Systematics and Phylogeography of The Non-Ethiopian Speckled-Pelage Brush-Furred Rats (Lophuromys Flavopunctatus Group) Inferred From Integrative Genetics and Morphometry

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Research Article

Keywords: East Africa, Kivumys, Lophuromys flavopunctatus group, Lophuromys, biogeography, integrative systematics

Posted Date: February 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-157741/v1

Version of Record: A version of this preprint was published on May 19th, 2021. See the published version at https://doi.org/10.1186/s12862-021-01813-w.

Abstract

Background: The speckled-pelage brush-furred rats (*Lophuromys flavopunctatus* group) has been difficult to define given conflicting genetic, morphological, and distributional records that combine to obscure meaningful accounts of its taxonomic diversity. In this study, we inferred the systematics, phylogeography, and evolutionary history of the *L. flavopunctatus* group using maximum likelihood and Bayesian phylogenetic inference, divergence times, historical biogeographic reconstruction, and morphometric discriminant tests. We compiled comprehensive datasets of three loci (two mitochondrial [mtDNA] and one nuclear) and two morphometric datasets (linear and geometric) from across the known range of the genus *Lophuromys*.

Results: The mtDNA phylogeny supported the division of the genus *Lophuromys* into three primary groups with nearly equidistant pairwise differentiation: one group corresponding to the subgenus *Kivumys* (*Kivumys* group) and two groups corresponding to the subgenus *Lophuromys* (*L. sikapusi* group and *L. flavopunctatus* group). The *L. flavopunctatus* group comprised the speckled-pelage brush-furred *Lophuromys* endemic to Ethiopia (Ethiopian *L. flavopunctatus* members [ETHFLAVO]) and the non-Ethiopian ones (non-Ethiopian *L. flavopunctatus* members [NONETHFLAVO]) in deeply nested relationships. There were distinctly geographically structured mtDNA clades among the NONETHFLAVO, which were incongruous with the nuclear tree where several clades were unresolved. The morphometric datasets did not systematically assign samples to meaningful taxonomic units or agreed with the mtDNA clades. The divergence dating and ancestral range reconstructions showed the NONETHFLAVO colonized the current ranges over two independent dispersal events out of Ethiopia in the early Pleistocene.

Conclusion: The phylogenetic associations and divergence times of the *L. flavopunctatus* group conform to demonstrated hypotheses surrounding the paleoclimatic and ecosystem refugium impacts on the evolutionary radiation of rodents dependent on stably humid conditions in the East Africa region. The overlap in craniodental variation between distinct mtDNA clades among the NONETHFLAVO suggests unraveling underlying ecomorphological drivers is key to reconciling taxonomically informative morphological characters. The genus *Lophuromys* requires a taxonomic reassessment based on extensive genomic evidence to elucidate the patterns and impacts of genetic isolation at clade contact zones.

Background

Well-resolved taxonomic accounts enable correct species delimitation, providing an objective framework for useful biodiversity quantification and management [1]. New developments in integrative morphologic, phylogeographic, genetic, and ecological analysis have increasingly complemented traditional reliance on morphological evidence to resolve taxonomic limits [2]. This 'integrative systematics' approach is most effective when delimiting cryptic species [3].

The genus *Lophuromys* contains between 15–34 valid species, with the variable number attributed to debatable morphological differences between species [4-7]. The genus was placed in the Murinae subfamily until recently when Steppan *et al.* [8] and Steppan *et al.* [9] noted genetic affinity between *Lophuromys, Uranomys, Deomys,* and *Acomys* that warranted their classification as a unique subfamily – Deomyinae. In deep phylogenetic relationships, morphology divides the genus into two subgenera; *Lophuromys,* with shorter tails and hindfeet, and *Kivumys,* with longer tails and hindfeet and unique gastrointestinal morphology [10, 11]. In the subgenus *Lophuromys*, three species groups have been previously defined based on pelage coloration and craniodental characterization; the *L. sikapusi* group with unspeckled dorsal pelage, the *L. flavopunctatus* group and *L. aquilus* group, both with speckled dorsal pelage coloration [6, 12]. Between the two speckled-pelage groups – *L. flavopunctatus* group and *L. aquilus* group – species are classified based on morphological affinities. However, it is not clear how morphology (external body features, pelage color, craniodental characters) explicitly delimitate species in the literature [6, 12]. Some Ethiopian endemics, such as *L. brunneus* and *L. chrysopus*, are included in the mainly non-Ethiopian *L. aquilus* group [12-14]. The inclusion of the unspeckled-pelage *L. dieterleni* and *L. eisentrauti* in the *L. aquilus* group and *L. pseudosikapusi* in the *L. flavopunctatus* group [6] further confounds how pelage coloration relates to phylogenetic relationships. There is a need to reassess to clearly define whether and how pelage coloration and morphological affinities relate to phylogenetic relationships in the genus *Lophuromys*. Hereafter, we use 'Ethiopian *L. flavopunctatus* members [ETHFLAVO]' to refer to the *Lophuromys* taxa endemic to the Ethiopian Highlands, the 'non-Ethiopian *L. flavopunctatus* members [NONETHFLAVO]' to refer to the remaining *Lophuromys* taxa [i.e., not belonging to the *L. sikapusi* group or the *Kivumys* group). The '*L. flavopunctatus* group' combines the ETHFLAVO and NONETHFLAVO.

In contrast to the relatively resolved taxonomy of the ETHFLAVO [15-17], the NONETHFLAVO generally lack broader phylogenetic and biogeographical understandings. A chronological review of the genus *Lophuromys* reveals persistent taxonomic controversy, especially concerning the morphological traits used to diagnose species, synonyms, and species groups [6]. Such controversy is most notable in the descriptions of several new species in checklists compiled before the 21st century, which relied exclusively on external morphology and craniodental characters for taxonomic designations [18-21]. Checklists compiled in the 21st century, employing more integrative techniques, also vary in the individual number of species recognized, and generally agree on an increasing number of valid species, ranging from 21 species [6] to 15 species [22], and most recently 34 species [5, 7]. The Musser and Carleton [6] checklist, which is one of the most cited taxonomic references, listed 21 valid species in the genus *Lophuromys* and mainly followed Verheyen *et al.* [12] in recognizing seven of these species under an *L. aquilus* group based on craniodental affinities (*L. aquilus* [23], *L. brunneus* [24], *L. chrysopus* [19], *L. dieterleni* [25], *L. eisentrauti* [26], *L. verhageni* [12], and *L. zena* [27]). Six other species were considered as synonyms of *L. aquilus* by Musser and Carleton [6]: *L. cinereus* [28], *L. laticeps* [29], *L. major* [29], *L. margarettae* [30], *L. rita* [31], and *L. rubecula* [27]. However, Dieterlen [22] recently considered *L. aquilus, L. cinereus, L. laticeps, L. major, L. margarettae, L. rita,* and *L. rubecula* as morphotypes/synonyms of *L. flavopunctatus*. Yet, in the most recent checklists – Monadjem *et al.* [7], Denys *et al.* [5], Burgin *et al.* [32], and Mammal Diversity Database [33] – virtually all species previously associated with the genus are considered as valid. This steady increase in newly recognized species suggests undescribed diversity in the genus *Lophuromys* and promotes debate over 'species concepts,' especially involving morphospecies, thus, demand further taxonomic and biogeographic reevaluations.

The NONETHFLAVO are among the most abundant small mammal fauna in forests of the Eastern Afromontane biodiversity hotspot south of the Ethiopian Highlands, including in the Kenya Highlands, Albertine Rift montane forests, Tanzanian Highlands, and the Southern Rift montane forests [5, 7, 11, 15, 22, 34-

36]. As such, they are an essential ecological component in these biodiversity hotspots, serving both as prey to raptors and small carnivores and as predators of invertebrates [11, 37]. Moreover, their association with ecosystems characterized by stable annual precipitation makes them models for investigating how changing ecosystem-climate processes impact phylogeographic and speciation trends. Altogether, they demand stable taxonomic accounts to guarantee accurate appraisal of their diversity, ecological roles, and ecosystem functions.

The current distribution of the genus *Lophuromys* reflects relatively well-structured phylogeographic patterns. The *L. sikapusi* group spans a pantropical African range, from western Guinea to western Kenya, the *Kivumys* group is restricted to the Congo Basin east of the Congo River and the Albertine Rift, and the *L. flavopunctatus* group is distributed primarily in eastern to central Africa [5, 7, 10, 11, 22]. Despite this remarkable geographic range, the spatiotemporal influence of geographical features and climatic oscillations on evolutionary radiation in the genus *Lophuromys* remains largely unknown [7]. Studies using larger genomic datasets, like Komarova *et al.* [16], have uncovered complex reticulate evolution and recurrent mitochondrial introgression among the Ethiopian *flavopunctatus* members, clearly illustrating that single-gene phylogenies, especially mitochondrial loci, should not serve as the exclusive basis for taxonomic assignment. For the non-Ethiopian *flavopunctatus* members, even knowledge of mitochondrial DNA (mtDNA) diversity is limited. There is a necessity first to test the extent to which the mtDNA reflects taxonomic units and biogeographical trends across its distribution, and then to contrast it with nuclear data.

In this study, we evaluated the taxonomic limits and biogeographic patterns in the genus *Lophuromys* using a comprehensive mtDNA (*Cytochrome b*; *CYTB*) dataset. We then focused on the non-Ethiopian *flavopunctatus* members and complemented the *CYTB* alignment with *Cytochrome c oxidase I* (*COI*), and *Interphotoreceptor retinol-binding protein* (*IRBP*), and two morphometric datasets (geometric landmarks and linear measurements). The specific aims were i) to elucidate the systematics of the NONETHFLAVO in the context of their position in the genus *Lophuromys* and ii) to investigate when and how the NONETHFLAVO diverged and dispersed to colonize their present ranges.

Methods

5.1. Sampling

We compiled three datasets for the combined genetic and morphometric analyses. Sampling across the genus *Lophuromys* was possible for *CYTB* only and covered the currently known range of the genus (Fig. 1). Sampling for the full dataset (*CYTB, COI, IRBP,* and linear and geometric data) was possible for the non-Ethiopian *L. flavopunctatus* members only, for which we sampled the currently known range, representing type localities (or their environs) of all species currently classified under or associated with the group (Fig. 1, Additional file 3 Fig. S1, Additional file 1 Table S1). The skulls are deposited at the Field Museum of Natural History, Chicago, USA (FMNH), Kunming Institute of Zoology, Kunming, China (KIZ), and National Museums of Kenya, Nairobi, Kenya (NMK).

5.2. Genetic data

Total DNA was extracted from muscle or liver tissue preserved in absolute ethanol at -80°C using the sodium dodecyl sulfate method [83]. The DNA was PCR-amplified using gene-specific primer pairs (Additional file 2 Table S2). The PCR reaction template comprised of 20 µl volumes (0.5 µl primer pairs, 10 µl PCR Master Mix, 8.5 µl water, and 0.5 µl DNA template); the cycling temperature, time settings, and primers were specified as shown in Additional file 2 Table S2. The amplified product was sequenced in forward and reverse directions using the ABI Genetic Analyzer (Applied Biosystems), assembled in Geneious Prime® 2020.2.4 (https://www.geneious.com, Accessed September 2020), and aligned in Aliview v.1.26 [84] using MUSCLE [85]. After dropping duplicates and sequences with a high ratio of gaps/ambiguous bases, we retained 803 *CYTB* sequences, of which 316 were newly generated, and the rest downloaded from GenBank [86] and the African Mammalia database [87] (Additional file 1 Table S1). From the new *CYTB* sequences, we subsampled from the unique haplotypes and extracted 138 *COI* and 100 *IRBP* sequences, which were aligned separately and concatenated in SequenceMatrix [88]. The alignment of concatenated loci was available for the non-Ethiopian *L. flavopunctatus* members only and comprised 91 sequences, 3088 bp long (1140 bp *CYTB*, 717 bp *COI*, and 1231 bp *IRBP*), after matching similar sample identifications. We confirmed that there were no premature stop codons, indels, or heterozygous bases in MEGA X v.10.1.8 [89] and resolved heterozygous bases in the *IRBP* alignment using PHASE [90] in DnaSP v.6 [91]. All the unique new sequences were submitted to GenBank (accession numbers MW464441 - MW464606).

