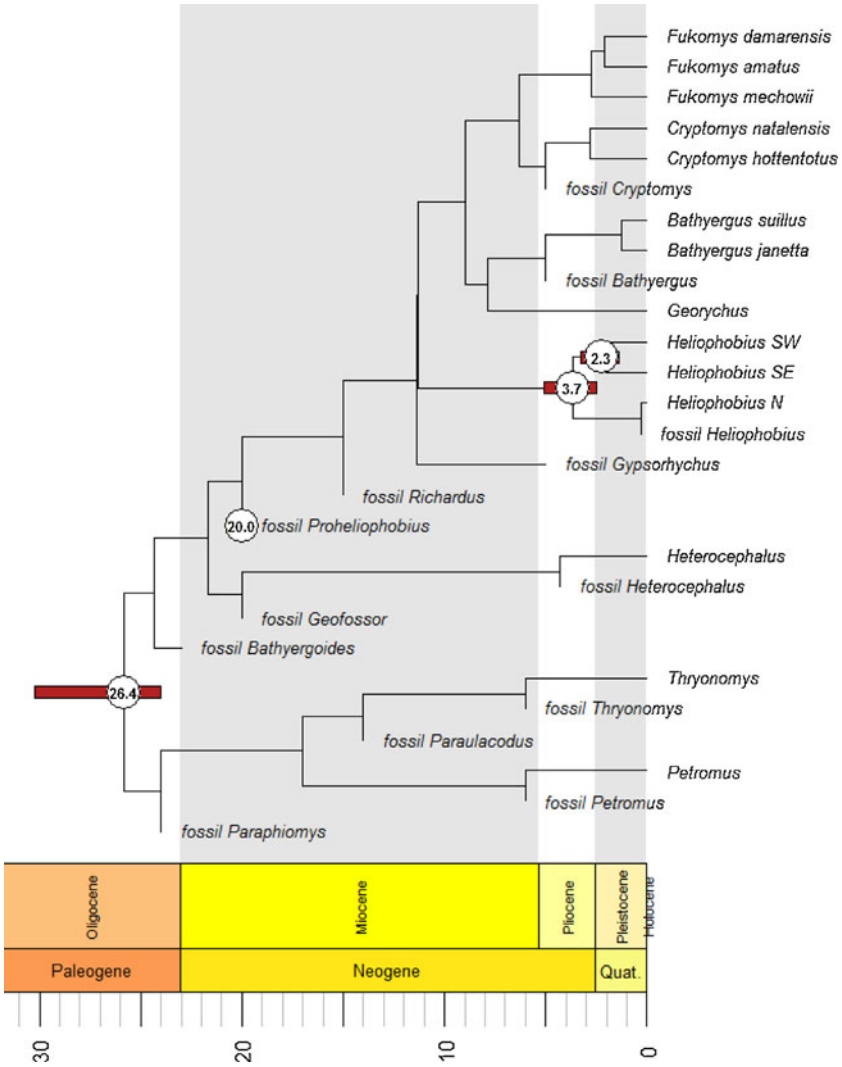

Fig. 4. Divergence dating of African bathyergids using the fossilized birth-death model in BEAST. Highlighted is the estimated time of Bathyergidae origin, the first occurrence of *Proheliophobius* treated as direct ancestor of *Heliophobius* by Faulkes et al. (2011) and the estimated divergence dates in *Heliophobius*. Positions of fossil *Geofossor*, *Proheliophobius*, *Richardus* and *Gypsorhychus* in the bathyergid clade are arbitrary and the same holds true for the fossil *Cryptomys* in the *Cryptomys*/*Fukomys* clade.

or SW2 in our study), because the type locality of "*angonicus*" lies in the distribution area of the "Southwestern" lineage, but both the "*argenteocinereus*" and "*angonicus*" type localities (and very probably also that of "*robustus*") are clearly in the range of mitochondrial clade SW3. Microsatellite data suggest gene flow between populations bearing mtDNA from the SW2 and SW3 lineages in Malawi and southwestern Zambia, as well as between SW2 and SE3 in southern Malawi, east of the rift. The name "*argenteocinereus*" is the oldest name in the genus and should be used at least for a biological species represented by populations with "Southwestern" and "Southeastern" mtDNA on both sides of EARS (with "*angonicus*" and "*robustus*" as junior synonyms).