5.3. Morphometric data

Morphometric variation among the non-Ethiopian *L. flavopunctatus* members was inferred using a linear dataset of 725 skulls [310 ♂, 363 ♀, and 23 unsexed specimens] and a geometric dataset of 635 two-dimensional cranial images [278 ♂, 338 ♀, and 19 unsexed specimens] (Additional file 1 Table S1). The samples were age-classified based on the stage and pattern of M^3 wear into three age classes: *young adults* – fully erupted M^3 but very little to no visible wear, *adults* – medium wear on M^3, and *old adults* – medium to extensive M^3 wear. Consequently, the geometric dataset comprised 29% young adults, 40% adults, and 31% old adults, while the linear dataset comprised 28% young adults, 41% adults, and 31% old adults. The samples' assignment to separate sex and age categories was used to illustrate the potential impacts of ontogenic variation and sexual dimorphism, which can confound differences between taxonomic units [92, 93]. We used TPSUtil v.1.74 and TPSDig2 v.2.30 [94] to digitize 37 landmarks on the 2-dimensional skull images (Additional file 3 Fig. S2) and processed the resulting dataset in MorphoJ v.1.07a using Generalized Procrustes Analysis (GPA). The GPA untangles shape and size to produce centroid size (CS) and Procrustes coordinates. We performed a regression analysis with CS as an independent variable and the Procrustes coordinates as a dependent variable and used the resulting regression residuals as shape variables, free of CS variation, for consequent analyses. For the linear craniodental variation analysis, we used the same measurements and extraction techniques as in Onditi *et al.* [72].

5.4. Data analysis

5.4.1. Phylogenetic analysis

The mitochondrial phylogeny of the genus *Lophuromys* was reconstructed from an alignment of 241 *CYTB* sequences (1140 base pairs long, 711 distinct patterns, 443 parsimony-informative, 88 singleton sites, and 609 invariant sites), which included single longest sequences of each haplotype identified in the initial 803 sequences. The alignment represented all the species currently recognized in the genus *Lophuromys* [5, 22], except *L. medicaudatus* and *L. eisentrauti,* and *L. dieterleni* for which we could not obtain representative new material or publicly available sequences. Sequences of *Acomys ignitus, Deomys ferrugineus,* and *Uranomys ruddi*, downloaded from GenBank, were used as outgroups. We used maximum likelihood (ML) and Bayesian inference (BI) methods for phylogenetic reconstructions, based on a GTR+F+G4 model of nucleotide substitution, which was identified as the best-fitting under the Bayesian information criterion (BIC) in ModelFinder [95]. The ML analysis was performed using IQ-TREE v.1.6.12 [96] in PhyloSuite v.1.2.2 [97] using 100,000 ultrafast bootstrap replicates [98] to estimate branch support (BS). The BI analysis was performed in MrBayes v.3.2.7a [99] with two independent runs involving 10 million generations each, sampled every 1000th run, using the reversible-jump Markov chain Monte Carlo (MCMC) [100] to estimate posterior probability support [PP]. The BI results were visualized in Tracer v.1.7.1 [101] to diagnose convergence using the effective sample size values (ESS), with values >200 considered adequate. The majority-rule consensus tree was annotated after discarding 25% as burn-in. The resulting trees from the ML and BI analyses were graphically edited in FigTree v.1.4.4 [102].

5.4.2. Species delimitation and genetic diversity analysis

Initial principal component analysis (PCA) tests of the morphometric datasets showed both the linear and geometric craniodental characters did not cluster samples consistent with current taxonomic units a priori. Therefore, we used the *CYTB* dataset to delimit operational taxonomic units (OTUs) representing valid species. We used species delimitation methods that can reliably identify common species units without prior assignment of samples to taxonomic units and implemented both tree-based and distance-based algorithms. For tree-based species delimitation, we used the branch-cutting method (BCUT, Mikula [103]), the multi-rate Poisson Tree Processes algorithm (mPTP, Kapli *et al.* [104]), and the single threshold general mixed Yule coalescent model (GYMC, Fujisawa and Barraclough [105]). We used the genus-wide ML tree as input in BCUT and mPTP analyses and a time-calibrated tree reconstructed in BEAST2 v.2.6.3 [106] for GMYC. The BCUT and GMYC analyses were performed in R v.4.0.3 [107] using functions provided by the author for the former and the splits package [108] for the latter. The mPTP analysis was implemented using the command-line options with four MCMC runs of 500 million generations, each sampled every 50,000 runs with a 10% burn-in, with convergence confirmed from a visual inspection of the combined likelihood plot. Finally, the distance-based delimitation was performed using the Automated Barcode Gap Discovery method (ABGD, Puillandre *et al.* [109]). The same genus-wide *CYTB* alignment for the phylogenetic reconstructions was used as input. The analysis was run in the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html, Accessed 10 November 2020) using the K80 Kimura measure of distance, 0.001 - 0.1 prior bounds for intraspecific divergence, and a 0.75 relative gap width.

We used haplotype networks to inspect further the genealogical relationships between the delimited OTUs. Haplotype networks visualize genetic relationships among haplotypes, and because they do not force branching schemes, they may reflect evolutionary relationships better than the phylogenetic trees [110]. The haplotype networks were reconstructed using haplotypes generated in DnaSP and visualized in PopART v.1.7 [111] based on the Median Joining Network algorithm [112]. The genetic divergence within and between the delimited OTUs was explored using various indices of genetic diversity estimated in DnaSP, including the number of haplotypes (*h*), the probability that randomly selected haplotype pairs are different – haplotype diversity (*Hd*), the mean number of nucleotide differences (θ), and θ per site between randomly selected sequence pairs – nucleotide diversity (π). The genetic distances between and within the resolved OTUs/clades were estimated in MEGAX based on the number of nucleotide differences per site averaged between sequence pairs (uncorrected *p*-distances).

5.4.3. Estimation of divergence times

The divergence between main clades in the genus *Lophuromys* was inferred using the genus-wide *CYTB* alignment based on the coalescent-based approach in BEAST2. We applied secondary calibrations of the most recent common ancestor (MRCA) since *Lophuromys* has no fossil record. Two secondary calibration points were specified; the divergence between the *L. sikapusi* and *L. flavopunctatus* groups, which was estimated by Aghova *et al.* [113] to ca. 3.71 million years ago [Mya] (confidence: 2.66-5.05) and the root node of the subfamily *Deomyinae* (having included sequences of *Acomys ignitus, Deomys ferrugineus, Uranomys ruddi* as outgroups). According to Aghova *et al.* [113], diversification within *Deomyinae* commenced ca. 13.8 Mya (95% highest posterior density interval [HPDI]: 12.04–16.01). We used lognormal priors with a mean of 1.31 and standard deviation (SD) of 0.1 (median 3.71 Mya) for the divergence between the *L. sikapusi* group and *L. flavopunctatus* group and a mean of 2.628 and SD of 0.06 (median 13.8 Mya) for the MRCA of the *Deomyinae* subfamily members. Because the three genes, *CYTB*, *COI*, and *IRBP*, were available for the non-Ethiopian *L. flavopunctatus* members only, we estimated a species tree of the group using the StarBEAST2 package [114] of BEAST2. The time-calibration was based on the divergence between the *L. sikapusi* and *L. flavopunctatus* groups as specified above, following the inclusion of an *L. sikapusi* sequence as an outgroup. Two separate and unlinked substitution, clock and tree models corresponding to the mitochondrial (*CYTB+COI*) and nuclear (*IRBP*) loci were set, fitted with uncorrelated lognormal clock and Yule speciation models. The time-calibrated phylogeny of the genus *Lophuromys* and the non-Ethiopian *L. flavopunctatus* species tree were implemented with two MCMC runs, each 100 million generations-long, sampled every ten thousand runs were performed. The sampling convergence was assessed in Tracer; all the parameters had ESS values >400. The runs, including tree and log files, were combined in LogCombiner after discarding 10% as burn-in. The trees were summarized in TreeAnnotator and graphically edited in FigTree.

5.4.4. Biogeographical analysis

We reconstructed species ancestral ranges in RASP v.4.2 [115, 116], based on the dispersal-extinction cladogenesis (DEC) model [117] which was selected with the BioGeoBEARS R package [118] best-fitting to our dataset. The DEC model uses a species tree (with branch lengths scaled to evolutionary divergence times) and the geographical areas where the species (tree tips) occur to estimate ancestral ranges. The input tree was reconstructed from a reduced (single sequences from each GMYC-delimited OTU) time-calibrated genus-wide *CYTB* tree based on the same secondary calibrations as above. The major biogeographic ecoregions were defined according to Dinerstein *et al.* [119] [https://ecoregions2017.appspot.com/ – Accessed 5th November 2020], with slight modifications. A total of six ecoregions were used; Albertine Rift montane forests, Guinea-Congo forests, East African montane forests, Eastern Arc forests, Ethiopian montane forests, and Southern Rift Montane forests. Because neither the ETHFLAVO nor NONETHFLAVO are monophyletic and range overlap exists between the *Kivumys* group, *L. sikapusi* group, and NONETHFLAVO, dispersal was allowed between all the ecoregions.

5.4.5. Morphometric analyses

The linear variables were initially transformed by natural logarithms to enhance their multivariate normality. The presence of outliers in the geometric dataset was checked for in MorphoJ for each species group, while in the linear dataset, we used Tukey's 1.5*IQR rule using a custom R script (http://goo.gl/UUyEzD, Accessed 1st October 2020). From the combined 725 skulls for linear morphometry, <2% outliers existed for any of the 14 measurements across species groups, therefore, they were simply replaced with the respective group mean for each measurement. In the geometric dataset, a single sample identified as an outlier and was simply excluded from consequent analyses. The craniodental differences between groups were explored using discriminant function analysis (DA) in IBM SPSS Statistics v25 based on the within-group covariance matrices. In the DA, each group was assumed to have equal prior probabilities, so that cases were equally assignable to any group regardless of sample size. To test how classification accuracy compared to random assignment, we used the leave-one-out cross-validation model, where a discriminant function classifies cases based on all other cases except itself. Discriminant analysis is preferable when delimitating interspecific morphological differences due to its ability to estimate the combination of characters that best distinguish groups [93, 120]. Statistical significance of between-clade differences was estimated with the multilevel pairwise comparison using permutational MANOVA (multivariate analysis of variance) in the pairwiseAdonis R package [121]. We also used dendrograms of group mean clusters following MANOVA (performed using the manovacluster MATLAB function [www.mathworks.com/help/stats/manovacluster.html?s_tid=srchtitle, Accessed 1st October 2020]) to visualize the multivariate craniodental relationships between clades.

Results

2.1. Mitochondrial (*CYTB*) phylogeny of the genus *Lophuromys* and the definition of the *L. flavopunctatus* group

The genus-wide *CYTB* alignment produced congruent gene tree topologies for the BI and ML analyses (Fig. 2, Additional file 3 Fig. S3). In both trees, the genus *Lophuromys* bifurcated into two main groups that corresponded to the current subgeneric divisions – *Lophuromys* and *Kivumys* (Fig. 2, Additional file 3 Fig. S3). The *Lophuromys* branch split further into two groups, representing the *L. sikapusi* group and the *L. flavopunctatus* group (Fig. 2, Additional file 3 Fig. S3). In the *L. flavopunctatus* group, the non-Ethiopian samples (NONETHFLAVO1 and NONETHFLAVO2 in Fig. 2 and Additional file 3 Fig. S3) and samples from the Ethiopian Highlands (ETHFLAVO1 and ETHFLAVO2 in Fig. 2 and Additional file 3 Fig. S3) did not form separately monophyletic clades.

The major clades in the *Kivumys* group and *L. sikapusi* group corresponded to currently recognized species except for a single clade in the *sikapusi* group (*L.* sp.1 in Fig. 2 and Additional file 3 Fig. S2) and were assigned names based on the corresponding identifications in literature. These included two clades in the *Kivumys* group (*L. woosnami* and *L. luteogaster*) and eight clades in the *L. sikapusi* group (*L. sikapusi, L. nudicaudus, L. roseveari, L. ansorgei, L. huttereri, L. angolensis, L. rahmi,* and *L.* sp.1). Similarly, the major clades in ETHFLAVO1 and ETHFLAVO2 matched recently clarified taxonomies [15-17], from which names were extracted (Fig. 2, Additional file 3 Fig. S3).

The three main species groups in the genus *Lophuromys* were well-supported (BS and PP >0.95) and occupied relatively specific geographic areas (Fig. 1). The *L. flavopunctatus* group was distributed primarily in highland regions of east and east-central Africa, with ETHFLAVO1 and ETHFLAVO2 being endemic to Ethiopia and the NONETHFLAVO1 and NONETHFLAVO2 spanning a broader range over the Eastern Afromontane Highlands south of Ethiopia (Fig. 1). The *L. sikapusi* group traversed the Guinea-Congo forest belt, with a primarily west to central Africa range (Fig. 1). The *Kivumys* group distribution, on the other hand, was restricted to the Albertine Rift and adjacent lowlands to the east of the Congo River (*L. luteogaster*), where it overlapped ranges with the *L. sikapusi* group and the NONETHFLAVO (Fig. 1).

Overall, the between-group genetic distances (uncorrected *p*-distance) was highest in *Kivumys* group versus *L. sikapusi* group (16.9%), the *Kivumys* group versus *L. flavopunctatus* group was comparably distant at 16.4%, and the *L. sikapusi* group versus *L. flavopunctatus* group were relatively less differentiated (11.5%).

2.2. Mitochondrial phylogeny of the *L. flavopunctatus* group

Within the *L. flavopunctatus* group, 12 main clades were resolved from the non-Ethiopian samples (NONETHFLAVO); three in the first subgroup – NONETHFLAVO1 – and nine in the second subgroup – NONETHFLAVO2 (Fig. 2). Among the Ethiopian samples (ETHFLAVO), the number of clades in the two subgroups differed between ML and BI trees, with either one (*L. chrysopus*) or two (*L. chrysopus* and *L. simensis* 1) in the first subgroup – ETHFLAVO1 – and the rest (9-10) in the second subgroup – ETHFLAVO2, (Fig. 2, Additional file 3 Fig. S3). The ETHFLAVO1 and ETHFLAVO2 subgroups were separated by an

8.74% genetic *p*-distance, slightly higher than the 5.8% *p*-distance that separated NONETHFLAVO1 and NONETHFLAVO2. Over-all, the *p*-distances between clades corresponding to the NONETHFLAVO (NONETHFLAVO1 and NONETHFLAVO2) were consistently higher than within clades, and were comparable, albeit averagely lower, to the *p*-distances between the ETHFLAVO clades – ETHFLAVO1 and ETHFLAVO2 (Table 2).

The first NONETHFLAVO subgroup, NONETHFLAVO1, was comprised of three clades – *L. aquilus, L. verhageni,* and *L. kilonzoi* (Fig. 2). The *L. aquilus* clade was separated by 2.84% *p*-distance from the sister clade, *L. verhageni,* and 4.81% from *L. kilonzoi*. The *L. verhageni* was separated by a 4.7% *p*-distance from *L. kilonzoi* clade, which was sister to the *L. aquilus + L. verhageni* clade (Fig. 2, Fig. 3) and more diverse than both (Table 2, Table 3).

The second NONETHFLAVO subgroup, NONETHFLAVO2, contained nine distinct clades – *L. machangui, L. sabuni, L. makundii, L. dudui, L. rita, L.* cf. *cinereus, L. laticeps, L. stanleyi,* and *L. zena* (Fig. 2 and Fig. 3). The phylogenetic relationships and divergence times between the clades are shown in Fig. 2 and Fig. 3, their respective geographic ranges in Fig. 1 and Additional file 3 Fig. S1, and their genetic and evolutionary diversity in Table 1 and Table 2.

Table 1 Estimates of evolutionary divergence between and within clades in the *L. flavopunctatus* group. The number of base substitutions per site from averaging over all sequence pairs between groups are shown for within-clade (shaded diagonal) and between-clade (upper matrix: actual average site differences, lower matrix: uncorrected *p*-distances) comparisons estimated in MEGA X [1]. The non-Ethiopian *L. flavopunctatus* members are highlighted in bold fonts.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *L. zena*	**0.94**	31.57	40.39	33.26	31.24	41.65	41.16	46.55	38.6	49.28	40.91	32.37	45.99	48.27	43.81	47
2 *L. stanleyi*	2.89	**1.36**	36.63	30.03	29.81	40.51	41.61	48.6	34.7	44.43	35.44	32.88	43.22	45.1	46.3	46
3 *L. laticeps*	3.82	3.41	**1.06**	28.76	27.81	36.27	40.32	47.67	39.49	46.63	37.28	32.29	42.08	46.95	43.56	45
4 *L. dudui*	3.61	3.24	3.14	**1.59**	20.09	30.99	30.98	33.93	30.89	36.21	29.88	23.96	28.26	39.13	35.2	39
5 *L. makundii*	3.39	3.2	3.04	2.39	**0.56**	27.51	28.06	31.92	28.15	36.23	30.57	25.74	31.29	36.7	32.65	37
6 *L. machangui*	3.85	3.7	3.4	3.35	2.96	**1.28**	29.41	46.17	34.8	42.22	39.17	33.17	43.23	46.7	45.48	43
7 *L. sabuni*	3.8	3.78	3.78	3.29	3.01	2.7	**0.63**	44.9	36.91	39.17	38.02	32.7	41.67	45.1	40.13	42
8 *L. rita*	4.24	4.37	4.43	3.64	3.42	4.2	4.07	**0.24**	44.12	44.52	42.93	36.28	43.5	50.93	47.4	49
9 *L.* cf. *cinereus*	3.52	3.11	3.65	3.3	3.04	3.16	3.35	3.95	**0.9**	41.58	36.59	33.16	44.59	46.34	41.74	44
10 *L. pseudosikapusi*	5.67	5.06	5.39	4.49	4.48	4.81	4.5	5.03	4.73	**0.28**	37.66	34.45	40.09	42.78	44	44
11 *L. melanonyx* 2	4.6	3.91	4.21	3.72	3.78	4.38	4.23	4.74	4.06	4.81	**0.7**	17.15	21.44	35.91	36.47	42
12 *L. menageshae*	3.79	3.79	3.78	2.98	3.23	3.85	3.79	4.17	3.84	4.41	2.26	**0.2**	15.5	30.35	31.14	39
13 *L. simensis* 2	4.66	4.34	4.31	3.22	3.52	4.36	4.21	4.35	4.47	4.61	2.51	1.82	**0.62**	38.48	37.84	46
14 *L. chercherensis*	5.14	4.74	5.05	4.65	4.3	4.95	4.79	5.34	4.9	5.2	4.41	3.77	4.25	**0.78**	43.24	41
15 *L. brunneus*	4.84	5.06	4.83	4.27	3.93	4.96	4.36	5.15	4.52	5.43	4.56	3.95	4.25	5.14	**0.66**	40
16 *L. flavopunctatus*	5.42	5.26	5.16	4.96	4.55	4.92	4.86	5.53	5.08	5.67	5.47	5.08	5.31	5.07	5	**1.**
17 *L. brevicaudus*	5.94	5.45	6.14	5.85	6.2	5.92	6.07	6.31	5.81	6.42	6.01	5.54	5.78	7.1	6.1	6.
18 *L. melanonyx* 1	5.76	5.7	6.31	5.49	5.11	5.78	5.98	5.6	5.48	6.48	5.75	6.13	5.86	7	5.8	6.
19 *L. simensis* 1	6.13	5.99	5.9	5.8	5.19	5.63	5.92	5.81	5.93	6.29	6.23	5.61	5.6	7.03	5.3	6.
20 *L. kilonzoi*	5.93	6.11	6	5.55	4.59	5.76	5.88	6.14	6.04	6.61	5.86	5.55	5.71	6.38	5.06	5.
21 *L. verhageni*	5.87	5.82	5.85	5.58	4.68	5.39	5.42	6.06	5.27	5.95	5.27	5.45	5.47	6.35	5	5.
22 *L. aquilus*	5.85	6	5.73	5.37	4.36	5.41	5.48	5.87	5.45	5.6	5.28	5.21	5.17	5.73	5.14	5.
23 *L. chrysopus*	8.44	7.93	8.89	8.5	8.09	8.58	8.74	9.14	8.12	9.72	8.83	8.77	8.77	10.01	8.33	8.

2.3. Concatenated mitochondrial and nuclear phylogeny of the non-Ethiopian *L. flavopunctatus* members

The concatenated mitochondrial tree of the NONETHFLAVO was generally congruent to the genus-wide *CYTB* topology, with minor differences in sister relationships between clades (Additional file 3 Fig. S4). The NONETHFLAVO1 subgroup was distinct from NONETHFLAVO2, each separately monophyletic (Additional file 3 Fig. S4). In the NONETHFLAVO1 subgroup, notable differences with the *CYTB* topology included the paraphyly of *L. zena* + *L. stanleyi* clade, which contrasted the monophyly in the *CYTB* tree. The *L. zena* and *L. stanleyi* clades were also positioned at the root of NONETHFLAVO2, unlike in the *CYTB* tree. Except for *L. laticeps* and *L. rita,* which were not successfully sequenced for *COI* and therefore not included in the concatenated mitochondrial analysis, the rest of the NONETHFLAVO clades maintained congruent topologies as in the *CYTB* tree (Additional file 3 Fig. S4). On the other hand, the nuclear (*IRBP*) phylogeny did not correspond to the *CYTB* or concatenated mitochondrial tree, with most clades included in polytomies (Additional file 3 Fig. S5). In the NONETHFLAVO1 subgroup, *L. aquilus* merged with *verhageni* in monophyly while the *L. kilonzoi* samples remained monophyletic but not sister to the *L. aquilus + L. verhageni* (Additional file 3 Fig. S5).

2.4. Mitochondrial species delimitation, genetic distances, and networks

Each of the four delimitation methods produced an incongruous number and topology of splitting OTUs based on the genus-wide *CYTB* trees (Fig. 2). The mPTP identified 25 OTUs which differed from the 39 identified by BCUT, 46 identified by ABGD, and 56 identified by GMYC (Fig. 2). The *L. sikapusi* and *L. chrysopus* clades were consistently split into at least three OTUs across the methods, except in mPTP (Fig. 2). Several other clades were split as multiple OTUs by at least one of the delimitation methods, including those of NONETHFLAVO2 [*L. zena, L. stanleyi, L. dudui,* and *L. machangui*] (Fig. 2). Based on currently recognized species in literature and the haplotype networks, we resolved the 25–56 delimited OTUs to represent 33 clades. Of these, there were two clades in the *Kivumys* group, eight in the *L. sikapusi* group, and 23 in the *L. flavopunctatus* group [12 clades corresponding to the NONETHFLAVO and 11 clades corresponding to the ETHFLAVO (Fig. 2)].

The evolutionary diversity (uncorrected *p*-distance) within the NONETHFLAVO clades (0.2% - 1.6%) was systematically lower than between-clade diversity (2.39% - 6.14%) (Table 2). The haplotype networks of the *L. flavopunctatus* group (combined ETHFLAVO and NONETHFLAVO) depicted composite genealogical relationships between clades that were not apparent in the phylogenetic trees, but altogether suggested a common evolutionary origin (Fig. 4). The NONETHFLAVO clades with lower π and θ, such as *L. rita*, *L. verhageni*, and *L. aquilus*, had fewer haplotypes (Fig. 4, Table 2). Also, the more broadly sampled clades such as *L. zena*, *L. stanleyi*, and *L. machangui* had more haplotypes and higher haplotype diversity than those from single/fewer localities such as *L. aquilus* and *L. verhageni* (Table 3). These broadly sampled clades also revealed that haplotype networks were only slightly influenced by sampling coverage, such that, within a clade, different localities were not uniquely systematically clustered (Additional file 3 Fig. S6).

Table 2 Genetic differentiation in the *L. flavopunctatus* group based on *Cytochrome b* gene sequences used in the study. The analysis involved 675 sequences representing the 23 clades resolved from the species delimitation. Sites with invariable based and alignment gaps were excluded, leaving a total of 403 sites in the analysis. *N* = number of sequences; *S* = number of segregating sites; *h* = number of haplotypes; *Hd* = haplotype diversity; θ = average number of differences; π = nucleotide diversity. The non-Ethiopian *L. flavopunctatus* members are highlighted in bold fonts.