(2) ***H. emini*** Noack, 1894 (type locality Simba Muënne, Tanzania):

Faulkes et al. (2011) putatively designated their clade 3 (=SE1 in our study) as *H. emini* on the basis of "*proximity of [their] sampling to the type locality*". However, the type locality of "*emini*" lies in the putative contact zone of N4 and SE1 (Fig. 5) and it is not possible to decide to which clade it belongs. Our new data rather suggest that the type locality of "*emini*" is closer to the distribution of the N4 lineage than to SE1. Further, Faulkes et al. (2011) incorrectly wrote (p. 335) that their clade 2b (=N4 in our study) and 3 (=SE1) are now in sympatry, because they used incorrect GPS coordinates for two localities of the N4 individuals (see Results). Microsatellite analysis suggests gene flow between SE1 (= *H. emini* sensu Monadjem et al., 2015), SE2, SE3 (none of the two reported by Faulkes et al., 2011 and Monadjem et al., 2015) and SW2 (= *H. argenteocinereus*). Using BSC, *H. emini* (sensu Monadjem et al., 2015) should thus be considered a junior synonym of *H. argenteocinereus*. Microsatellite data, however, also provide evidence for gene flow between populations with mtDNA of N4 and SE1 in their contact zone in central EAM (i.e. exactly in the proximity of the type locality of "*emini*"). The intensity of this gene flow should be studied in more detail and the results used in a qualified decision on the conspecificity of populations represented by the "Northern" and "Southeastern" mitochondrial clades.

(3) ***H. kapiti*** (Heller, 1909) (type locality: Potha, Kapiti plains, southern Kenya):

This taxon is considered as the only Kenyan *Heliophobius* by Monadjem et al. (2015), obviously overlooking the type locality of "*spalax*" (see below). It is represented by the clade 2a (= N1 in our study) in Faulkes et al. (2011), who genetically analysed material from very close to the type locality. We found an individual from the same lineage also in the Taita Hills, close to the type locality of "*spalax*" (Fig. 5), suggesting that