	N	*S*	*h*	*Hd*	*θ*	*π*
L. chrysopus	38	44	24	0.9474	8.588	0.0213
L. brevicaudus	19	6	7	0.8012	1.216	0.0030
L. melanonyx 1	3	3	2	0.6667	2.000	0.0050
L. simensis 1	29	16	15	0.8990	2.232	0.0055
L. verhageni	**6**	**2**	**2**	**0.3333**	**0.667**	**0.0017**
L. aquilus	**5**	**3**	**3**	**0.8000**	**1.400**	**0.0035**
L. kilonzoi	**38**	**13**	**12**	**0.8563**	**2.211**	**0.0055**
L. pseudosikapusi	10	4	4	0.6444	1.467	0.0036
L. flavopunctatus	50	36	20	0.8702	4.008	0.0100
L. brunneus	7	8	5	0.9048	2.476	0.0061
L. chercherensis	12	7	6	0.7576	1.621	0.0040
L. menageshae	13	2	3	0.2949	0.308	0.0008
L. simensis 2	10	11	7	0.9111	2.778	0.0069
L. melanonyx 2	20	18	7	0.7316	3.432	0.0085
L. sabuni	**17**	**9**	**6**	**0.5882**	**2.162**	**0.0054**
L. machangui	**61**	**32**	**27**	**0.9372**	**4.412**	**0.0110**
L. laticeps	**18**	**15**	**10**	**0.8497**	**3.771**	**0.0094**
L. rita	**6**	**1**	**2**	**0.3333**	**0.333**	**0.0008**
L. dudui	**18**	**18**	**11**	**0.9346**	**4.588**	**0.0114**
L.* cf. *cinereus	**20**	**21**	**16**	**0.9790**	**4.374**	**0.0109**
L. stanleyi	**48**	**36**	**34**	**0.9805**	**4.491**	**0.0111**
L. zena	**207**	**51**	**80**	**0.9550**	**3.464**	**0.0086**
L. makundii	**20**	**10**	**10**	**0.9211**	**2.316**	**0.0058**

2.5. Divergence dating – Time-calibrated trees

The *CYTB* divergence time estimates and phylogenetic associations between clades in the genus *Lophuromys* are presented in Fig. 3. Although deep divergences were well supported (PP >0.95), most of the recent splits had low posterior support [PP <0.95]. Divergence within the genus *Lophuromys* commenced ca. 7.12 Mya (HPDI: 5.86-8.42), resulting in the split of the genus into the two subgenera – *Kivumys* and *Lophuromys*. In the *Kivumys* subgenus, *L. luteogaster* and *L. woosnami* diverged ca. 4.38 Mya (HPDI: 3.38-5.38 Mya) while in the *Lophuromys* subgenus, the *L. sikapusi* and *L. flavopunctatus* groups diverged ca. 4.5 Mya (HPDI: 3.85-5.14). The earliest divergence in the *L. sikapusi* group occurred ca. 4.13 Mya (HPDI: 3.52-3.05) when *L. nudicaudus* split from the ancestor the rest of the group, within which divergences between ca. 2.49 Mya to ca. 1.78 Mya resulted in seven clades (Fig. 3). Internal divergences within the *L. flavopunctatus* group were more recent than in the *Kivumys* group and *sikapusi* group; with the oldest lineage, *L. chrysopus*, appearing ca. 2.76 Mya (HPDI: 2.27-3.32) but all other species appearing after the last divergence in the *L. sikapusi* group (Fig. 3). The ancestor of NONETHFLAVO1 diverged ca. 0.91 (HPDI: 0.69 - 1.04) Mya from *L. simensis* 1 while NONETHFLAVO2 diverged ca. 0.7 (HPDI: 0.55 - 0.86) Mya from *L. pseudosikapusi*. Internal divergences within NONETHFLAVO1 ca. 0.45 - 0.79 Mya led to three clades (*L. aquilus, L. verhageni,* and *L. kilonzoi*). Divergences within NONETHFLAVO2 ca. 0.41 – 0.61 Mya led to nine clades (*L. makundii, L. stanleyi, L. rita, L. zena, L. laticeps, L. dudui, L.* cf. *cinereus, L. machangui,* and *L. sabuni*) – Fig. 5. The divergence times of the NONETHFLAVO based on the species tree of the three genes (*CYTB + COI + IRBP*) was generally more recent than the genus-wide *CYTB* estimates but maintained the relationships between clades (Additional file 3 Fig. S7). Because the time-calibrated phylogeny using the three genes was possible for the NONETHFLAVO only, we relied on the *CYTB* divergence dates for all divergence references.

2.6. Historical biogeography of the genus *Lophuromys*

Divergence within the genus *Lophuromys* likely originated in the Guinea-Congo/Albertine Rift forests, from where several dispersal events (28 dispersals versus six vicariance events) led to the colonization of current ranges (Fig. 5). These dispersals mostly occurred within ecoregions (mainly in the Guinea-Congo and Ethiopian Highlands forests) than between ecoregions (Fig. 5). The divergence in the *Kivumys* group likely originated in the same area as the genus, while in the *Lophuromys* subgenus, the Guinea-Congo forests formed the ancestral range, after which the *L. sikapusi* group remained in the Guinea-Congo forests while the *L. flavopunctatus* group dispersed to the Ethiopian Highlands. From the Ethiopian Highlands, the NONETHFLAVO species colonized current ranges over two southward dispersal events (Fig. 5). The first dispersal was by the NONETHFLAVO1 ancestor to the East African montane and Eastern

Arc forests after which vicariance caused consequent divergences (Fig. 5). The NONETHFLAVO2 ancestor later dispersed to the Albertine Rift forests, from where both dispersal and vicariance events resulted in the colonization of the Congolian forests, East African montane forests, Eastern Arc forests, and the Southern Rift Montane forests (Fig. 5).

2.7. Morphometric analysis of the non-Ethiopian *L. flavopunctatus* members

Morphometric analyses were conducted on combined datasets (age and sex) because sexual dimorphism and ontogenic variation did not impact discriminant tests between the *CYTB* clades. Overall, *L. dudui* had the smallest skull (based on CI and CS), while the *L. aquilus* skulls were the largest (Fig. 6, Additional file 2 Table S3). The combined-clades' morphospace following linear (DA^{LIN}) and geometric (DA^{GEO}) discriminant tests overlapped randomly with no evident systematic pattern delimiting the *CYTB* clades. Therefore, we partitioned the datasets into two groups around the two phylogenetic subgroups; NONETHFLAVO1 (*L. aquilus*, *L. verhageni*, *L. kilonzoi*) and NONETHFLAVO2 (*L. sabuni, L. makundii,* and *L. machangui, L. stanleyi*, *L. dudui*, *L. laticeps, L.* cf. *cinereus*, and *L. zena*).

The DA^{LIN} and DA^{GEO} classification results were consistent over-all, however, most clades were more correctly classified (more distinguishable) by DA^{GEO}, especially in NONETHFLAVO2 (Fig. 6, Additional file 3 Fig. S8). Between-clade differences between the two skull datasets were not unidirectional, with DA^{GEO} achieving higher correct classification than DA^{LIN} in NONETHFLAVO1 but not in NONETHFLAVO2 (Table 3). In NONETHFLAVO1, all three clades were distinct, with DA correctly classifying >85% of each clade into the respective given group (Table 3). The *L. aquilus* and *L. verhageni* were the most correctly classified by either DA^{LIN} or DA^{GEO} (Table 3, Fig. 6). The *L. verhageni* skulls were smaller than the adjacent *L. aquilus* or *L. kilonzoi* (Additional file 2 Table S2). The *L. verhageni* and *L. aquilus* skulls were more closely related to each other more than to *L. kilonzoi* (Fig. 6, Additional file 3 Fig. S8, Table 3).

In NONETHFLAVO2, the morphospace of *L. zena* and *L. stanleyi* markedly overlapped in DA^{LIN} and DA^{GEO}, between themselves and with several other clades, mainly *L.* cf. *cinereus, L. dudui,* and *L. laticeps* (Fig. 6, Table 3, Additional file 3 Fig. S8). The *L. laticeps* skulls were highly indistinguishable from other clades, being least correctly classified in the NONETHFLAVO2 subgroup and the combined pool of all clades.

The range-restricted clades, such as *L. verhageni, L. aquilus,* and *L. makundii*, were less ambiguously delimited and highly correctly classified by DA^{LIN} and DA^{GEO} (Fig. 6, Table 3, Additional file 3 Fig. S8). In contrast, more broadly sampled clades such as *L. zena* and *L. stanleyi* were less distinctly discriminated against from other clades (Fig. 6, Table 3). Statistical differences between clades based on multilevel pairwise comparison were significant, even for clades with overlapping morphospace (Additional file 2 Table S4).

Table 3 – The classification of the non-Ethiopian *L. flavopunctatus* members based on discriminant analysis of linear and geometric craniodental characters. Values are the cross-validated (leave-one-out bootstrapping) percentage success by which samples were predicted into the corresponding species. The shaded diagonal values indicate the success by which samples were predicted into their own groups which correspond to the distinct *Cytochrome b* clades shown in Fig. 2. The classification of the combined clades is shown in Fig. S8. σ = overall classification success, *N* = number of samples.

		1	2	3	*N*	*σ*					
Linear	1 *L. aquilus*	92.3	0	7.7	13	95.2					
	2 *L. kilonzoi*	2.7	94.6	2.7	74						
	3 *L. verhageni*	0	0	100	17						
Geometric	1 *L. aquilus*	84.6	0	15.4	13	87.4					
	2 *L. kilonzoi*	0	88.1	11.9	67						
	3 *L. verhageni*	13.3	0	86.7	15						
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*N*	*σ*
Linear	1 *L.* cf. *cinereus*	57.4	4.9	16.4	4.9	4.9	1.6	8.2	1.6	61	44.6
	2 *L. dudui*	13.8	55.2	20.7	0	0	0	6.9	3.4	29	
	3 *L. laticeps*	23.1	3.8	38.5	7.7	0	3.8	19.2	3.8	26	
	4 *L. machangui*	5.9	4.7	4.7	60	0	10.6	5.9	8.2	85	
	5 *L. makundii*	3.3	0	0	0	83.3	0	6.7	6.7	30	
	6 *L. sabuni*	0	0	0	13.6	0	81.8	0	4.5	22	
	7 *L. stanleyi*	13.7	10.9	12.8	6.6	7.6	1.9	35.5	10.9	211	
	8 *L. zena*	3.2	10.2	7.6	13.4	20.4	5.7	9.6	29.9	157	
Geometric	1 *L.* cf. *cinereus*	47.4	8.8	10.5	5.3	5.3	0	19.3	3.5	57	59.4
	2 *L. dudui*	24.1	51.7	6.9	0	0	3.4	13.8	0	29	
	3 *L. laticeps*	14.3	9.5	42.9	0	0	4.8	19	9.5	21	
	4 *L. machangui*	2.5	1.3	0	79.7	1.3	8.9	3.8	2.5	79	
	5 *L. makundii*	0	3.6	0	0	85.7	0	0	10.7	28	
	6 *L. sabuni*	0	5.3	0	15.8	0	63.2	5.3	10.5	19	
	7 *L. stanleyi*	13.1	6	9	2.5	3	1.5	53.8	11.1	199	
	8 *L. zena*	3.7	3.7	3.7	6.5	7.5	5.6	10.3	58.9	107	

Discussion

3.1. Phylogenetic relationships within the genus *Lophuromys*

The deeper phylogenetic relations in the genus *Lophuromys,* including the validity of the subgeneric divisions (*Lophuromys* and *Kivumys*) and older lineages (*Kivumys* group and *L. sikapusi* group), and their respective monophyly have been relatively uncontested in recent checklists [5, 6, 22]. In contrast, species accounts in the 'speckled pelage' groups, the *L. flavopunctatus* group, combining the Ethiopian endemics (herein as Ethiopian *L. flavopunctatus* members – ETHFLAVO) and the non-Ethiopian ones (herein as non-Ethiopian *L. flavopunctatus* members – NONETHFLAVO), have changed rapidly recently. In consensus, our genus-wide phylogenetic inference based on the *CYTB* gene supports the deep divergence of the genus *Lophuromys* into three distinct deeply-diverged groups that correspond with the widely recognized species groupings; i) *Kivumys* group (*Kivumys* subgenus), ii) *L. sikapusi* group (*Lophuromys* subgenus), and iii) *L. flavopunctatus* group (*Lophuromys* subgenus). The two *Lophuromys* groups – *L. sikapusi* group and *L. flavopunctatus* group – are separated by a much lower mtDNA divergence (*p*-distance) compared to the almost equidistant *p*-distance separating them from the *Kivumys* group. Based on the *CYTB* divergence, the *Kivumys* group appear distinct enough to be classified in a distinct genus, with the *L. sikapusi* group and *L. flavopunctatus* group also distinct enough to be included in separate subgeneric groups. However, testing these conjectures hinges on broader nuclear loci sampling and morphological characterizations across the genus. Overall, these findings broadly agree with the current classification of species into the *Kivumys* group [6]. However, we importantly highlight that classifying species into the *L. sikapusi* and *L. flavopunctatus* groups based on morphological characterization is essentially ambiguous unless backed up by genetic evidence.

Within the *Kivumys* group (subgenus *Kivumys*), high *CYTB* differentiation (13.39% *p*-distance) between the two species represented in our dataset – *L. woosnami* and *L. luteogaster* – clearly delimitate them as distinct lineages. Together with *L. medicaudatus,* whose *CYTB* sequences were not included in the study, all three species in the *Kivumys* subgenus have been recorded from overlapping ranges, i.e., in the northeastern and eastern DRC forests and bordering montane forests of the Albertine Rift, with *L. woosnami* extending into western Burundi, Rwanda, and Uganda [5, 6, 22, 35, 38]. A thorough investigation of niche partitioning and other ecomorphological strategies inherent in gene flow and adaptive genetic divergence within the *Kivumys* subgenus is necessary to clear up their evolutionary history. Such a study would also illuminate the precise nature and limits of their ranges (whether sympatric, syntopic, or parapatric).