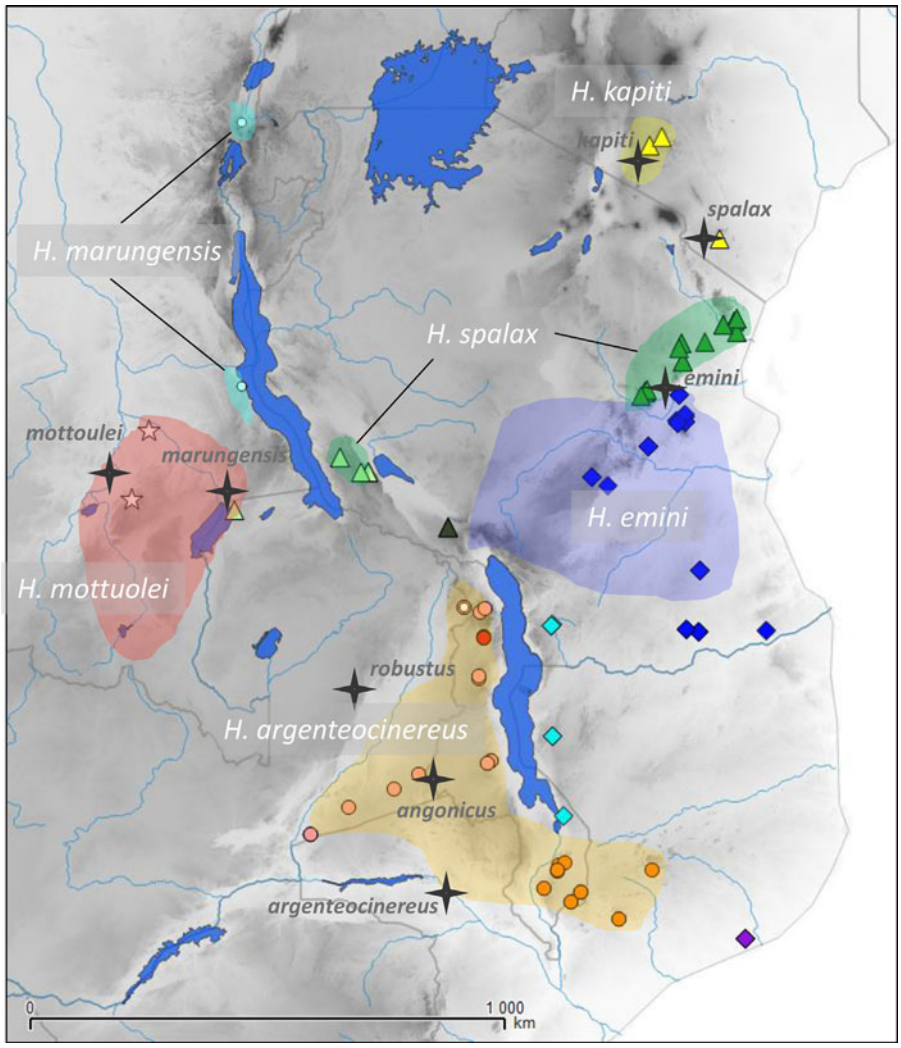

Fig. 5. Taxonomic summary. The distribution of mtDNA clades is identical to Fig. 1. Eight type localities of *Heliophobius* are shown by black crosses and available names in bold grey. Areas shaded by different colours indicate presumable distribution of six species in Rodents of Sub-Saharan Africa (Monadjem et al., 2015), their names are in white. See the text for more details on taxonomic implications.

"*spalax*" is a junior synonym of "*kapiti*", if we would name this taxon as a separate species. The Kenyan population is very similar at the nuclear microsatellites to that from the Poroto Mts. in southern Tanzania (= N3; see Figs. 2 and 3), suggesting their conspecificity (see also *H. spalax* account below). The level of hybridization between the "Northern" and "Southeastern" lineages along the Tanzanian EAM and in the Southern Highlands near the Tanzania/Malawi/Zambia borders should be studied in a more detailed study, but it seems that, despite pronounced genetic differences at mtDNA, they are not completely reproductively isolated (see Fig. 2 and Results). The Kenyan individuals have also a slightly distinct karyotype (2N = 60; George, 1979), compared to the Zambian and Malawian "*argenteocinereus*" (2N = 62; Scharff et al., 2001; Šumbera et al., 2007).

(4) ***H. marungensis*** Noack, 1887 (type locality: Marungu, southeastern DRC, very close to Zambian border):

Faulkes et al. (2011) suggested this name for their very distinct clade 5, identified only on the basis of sequences from old museum material. The three localities with this clade "*represent a wide geographical spread and potentially overlap the ranges of populations in clades 1 and 4*" (Faulkes et al., 2011). We showed (Supplementary material S2) that the genetic distinctiveness of this clade was likely caused by a laboratory contamination, because the central part of the *CYTB* sequences obtained from the museum material in fact represented another bathyergid genus *Cryptomys*. If the genetic delimitation of this taxon was based on incorrect sequences, the whole discussion about *H. marungensis* in Faulkes et al. (2011) as well as the disjunct distribution of this species shown in Monadjem et al. (2015) (see also Fig. 5) are nonsensical. At the same time, the presence and species assignment of silvery mole-rats westward of the Albertine rift remains obscure and must be confirmed by new sampling and genotyping. The type locality of "*marungensis*" is just several kilometres distant from the Zambian locality Chieng, from where we documented the presence of the N2 clade (albeit only by analysis of museum material, so we are cautious; see below). If future taxonomic work proves a separate species status for populations with "Northern" mtDNA (as suggested e.g. by the STACEY approach in our study; see Fig. 3), ¨*marungensis* Noack, 1887s̈hould be considered as the oldest available name. Importantly, Faulkes et al. (2011) cited Noack, who distinguished "*marungensis*" from "*argenteocinereus*" by their different number of molars (four vs. six). However, the number of molars is not a good diagnostic trait because *Heliophobius* is the only bathyergid genus and one of the few mammals with the presence of a continuous dental replacement (Gomes Rodrigues et al., 2011).