The eight clades in the *L. sikapusi* group correspond to seven described species (*L. angolensis, L. ansorgei, L. huttereri, L. nudicaudus, L. rahmi, L. roseveari,* and *L. sikapusi*) and the unidentified taxon (*L.* sp.1 in Fig. 2). The *L.* sp.1 clade is separated by 10.47-13.85% *p*-distance from all other species in the *L.*

sikapusi group and forms a sister relationship with *L. sikapusi* (separated by 11.42% *p*-distance), representing a potentially undescribed species. While describing rodents of western and southwestern Guinea, Denys *et al.* [39] considered their *Lophuromys* samples from Mankountan, Boffa prefecture, Guinea, as a tentative new species in the *sikapusi* group due to its unique head and body length, pending morphometric and genetic evidence for a taxonomic description. Our *CYTB* evidence suggests that the *Lophuromys* from the Conakry region of Guinea might belong to their undescribed taxa. Still, without more the morphometric evidence to confirm its morphological relationships in the *sikapusi* group and range limits, we retain a similar provisional species status for *L.* sp.1.

The utilization of pelage coloration to resolve the systematic grouping of species is rather debatable in the genus *Lophuromys*. For instance, the inclusion of *L. dieterleni* (Mt Oku, Cameroon) and *L. eisentrauti* (Mt Lefo, Cameroon) in the *L. flavopunctatus* group by Musser and Carleton [6] (citing Verheyen *et al.* [25]) is quite doubtful. Our *CYTB* tree shows all unspeckled-pelage taxa clusters in the *L. sikapusi* group (*L. angolensis, L. ansorgei, L. huttereri, L. nudicaudus, L. rahmi, L. roseveari, L. sikapusi,* and *L.* sp.1), well distinct from the speckled-pelage *L. flavopunctatus* group. From an ecomorphological outlook, the craniodental relationship between *L. dieterleni* and *L. eisentrauti* and the *L. flavopunctatus* group [25, 26] might simply be signals of convergent adaptive responses to local environments [40, 41], which are taxonomically uninformative without genetic evidence. Then again, the genetic and craniodental affinity of the unspeckled *L. pseudosikapusi* to the Ethiopian endemics [13] confounds further the overall phylogenetic relationships within and between the speckled-pelage (*L. flavopunctatus* group) and unspeckled-pelage (*L. sikapusi* group) species. From our findings, assigning clades to either the *L. sikapusi* group or the *L. flavopunctatus* group is unambiguous based on genetic relationships, and future genetic studies are likely to resolve the *L. dieterleni* and *L. eisentrauti* membership.

The assignment of ETHFLAVO clades to corresponding species is a nontrivial task due to the recently clarified taxonomic accounts of the Ethiopian *Lophuromys* [15-17]. For example, the pairs of highly divergent *CYTB* clades of *L. simensis* (*L. simensis* 1 and *L. simensis* 2) and *L. melanonyx* (*L. melanonyx* 1 and *L. melanonyx* 2) comprise the multiple haplogroups within the same species due to past mtDNA introgression events. Such introgressions have also been confirmed in *L. brunneus,* of which we only sampled one haplogroup, summing up to the 12 mtDNA *Lophuromys* lineages endemic to Ethiopia. Nuclear genomic data support these 12 lineages represent nine species (*L. chrysopus, L. melanonyx, L. simensis, L. flavopunctatus, L. brunneus, L. pseudosikapusi, L. menageshae, L. chercherensis,* and *L. brevicaudus*) which differ by karyotypes, morphology, and preferred elevation, i.e., types of ecosystems [13, 15, 16]. Interestingly, mitochondrial introgression, apparently common in the *flavopunctatus* group, was not detected in the rest of the genus *Lophuromys*, although it should be noted that nuclear genetic data are relatively scarce outside the Ethiopian *flavopunctatus* members.

Among the NONETHFLAVO, three clades, *L. aquilus, L. verhageni,* and *L. kilonzoi*, form a distinct subgroup (NONETHFLAVO1) that is phylogenetically isolated from a second subgroup, NONETHFLAVO2 (*L.* cf. *cinereus, L. dudui, L. laticeps, L. machangui, L. makundii, L. rita, L. sabuni, L. stanleyi,* and *L. zena*). The NONETHFLAVO1 and NONETHFLAVO2 are deeply nested within the Ethiopian endemics to form a monophyletic '*L. flavopunctatus* group', agreeing with previous conjectures that the NONETHFLAVO colonized the current ranges following dispersals out of Ethiopia [13, 14].

3.2. Species divergence and biogeography

The nested phylogenetic relationships between the ETHFLAVO and NONETHFLAVO conform generally to evolutionary processes speculated previously [13, 14]. While our findings support the Ethiopian highlands as the cradle of the speckled-pelage *Lophuromys*, the precise nature of their evolutionary radiation, including processes characterizing the observed differentiation between clades remains a matter for speculation, mainly owing to the strong effect of mtDNA on the inferred phylogeny. In any case, it is currently not possible to ascertain whether long-distance dispersal and/or montane-forest bridges promoted the divergence and dispersal of non-Ethiopian *flavopunctatus* members out of the Ethiopian Highlands. Dispersal along a north-south axis, i.e., out of Ethiopia to southern Afromontane Highlands is relatively like that of other montane-adapted rodents [42-44] and attributed to montane forest expansion during Pliocene-Pleistocene interglacials.

The timing of the NONETHFLAVO and NONETHFLAVO out-of-Ethiopia dispersals, albeit based on a single mitochondrial locus, coincide with the repeated expansion and contraction/isolation of montane forests and their faunal assemblages during the humid intervals of Pleistocene glacial-interglacial cycles [17, 45-48]. Within the *L. flavopunctatus* group, these events likely connected the southern Ethiopian Highlands with Albertine Rift montane forests, and Kenyan and Tanzanian Highlands across the currently arid Turkana depression [43, 45, 49-51]. The *L. flavopunctatus* group is primarily restricted to humid/wet habitats which are currently confined to montane areas in East Africa. These species could only have dispersed when the East Africa Highlands were connected with similarly suitable habitats. The first out-of-Ethiopia dispersal by the NONETHFLAVO1 ancestor and consequent range retention in the northern EAMs concur with their prolonged stability that preceded the formation of most of the Kenya Highlands, Tanzanian Highlands, and Albertine Rift montane forests. The split and dispersal of the *L. aquilus* + *L. verhageni* clade from *L. kilonzoi*, the consequent split of *L. aquilus* from *L. verhageni,* and the appearance of several clades in the NONETHFLAVO2 subgroup, all happened in the mid-late Pleistocene. This coincides with wet climate periods that made it possible to cross currently dry valleys such as those isolating Mt. Kilimanjaro and Mt. Meru and the Turkana depression in northern Kenya and southern Ethiopia [44, 52]. The absence of genetic evidence of this first dispersal in East African highlands such as the Kenyan Highlands, suggests these mountains served as 'stepping-stones'. The 'first colonizers' – NONETHFLAVO1 – were presumably replaced by the more successful 'second colonizers' – NONETHFLAVO2, such as *L. zena* in the Kenyan highlands. Whether or not the second colonizers hybridized with the first ones remains unclear from mtDNA, and genomic analyses should be applied to investigate this possibility.

The Albertine Rift Valley is a crucial biogeographical feature in the radiation of the NONETHFLAVO and is likely an active barrier to gene flow on either side. The four distinct clades whose ranges are separated by the Albertine Rift (*L. dudui, L. stanleyi, L. cf. cinereus,* and *L. laticeps*) suggest that they are not able to cross and have not experienced gene flow since their divergence. While *L. stanleyi* occurs widely eastward of the Rwenzori Mountains, it does not extend west of the mountains, whereas the range of *L. dudui* begins in Virunga National Park, and only extends westwards. The Albertine Rift might have been a barrier to

L. stanleyi's westward dispersal and *L. dudui*'s eastward dispersal. This hypothesis is also consistent with the occurrence of *L. laticeps* on the eastern and *L. cf. cinereus* on the opposite western side of the Albertine Rift around Lake Kivu and Lake Tanganyika, with either presumptively unable to cross. Notably, *L. sabuni*, which is the only clade whose occurrence span both flanks of the Albertine Rift, appear to have dispersed between the Rukwa Rift and Lake Tanganyika and then southwards to Chishimba Falls (Northern Zambia), where it was recently recorded (Sabuni *et al.* [53]. Other forest rodents have ranges that span the Albertine Rift, unlike observed here for *L. dudui, L. stanleyi, L. cf. cinereus,* and *L. laticeps*. For instance, the *Malacomys longipes* [54] and the *Praomys jacksoni* [55] occur on both sides of the Albertine Rift Valley.

3.3. Morphological variation within the non-Ethiopian *flavopunctatus* members

Most species in the NONETHFLAVO have overlapping craniodental characters in morphospace, making our large dataset of linear measurements and geometric landmarks unreliable as the exclusive evidence to infer species limits. For instance, the range of skull morphology of *L. stanleyi* and *L. zena* (both linear and geometric) significantly resembles the skull forms of all other clades in the NONETHFLAVO, except *L. verhageni* and *L. aquilus,* which unambiguously cluster and have the least overlap with any other species in the group. The *L. stanleyi* and *L. zena* clades exemplify a typical systematic problem in the NONETHFLAVO, where morphological evidence cannot classify samples to meaningful species units using taxonomically informative characters. Accounting for phenetic variation in the NONETHFLAVO, beyond their common ancestry, requires more comprehensive genomic analyses to disentangle the underlying ecomorphological processes among species occurring in similar habitats. Without such genomic evidence, the taxonomic accounts of several clades are best not considered reliably resolved when based on linear or geometric morphometrics only.

Divergence dates and biogeographic patterns in the NONETHFLAVO suggest that the drivers of craniodental variation fit multiple non-exclusive hypotheses associated with the correlation of ecomorphological divergence with speciation [56]. The relatively recent divergence of most of the clades suggests ecologically-mediated adaptive evolution might not be predominant speciation drivers between congeners in sympatry [57-59]. Except for *L. aquilus* and *L. verhageni* which are restricted to single mountain ecosystems, all the NONETHFLAVO species appear to have non-specialized niches as they are not restricted to high montane habitats. They are, thus, more likely to exhibit non-specialized morphological traits that are taxonomically uninformative [60]. The treatment of the *L. flavopunctatus* group by Verheyen *et al.* [12] and Verheyen *et al.* [14] highlights the use of craniodental and external morphology data to recognize populations as unique species with minimal use of genetic data. However, inter/intraspecific taxonomic delimitation among rodents often have fewer taxonomically informative stable morphological states, possibly due to nonadaptive and or rapid adaptive radiations [61]. These influences might hinder a replicable definition of taxon-specific phenotypic traits [62-64], leading to the subjective interpretation of valid species. While our geometric morphometry appears generally more sensitive at detecting variabilities between clades compared to linear measurements, just like in other cases [65], over-all, both datasets produced virtually similar results.

3.4. Taxonomic assessment of the non-Ethiopian *flavopunctatus* members

While most of the OTUs recovered in the *L. flavopunctatus* group represent species currently named, the between-clade genetic distances and *CYTB* incongruence with morphometric and *IRBP* gene results raise more taxonomic questions than resolution. For instance, only a few mutations at *CYTB* separate *L. aquilus* from *L. verhageni* (2.8% p-distance) and *L. stanleyi* from *L. zena* (2.9% p-distance), which is among the closest between-species *CYTB* divergences in the *L. flavopunctatus* group. While such low sequence divergence between these sister clades indicates a recent separation of gene pools (at least at mtDNA), it nonetheless, raises concerns about the species' taxonomic validity, suggesting the need to synonymize them in future taxonomic revisions, without more genetic support, especially since no clear diagnostic morphological differences delimit them. Moreover, the *IRBP* failure to delimitate several distinct mtDNA clades in the NONETHFLAVO might relate to its slow mutation rate which makes it unable to resolve deeper and or short branches among rodents [66, 67]. Nevertheless, future taxonomic reassessments of the genus *Lophuromys* should utilize more comprehensive genomic analysis (such as multiple nuclear loci) which are likely to be more informative in delimitating phylogenetic relationships. Such genomic evidence would also quantify the level of distinctiveness between close relatives that are allopatric such as *L. aquilus* and *L. verhageni* and the level/absence of gene flow between parapatric ones such as *L. stanleyi* and *L. zena* as is prevalent in the ETHFLAVO [16].

The genetic diversity within lineages such as *L. sikapusi* and *L. chrysopus,* for instance, showed the delimited OTUs within them were similarly distinguishable based on *CYTB* comparably to several clades in the NONETHFLAVO. It appears that taxonomic classifications of *Lophuromys* species that is not based on extensive nuclear evidence, should yet be regarded inconclusive (i.e., within the *Kivumys* group, *L. sikapusi* group, and NONETHFLAVO).

The NONETHFLAVO1 subgroup – *L. aquilus, L. verhageni,* and *L. kilonzoi*

The samples from Mt. Kilimanjaro, Mt. Meru, and northeastern Tanzanian Eastern Arc Mountains form distinct monophyletic lineages, representing species currently recognized as valid. *L. aquilus* was described by True [23] from Mt. Kilimanjaro and confirmed by Verheyen *et al.* [14] to be the only *Lophuromys* along the entire elevation gradient. *Lophuromys verhageni* was described by Verheyen *et al.* [12] as an endemic of Mt Meru, while *L. kilonzoi* was described by Verheyen *et al.* [14] from the Magamba, East Usambara. Perhaps because of fewer informative sites in shorter sequences, *L. aquilus, L. verhageni,* and *L. kilonzoi* had a different phylogenetic topology in Verheyen *et al.* [14]. Our expanded *CYTB* sampling supports the three species are minimally differentiated, forming a sister clade to one of the haplogroups of *L. simensis*. The current *CYTB* phylogeny, therefore, provides a clearer picture of the phylogenetic relationship between *L. aquilus, L. verhageni,* and *L. kilonzoi* and their position in the genus. The historical biogeographical reconstruction suggested the colonization and divergence of *L. aquilus* and *L. verhageni* resulted from vicariance events that coincide with the Pleistocene climatic oscillations which fragmented humid montane forests in East Africa [45, 54, 55, 68, 69]. The savannas separating their current ranges were substantially stable even across glacial cycles in the late Pleistocene [69]. The occurrence of *L. aquilus* and *L. verhageni* is also consistent with the endemism of *Crocidura newmarki* on Mt. Meru [70] and *Myosorex zinki* on Mt Kilimanjaro [71]. The divergence and dispersal of the NONETHFLAVO1 ancestor coincided with temporary

biogeographical contacts between the Ethiopian Highlands and other Afromontane forests in the early Pleistocene [69]. After initial colonization, montane forests were again fragmented by climatic oscillations, in the process facilitating allopatric speciation. Similarly, the patchy distribution of *Praomys delectorum* across the Eastern Arc Mountains and Southern Montane Forests was probably driven by comparable vicariance events [68]. The higher genetic diversity within *L. kilonzoi* suggests that it remained in the ancestral range after diverging from the ancestor of *L. aquilus* + *L. verhageni*. Similar divergence and diversity patterns were observed for the forest-dependent *Praomys delectorum* [68], where the MRCA of populations from the Eastern Arc Mountains predated those from Mt. Kilimanjaro and Mt. Meru and correlated with genetic diversity.

The northern part of NONETHFLAVO: *L. stanleyi* and *L. zena*

The sister clades from the Kenyan and Ugandan highlands, *L. zena* and *L. stanleyi,* comprise the northern part of the non-Ethiopian *flavopunctatus* members. Our sample coverage of these two clades was the most comprehensive to date and substantially extend their known ranges. *Lophuromys zena* [31], thought to be endemic to the higher elevations of central Kenya [12, 14], occurs in all the stably humid ecosystems in Kenya, including Loita Hills forests in the southeast of their range to western (Mt. Elgon, Cherangani Hills, and Kakamega Forest) and southwestern (Victoria Basin – Yala Swamp) Kenya. The distribution of *L. zena* overlaps with *L. stanleyi* and *L. ansorgei* in the Kakamega Forest and with *L. ansorgei* in Yala Swamp. This distribution supports the conclusions of Onditi *et al.* [72] that *L. zena* could have been much more widespread in Kenya than previously known [12, 14]. The range of *L. stanleyi* is also much more extensive than previously described. Sabuni *et al.* [53] extended the range of *L. stanleyi* (delimited by mtDNA) into northwestern Tanzania beyond its Mt Rwenzori type locality [12], where it was thought to be restricted. Here we provide evidence that *L. stanleyi* occurs through much of Uganda, spanning southeastern South Sudan and northeastern Uganda forests eastwards to the Kakamega Forest in Kenya (its eastern limit) and south into northern Rwanda. The 'Karamoja/Uganda gap' [47, 73] was not a barrier to the dispersal of *L. stanleyi* through Uganda to connect the Kenya Highlands and Albertine Rift montane forests, as was the case for the forest-dependent *Hylomyscus* [47, 74]. Generally, the sister relationship of *L. zena* and *L. stanleyi* (minimal *CYTB* divergence) reinforce biogeographic affinities between the Albertine Rift montane forests and the Kenya Highlands [47-49]. Furthermore, the occurrence of *L. zena* and *L. stanleyi* in both lowland and highland forests suggest a phylogeographic pattern shaped also by an opportunistic ecological strategy, unlike true forest-specialists such as the *Hylomyscus denniae* and *Sylvisorex granti* groups that are restricted to high-elevation forests [47, 48]. The biogeographies of *L. zena* and *L. stanleyi* mirror similar patterns as the more widespread *Praomys jacksoni* which colonized both montane and lowland forests. However, the absence of a taxonomic structure based on *IRBP* further reinforces the need to apply genomic analyses, especially in zones of secondary contacts, such as in Kakamega, to shed light on the level of their reproductive isolation and the taxonomic validity.

The southern part of NONETHFLAVO: *L. machangui* and *L. sabuni*

These two significantly supported sister clades correspond to *L. machangui* and *L. sabuni,* both described by Verheyen *et al.* [14] from Mount Rungwe and the Mbizi Mountains (Ufipa Plateau), respectively. Their sister relationship and late Pleistocene divergence coincide with the split of *L. verhageni* and *L. aquilus,* attributable to the late Pleistocene expansion of moist forests that likely enabled them to disperse to the current ranges, whose suitability was later restricted to highland forests. Overall, the distribution of *L. machangui* and *L. sabuni* reveal biogeographical trends that both coincide and contrast with other small mammals in the region, suggesting that other taxon-specific functional traits, such as dispersal ability and habitat specificity versus generality also influenced their evolutionary radiation. For instance, the distribution of *L. machangui* suggests the Makambako Gap has not barred its dispersal, similar for other small mammals including *Myosorex kihaulei* [75], but has barred the dispersal of *Praomys delectorum* [68] and *Otomys lacustris* [76]. Within the range of *L. sabuni*, Kerbis Peterhans *et al.* [73] recently described two species in the genus *Hylomyscus, Hylomyscus stanleyi* from Mbizi Forest Reserve and *Hylomyscus mpungamachagorum* from Mahale National Park, suggesting that the so-called Karema Gap was a barrier to the dispersal of these *Hylomyscus* species but not to the dispersal of *L. sabuni*. Overall, the close craniodental and genetic affinity between *L. sabuni* and *L. machangui* to each other in comparison with members from other clades in the NONETHFLAVO2 subgroup suggests they have experienced somewhat similar ecological selection resulting in similar ecomorphological characteristics [77]. The craniodental and genetic affinities between *L. sabuni* and *L. machangui* also concurs with the floral and faunal affinity between the Southern highlands of the northern end of Lake Malawi and the Mbizi Forest, attributed mostly to the absence of a substantial biogeographical barrier between them. More studies are needed to delineate genetic differentiation across the range of *L. machangui* and *L. sabuni*, and detail how isolation by distance and geographical features have impacted dispersal.

The western part of NONETHFLAVO: *L. dudui* and *L. rita*

The *L. dudui* clade comprised samples from the northeastern DRC montane highlands of the Albertine Rift –Rwenzori Mountains, westwards to the Kisangani – Bomane – Yaenero areas. This distribution leaves a ca. 480 km sample gap between the eastern limits (Epulu – Tshiabirimu – Ituri) and western limits near Baliko, Boende on the left bank of Congo River. The inclusion of samples from both sides of the Congo River in the *L. dudui* clade modifies the original description as well as consequent accounts of *L. dudui*, where it has been thought to be restricted between the right bank of the Congo River and the western foothills of the Albertine Rift mountains [12, 14]. The current range of *L. dudui* resembles that of *Praomys mutoni* and *Praomys jacksoni* [55] both of which occupy lowland forests on both banks of Congo River in the Kisangani region [55, 78]. Morphologically, *L. dudui* is easily diagnosable from the nearby NONETH2 members due to its distinctly small skull (Fig. 6, Additional file 2 Table S3), consistent with previous findings [12, 14]. The *L. dudui* range overlaps with that of *L. rita*, which was assigned to samples spread over an expansive area in the Congo Basin, spanning southwestern DRC (Kinshasa) to the northeast (Kisangani, left bank of Congo River) and southwards to northwestern Zambia. Although we are unable to make skull comparisons with the holotype, this clade forms a well-defined mtDNA lineage, probably representing the *L. rita* described by Dollman [31] from south of Lake Tanganyika in NE Zambia (Mporokoso) and Lufupa River, Katanga, DRC. Despite its expansive range, *L. rita* appears bound to the central Congo basin by the Congo and Lualaba Rivers, which have likely limited its dispersal, like *Praomys minor* in the central Congo Basin [55]. Our geographic sampling of *L. rita* is notably sparse relative to its distribution and more surveys are necessary to resolve the full range and genetic diversity within the *L. rita* clade. Importantly, a formal taxonomic reassessment is required to validate the morphological relationship of the *L. rita* clade with the holotype and topotypes.

L. makundii

Specimens attributed to the monophyletic *L. makundii* derive from the Mount Hanang (type locality) northwards over the Lake Manyara and Ngorongoro crater to Mt Kitumbeine. Several 'unsuitable' dry corridors which currently isolate *L. makundii* from Eastern Arc Montane forests, Albertine Rift Mountains, Kenyan Highlands, and even the nearby Mount Kilimanjaro and Mount Meru seem to have impacted its dispersal after the initial colonization event. However, the occurrence of *Crocidura montis, Crocidura hildergadea, Otomys angoniensis, Grammomys dolichurus/macmillani, Graphiurus murinus,* and *Praomys taitae* in similar habitats as *L. makundii* in the north-central Tanzania region [53, 79] suggest that its biogeographical affiliation to other Eastern Afromontane forests in the region is recent. The relatively isolated range of *L. makundii* likely imposed a more rigid barrier to genetic exchange with other lineages after divergence [80], and might explain why it is the only other clade in the NONETHFLAVO, besides *L. kilonzoi,* that retains monophyly in the *IRBP* tree. Still, the minimum divergence time and possible sister relationship to either *L. dudui* or *L. laticeps* show that it is more closely affiliated to the Albertine Rift than the NONETHFLAVO1 range. As such, *L. makundii* probably colonized its current range when moist forests connected the currently isolated volcanic mountains during the late Pleistocene climate fluctuations.

L. cf. *cinereus* and *L. laticeps*

The *L.* cf. *cinereus* samples overlap the Kahuzi-Biega National Park locality from where Dieterlen and Gelmroth [28] described *L. cinereus*. Following the initial proposal by Dieterlen [11] that the external and craniodental distinctness used by Dieterlen and Gelmroth [28] to describe *L. cinereus* were, in fact, morphotypes of *L. laticeps,* there has since been no formal taxonomic reassessment of its validity, with the foundational references [6, 12] maintaining its synonymy to *L. laticeps*. Our mtDNA, nuclear (*IRBP*), and craniodental tests showed similar differences between the *L.* cf. *cinereus* and *L. laticeps* clades, comparable to the distances within and between other NONETHFLAVO2 clades, including the sister clade, *L. rita*. The *L.* cf. *cinereus* skulls overlapped most with *L. laticeps, L. dudui,* and *L. stanleyi,* consistent with the earlier rationale for its synonymy [6, 12]. A formal taxonomic revision of *L.* cf. *cinereus,* is needed to validate and update its distribution, and genetic and phenetic relationship to other non-Ethiopian *L. flavopunctatus* members. Such a revision would importantly update the occurrence of *L. cinereus* (herein as *L. cf. cinereus*), which was perceived restricted to the type locality [28, 81], to extend from Kahuzi-Biega NP to the Itombwe Massif and southwards ca. 300 kilometers to Mt. Kabobo – on the western shore of Lake Tanganyika. Thomas and Wroughton [29] considered *L. laticeps* as a morphologically unique lineage among its close relatives allied to *L. aquilus* [23] due to a broader lower braincase and shorter palatal foramina. In agreement, our *L. Laticeps* skulls had the broadest BBC and one of the shortest PPL in NONETHFLAVO2 dataset (Additional file 2 Table S3). The *L. laticeps* clade is also genetically well-differentiated, comparably to close relatives – *L.* cf. *cinereus, L. stanleyi,* and *L. laticeps*. There is a need to formally reassess the taxonomy of *L.* cf. *cinereus* and *L. laticeps,* to clarify and update their distinctness from other lineages in the NONETHFLAVO, not to be synonymized under *L. aquilus*.