(5) ***H. mottoulei*** (Schouteden, 1913) (type locality: Kilongwe near the Upemba Swamps, Lualaba river, Katanga region, DRC):

Faulkes et al. (2011) obtained very distinct short sequences from museum material (RMCA in Tervuren) collected at two localities in the Katanga region (DRC). These populations were considered as a separate species, *H. mottuolei*, by Monadjem et al. (2015), because the type locality of ¨*mottouleï*lies also in the Katanga region (note that there is an obvious typing error in the species name in Monadjem et al., 2015). When we included these sequences into our preliminary analysis, we also recovered a very distinct clade, sister to all remaining *Heliophobius* (Supplementary material S2). Even if these sequences might represent a new, genetically very distinct taxon, given the problems with sequencing old museum material (see Supplementary material S2) we adopted a more cautious approach and excluded them from our final analysis. Awaiting more detailed multi-locus analyses from fresh samples collected in the Katanga region of DRC, we prefer to consider *H. mottoulei* provisionally as a dubious taxon.

(6) ***H. spalax*** (Thomas, 1910) (type locality: Taveta, south-eastern Kenya):

Faulkes et al. (2011) hypothesized that their clade 2b (=N2 and N4 in our study, but possibly also N3 which was not sampled in their study) represents *H. spalax*. The type locality of this species lies close to the Taita Hills, where we sampled *Heliophobius* with mtDNA from the N1 lineage (= *H. kapiti* sensu Faulkes et al., 2011). The use of the name ¨*spalax*for N2–N4 without genotyping the type material is therefore very probably erroneous (Fig. 5). Monadjem et al. (2015) present a map with a discontinuous distribution of this taxon living in northern EAM and the Mbizi Mts. in south-western Tanzania. New sampling, e.g. on the north-western slopes of the Udzungwa Mts., could be very informative for assessing the level of geographic and genetic fragmentation of populations in highlands circumscribing the arid Massai steppe in central Tanzania. We also found that the mitochondrial N2 lineage is present in northernmost Zambia, on the western side of the Albertine rift. This is not so surprising, because a similar biogeographical link was recently discovered in another rodent genus *Lophuromys*, where *L. sabunii* was described as an endemic species of the Mbizi Mts. (Verheyen et al., 2007), but it was recorded also in the highlands of northern Zambia (Sabuni et al., 2018). All populations with N̈orthernm̈itochondrial DNA are well distinguished also at the nuclear markers (see Figs. 2A or 3) and are the best candidates for separate species, if *H. argenteocinereus* would be split, with the oldest available name being *H. kapiti* (Heller, 1909) (but see above the account on *H. marungensis*).

We think that a split of *H. argenteocinereus* into six species is not well supported and strongly suggest retraction of recent taxonomic changes (used by Monadjem et al., 2015). It is clear that populations of silvery mole-rats are significantly structured (as could be expected in an animal with low mobility), which can have consequences in their adaptations to local environments. However, the observed genetic structure is a result of much more recent (Plio-Pleistocene) evolutionary processes than proposed by a previous study (Faulkes et al., 2011). Awaiting detailed multi-locus and/or multi-disciplinary studies focussed on particular populations (e.g. studies of reproductive isolation at secondary contacts of genetically differentiated populations and morphological or behavioural analysis of genetically characterized animals), we advocate for the use of the single name *H. argenteocinereus* for all populations belonging to the genus *Heliophobius*.

Authors' contributions

R.S. and J.B. conceived the ideas; H.K., R.S., O.M., R.M., W.N.C and J.B. collected material in the field; H.K. and A.B. genotyped the material, J.B., O.M. and H.K. analysed the data, J.B. and H.K. wrote the first version of the manuscript that was commented and improved by all authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mambio.2018.08.006.

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