L. margarettae, L. rubecula, and *L. major*

No genetic OTUs could be matched to *L. margarettae, L. rubecula,* or *L. major*, despite sampling from their respective ranges – Mathews Range, Mount Elgon, and proximity of Ubangi River. *L. margarettae* was described by Heller [82] from the Mount Gargues (Mathews range), north-central Kenya, with Verheyen *et al.* [12], Verheyen *et al.* [14] confirming its presence on the lower elevations of the Kenya highlands. However, Onditi *et al.* [72] did not record *L. margarettae* in the entire elevation gradient of Mount Kenya (ca. 1,700 – 4,000 meters). In the current study, the samples from Kaptagat that Verheyen *et al.* [14] assigned to *L. margarettae* are completely nested within the *L. zena* clade, including those from the nearby Mau Forest fragments. During this study, despite ~500 trap nights (standard trapping protocol using Sherman live traps) at intermediate elevations (1,210-1,930 meters) of the Mathews Range, not a single *Lophuromys* was captured. Although we cannot challenge the taxonomic validity of *L. margarettae* in the Mathews Range yet, it is absent from all the localities where *L. zena* was sampled – virtually all the wet highlands of Kenya. It may be that ongoing forest degradation and changing climates might have led *L. margarettae* to shift range and thus become rarer. More surveys of the higher, more intact forest of the Mathews Range are required to resolve with certainty whether *L. margarettae* is still resident in the area or is simply an *L. zena* variant.

Similarly, *L. rubecula* described by Dollman [27] is another species we are unable to confirm without new material. Our Mt. Elgon samples cluster genetically and craniodentally with *L. zena.* However, we lacked samples from other parts of the Mt Elgon ecosystem, without which we cannot dismiss *L. rubecula*'s occurrence or its validity. Future surveys of Mt Elgon should employ elevational stratified sampling transects on the Kenya and Uganda sides to substantiate the occurrence limits (or the absence thereof) of *L. rubecula*.

Finally, we were also unable to verify the validity of *L. major,* which was described by Thomas and Wroughton [29] from the Bwanda area, Ubangi River, DRC. The ranges of the presupposed nearest congeners – *L. dudui* and *L. rita* are considerably south of its type locality and without new material from the area, we cannot verify the validity of *L. major* or approximate its relationship to other species in the *Lophuromys* genus.

Conclusion

Despite being one of the most widely occurring and abundant rodents in east-central and East African montane and lowland rain forest habitats, the taxonomy and biogeography of the non-Ethiopian *L. flavopunctatus* members (NONETHFLAVO) remain poorly understood. Our utilization of the *CYTB* gene to reconstruct the phylogenetic relationships of the genus *Lophuromys* and combined mitochondrial and nuclear genes and morphometrics (geometric and linear characters) to analyze the systematics of the NONETHFLAVO revealed a substantial number of novel findings. Most of the species recognized previously based on morphology are supported as well geographically structured mtDNA lineages but lack stable informative craniodental characters capable of reliably assigning samples to putative species units a priori. The NONETHFLAVO colonized its current range over two independent dispersal events out of Ethiopia in the early Pleistocene, with the two resulting subgroups remaining respectively monophyletic but nested in the Ethiopian *flavopunctatus* members. Despite our study providing a comprehensive scenario for the evolution, phylogeography, and genetic differentiation of the NONETHFLAVO, a formal taxonomic harmonization based on more comprehensive genomic characterization of the genus is required to ascertain the full extent and influence of

mitochondrial-nuclear phylogenetic incompatibilities, as done recently for the Ethiopian *L. flavopunctatus* members [16]. Ultimately, such a comprehensive genomic phylogenetic approach, even in the absence of craniodental data, is likely to reliably delimit the unique population pools corresponding to valid species and the resolution of species groups. Currently, NONETHFLAVO occurs in ecosystems with stable annual precipitation which are susceptible to changing climates, meaning that ongoing climate fluctuations, warming climates, and continued habitat fragmentation are likely to fragment further their distributions, resulting in substantial range shifts and or loss of habitat. This would probably drive divergent ecomorphological adaptations between sympatric and parapatric close relatives, with taxonomic implications that are essential from a conservation point of view.

Abbreviations

ABGD – Automated Barcode Gap Discovery method

BCUT – branch-cutting species delimitation method

BI – Bayesian inference

BIC – Bayesian information criterion

BS – bootstrap support

COI – Cytochrome c oxidase I

CS – centroid size

CYTB – Cytochrome b

DA – discriminant function analysis

ESS – effective sample size values

FMNH – Field Museum of Natural History, Chicago, USA

GPA – Generalized Procrustes Analysis

GYMC – single threshold general mixed Yule coalescent model

HPDI – highest posterior density interval

IRBP – Interphotoreceptor retinol-binding protein

KIZ – Kunming Institute of Zoology, Kunming, China

M^3 – third molar tooth

MANOVA – multivariate analysis of variance

MCMC – Markov chain Monte Carlo

ML – maximum likelihood

mPTP – multi-rate Poisson Tree Processes algorithm

MRCA – most recent common ancestor

Mt(s) – Mountain(s)

mtDNA –mitochondrial Deoxyribonucleic Acid

Mya – Million years ago

NMK – National Museums of Kenya, Nairobi, Kenya

OUT – operational taxonomic unit

PCR – Polymerase chain reaction

PP – posterior probability support

Declarations

Ethics approval and consent to participate

The collection and handling of animals adhered to the wildlife research regulations of the respective countries. New fieldwork in Kenya conducted by joint teams from the National Museums of Kenya and Kunming Institute of Zoology was permitted by Kenya Wildlife Service and Kenya Forest Service, whose offices also provided key logistical support. Other new material was obtained from The Field Museum of Natural History (Chicago, USA) and we are grateful to the curators for facilitating access to study their collections.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests

Funding

The work was supported by the Sino-Africa Joint Research Centre – Chinese Academy of Sciences (SAJC201612) to JXL and The Council on Africa – Field Museum of Natural History to KOO. Partial support was provided by the Czech Science Foundation (projects no. 20-07091J) to JB, the Russian Foundation for Basic Research (project no. 19-54-26003) to LAL, and the COBIMFO Project (Congo Basin integrated monitoring for forest carbon mitigation and biodiversity, contract no. SD/AR/01A) funded by the Belgian Science Policy Office (BELSPO) to EV. KOO was sponsored by CAS-TWAS President's Fellowship. The funding bodies were not involved in designing the study, collecting and analyzing data, or writing the manuscript.

Authors' contributions

KOO, JKP, TD, JB, and JXL conceived, designed, and planned the study; KOO analyzed the data and wrote the manuscript with contributions from all authors; JKP, TD, JB, LAL, and EV provided materials; CZ and SM contributed to the study design and provided materials. All authors read and approved the final version of the manuscript

Acknowledgments

We are grateful to the personnel of the Mammal Ecology and Evolution Research group at Kunming Institute of Zoology – Chinese Academy of Sciences and Mammalogy Section – National Museums of Kenya who carried out most of the Kenyan fieldworks. We are also grateful to He Shui-Wang (Kunming Institute of Zoology) for help with laboratory protocols, Adam Ferguson and John Phelps (Field Museum of Natural History) for facilitating access to collections and the provision of tissue samples. We owe a great debt of thanks to several researchers whose efforts over the years enabled us to sample from across the genus *Lophuromys,* and to Dr. Fredrik van de Perre and Dr. Dudu Akaibe Migumiru for sequences from The Democratic Republic of the Congo.

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Figures

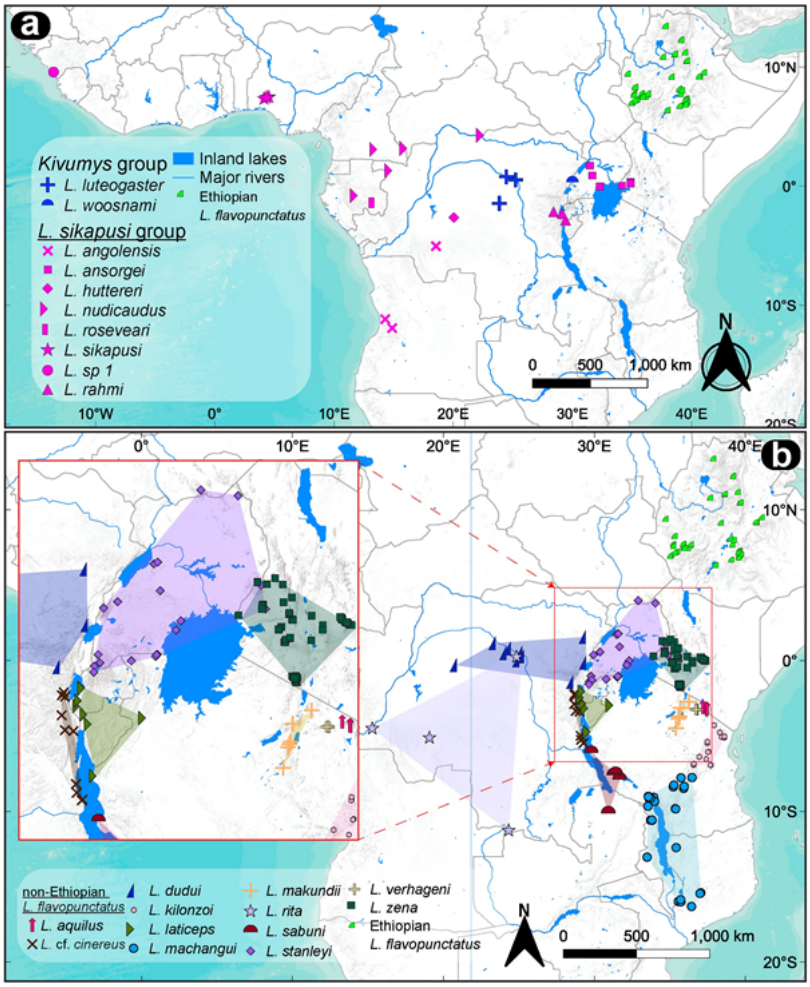

Figure 1

Topographic maps showing the geographical distribution of samples used in the study. a) sampling points of Kivumys group, L. sikapusi group, and Ethiopian L. flavopunctatus members. See Komarova et al. [16] for the detailed per-species sampling points of the Ethiopian flavopunctatus group members; b) sampling points for the non-Ethiopian L. flavopunctatus members, with convex hulls indicating distribution extents (inset map zooms in on the red-outlined area for clarity); their type localities are shown in Fig. S1. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

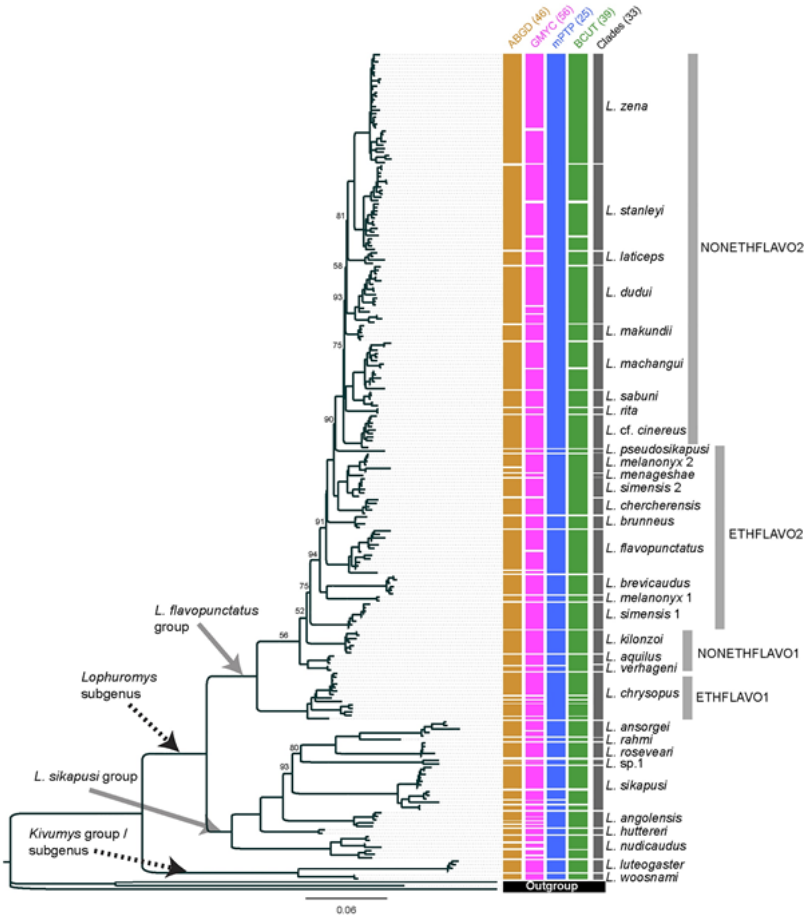

Figure 2

The phylogeny of the genus Lophuromys inferred from Cytochrome b gene in IQ-TREE using Maximum Likelihood phylogenetic inference. The analysis involved 239 sequences representing all unique haplotypes from the initial 803 selected sequences. Values above branches represent bootstrap support values <95percentage. The taxa labels 'Clades' represent the species identities of main clades resolved following operational taxonomic units (OTUs) suggested by the various species delimitation methods shown, with the corresponding number of OTUs in brackets.

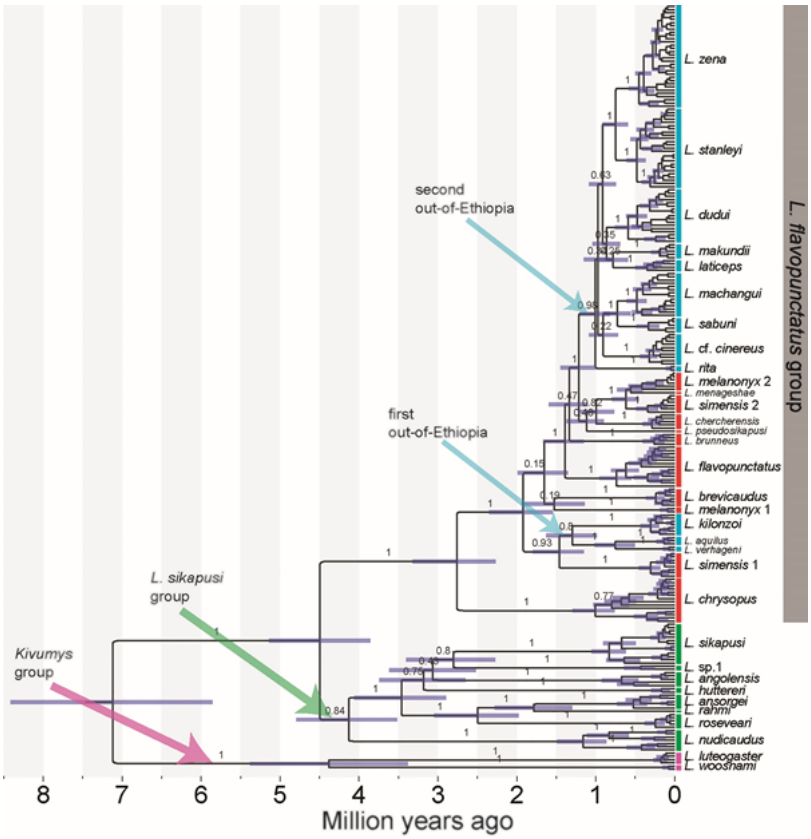

Figure 3

Time-calibrated maximum clade credibility tree showing the evolutionary relationships and divergence times in the genus Lophuromys. The tree was reconstructed based on Cytochrome b using secondary 'most recent common ancestor' calibrations. Branch labels show the posterior probability support values for main branches only. Node bars represent the highest posterior density interval of median ages.

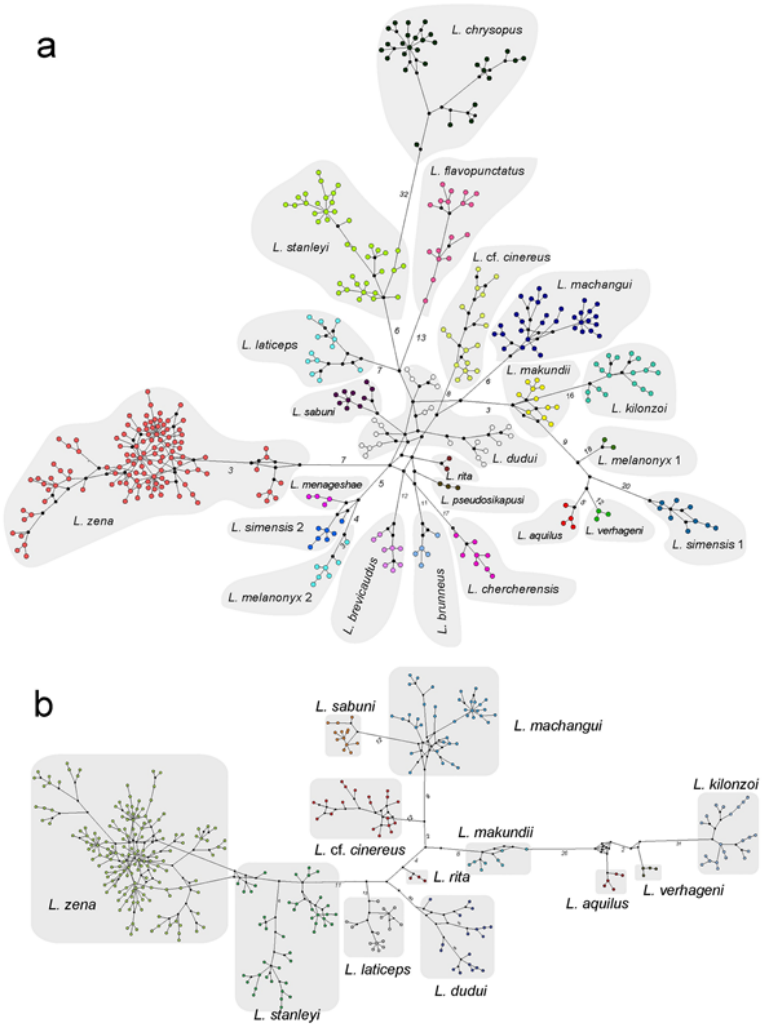

Figure 4

Haplotype network structure in the L. flavopunctatus group inferred from Cytochrome b using the Median Joining Network algorithm in PopART. The networks are illustrated separately for the Ethiopian L. flavopunctatus members (a) and non-Ethiopian L. flavopunctatus members (b). The number of base substitutions between haplotypes is shown as numbers for some of the main branches. The node sizes are fixed and do not correspond to the haplotype frequency (number of samples per haplotype) and branch lengths are relative but not proportional to the number of mutations between haplotypes.

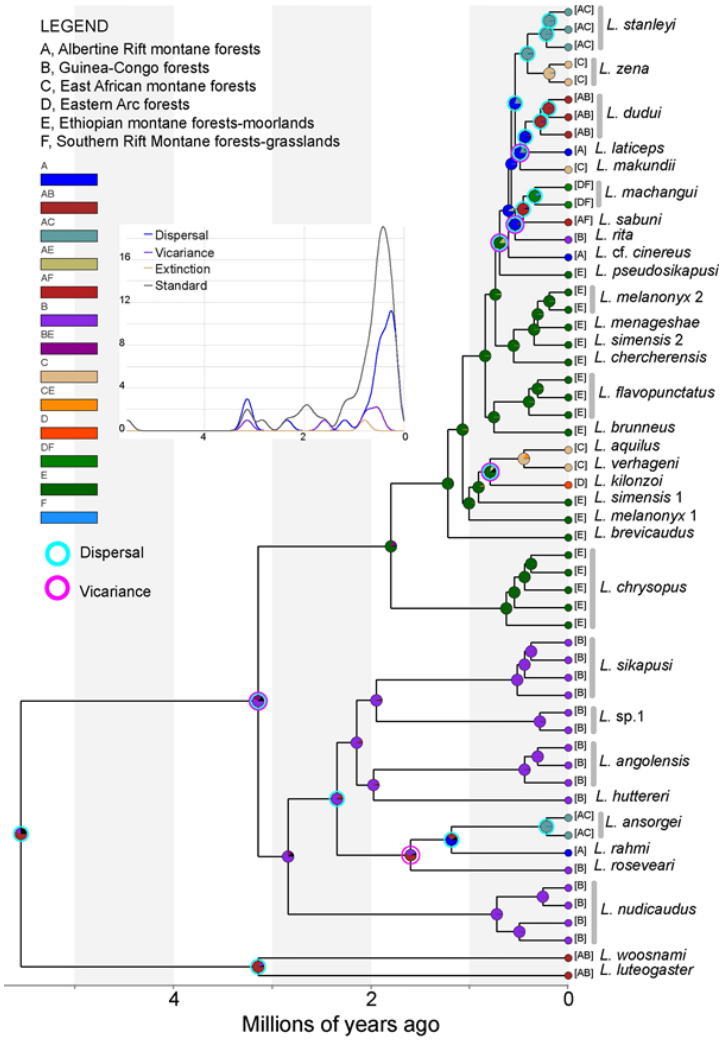

Figure 5

The historical ancestral areas and biogeography of the genus Lophuromys. The node shapes illustrate the suggested historical range of at divergence, marked as color proportions of the biogeographic ecoregions in the legend. The suggested vicariance and dispersal events are also shown as node shapes. The inset graph shows the frequency of various ancestral origins (y-axis) against divergence time (x-axis).

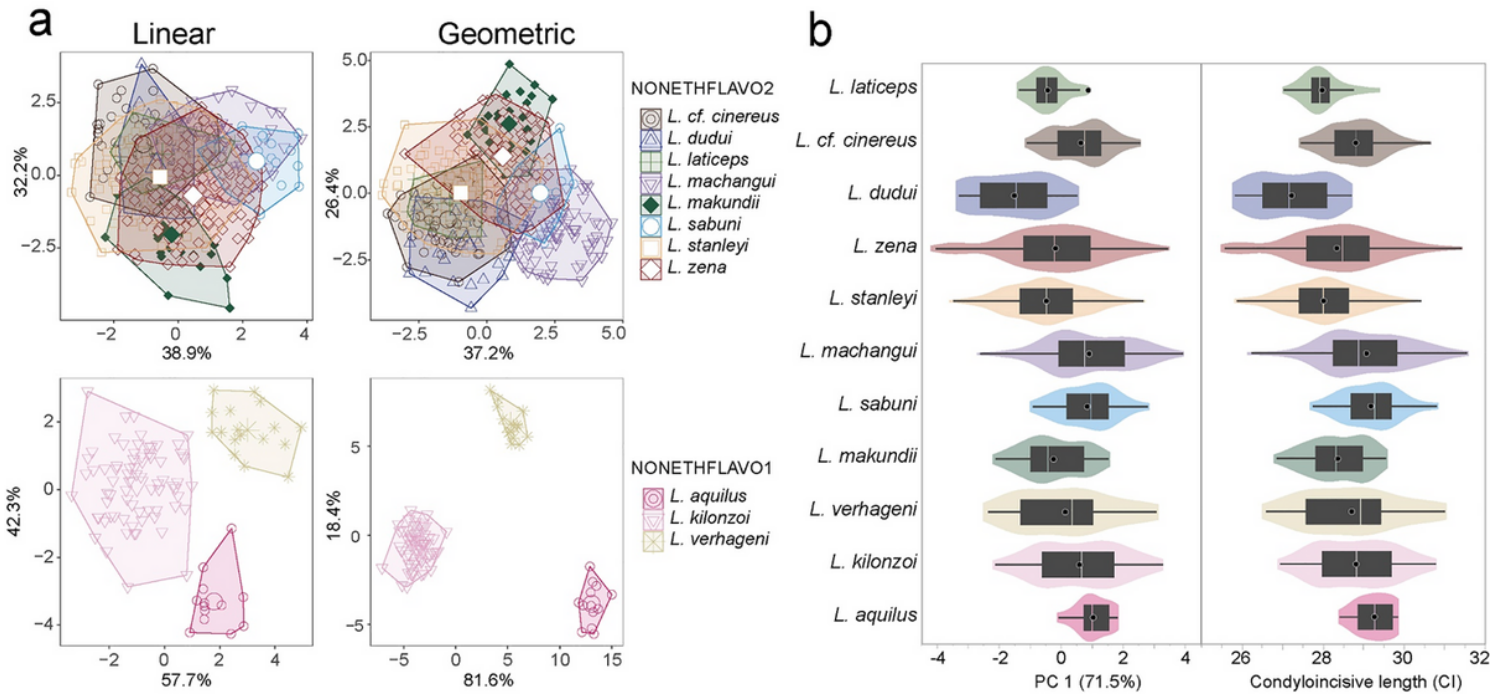

Figure 6

Craniodental variation between non-Ethiopian L. flavopunctatus members. The scatterplots (a) indicate discriminant function analysis of linear and geometric morphometric characters with the x-axis and y-axis showing the percentage variance accounted for by the first and second discriminant scores, respectively. The plots are partitioned based on the two subgroups of the non-Ethiopian L. flavopunctatus members. The box/violin plots (b) show how the condyle-incisive skull length and the first principal component analysis axis compare between clades. The violin breadth illustrates the spread of individual samples around the mean (white outlined black dot) and median (transparent line dividing boxes).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.TableS1Detailedlistofsamplesused.xlsx
- Additionalfile2.SupplementaryTablesTableS2toTableS4.pdf
- Additionalfile3.SupplementaryFiguresFig.S1toFig.S8.pdf