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Phylogeography and evolutionary lineage diversity in the small-eared greater galago, *Otolemur garnettii* (Primates: Galagidae)

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Assessing the true lineage diversity in elusive nocturnal organisms is particularly challenging due to their subtle phenotypic variation in diagnostic traits. The cryptic small-eared greater galago (*Otolemur garnettii*) offers a great opportunity to test if currently recognized subspecies, suggested by discontinuities in coat colour pattern and geographic barriers, represent distinct evolutionary lineages. To answer this question, we conducted the first population-level phylogeographic study of the species, sampling wild specimens from across almost its entire latitudinal range, including the Zanzibar Archipelago. We applied five species-delimitation algorithms to investigate the genetic diversity and distribution pattern of mitochondrial DNA across the geographic range of three out of four subspecies. Our results suggest that far-northern populations of *O. g. lasiotis* potentially represent an independently evolving lineage, but populations assigned to *O. g. garnettii* from Zanzibar Island and of *O. g panganiensis* from mainland Tanzania do not constitute two independent lineages. A dated phylogeny suggests that this northern clade diverged from all remaining samples approximately 4 Mya. Such old divergence age is in line with the split between many galagid species. This northern lineage could potentially represent an incipient species; however, there is not yet enough evidence to support a new taxonomic status for this unique mitochondrial group.

ADDITIONAL KEYWORDS: cryptic species – East Africa – nocturnal primates – speciation – species delimitation – subspecies – taxonomy.

INTRODUCTION

Diagnostic morphological traits and occurrence data constitute the core of traditional taxonomic classification – a surrogate for species boundaries. Therefore, biological diversity can be wrongly estimated if intraspecies phenotypic variation and geographic ranges are unclear or poorly characterized. For instance, diversity tends to be underestimated if different evolutionary lineages show limited to no phenotypic divergence (e. g. 'cryptic species'; Fišer *et al.*, 2018; Korshunova *et al.*, 2019). On the other hand, diversity can be overestimated when a polymorphic trait that does not reflect evolutionary divergence is interpreted as diagnostic, or if elements of the landscape (e. g. rivers, islands and mountain ranges) are erroneously assumed to be barriers to population gene-flow (Patton *et al.*, 2000; Rabosky *et al.*, 2014). Making matters more complex, different authors often disagree on how to interpret observed patterns of population variation, which may lead to conflicting taxonomic classifications (e.g. the 'lumpers vs. splitters debate'; Groves, 2001; Tattersall, 2007; Gippoliti *et al.*, 2018). Despite these operational challenges to species delimitation, modern systematists have increasingly agreed on the view of species as separately evolving metapopulation lineages, formalized in the general lineage concept of species (de Queiroz, 2007). This view predicts that diverging lineages will acquire distinctive ecological, behavioural, morphological and

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genetic features over evolutionary time. Therefore, efforts to characterize population genetic structure in the light of phenotypic and geographic patterns can help to recognize diverging lineages and thus guide species delimitation (Padial *et al.*, 2010).

Genetic and phenotypic trait variations have been used to inform taxonomic classifications at the species level and infraspecific categories. Among these categories are subspecies, traditionally defined and diagnosed on non-overlapping geographic discontinuities in phenotypic (more often, morphological) traits within the range of a polytypic species (Mayr, 1963; Patton & Conroy, 2017). By definition, subspecies are capable of interbreeding but do not co-occur in sympatry. As such, they correspond to geographic segments of the distribution of a species, assuming the form of geographically adjacent populations, often with limited level of gene flow and phenotypic intergradation (Groves, 2001). Characterizing patterns of genetic structure among morphologically defined subspecies can help to understand how spatial morphological transitions originated, for instance, through genetic clines, divergence with gene flow or secondary contact (O'Connell *et al.*, 2021). Moreover, genetic information can confirm that populations assigned to different subspecies are genetically interdependent and, thus, are a group of phylogenetically concordant phenotypes relative to other subdivisions of the species (O'Brien & Mayr, 1991). This step is crucial to distinguish subspecies from species, since species are expected to correspond to evolutionarily separate (or independent) lineages (de Queiroz, 2020; see also below). In the absence of genetic information, or if only limited data on population-level trait variation and geographic range are available, taxonomic schemes at and below species-level should be viewed with caution.

Assessing lineage diversity is particularly challenging for nocturnal organisms due to their elusive behaviour and generally subtle phenotypic variation (Groves, 2001a). Because nocturnal animals often hide during the day and forage at night, they rely more on acoustic and olfactory signals rather than visual communication for conspecific recognition (Zimmermann, 1995; Gursky & Nekaris, 2019). As a result, fur colour patterns are less variable and often limited to shades of grey and brown, which aid in camouflaging during the day (Caro, 2005; Munds *et al.*, 2021). Therefore, identifying external diagnostic traits can be challenging in nocturnal organisms (but see: Masters & Bragg, 2000). Furthermore, nocturnal animals have been historically neglected in field surveys and intraspecific phenotypic variation is often poorly characterized. In the face of these challenges, an increasing number of studies have incorporated genetic information to test for unrecognized evolutionary lineage diversity in nocturnal organisms. In mammals, for instance, many 'cryptic species' (i.e. morphologically similar) have been recently identified based on evidence of deep genetic divergence among phenotypically conserved populations (Taylor *et al.*, 2018). This is particularly true for the Strepsirrhini clade, which include most nocturnal primates, such as the mouse and dwarf lemurs, galagos and slow lorises, among others. By clarifying species boundaries and diversity, genetic assessments of strepsirrhine primates may also inform patterns of biogeography, macroevolution and conservation measures (Groves *et al.*, 2017; Coates *et al.*, 2018; Gippoliti *et al.*, 2018).

Despite these advances, genetic patterns remain uncharacterized for many nocturnal primates. This limitation leads to substantial knowledge gaps on the taxonomic status of species and subspecies, as well as their associated geographic ranges. This is the case, for instance, of the small-eared greater galago (*Otolemur garnettii* Ogilby, 1838), a species endemic to the coastal forests of eastern Africa, one of the world's biodiversity hotspots (Burgess *et al.*, 2004). The genus *Otolemur* currently includes only two species, *O. garnettii and O. crassicaudatus* (É. Geoffroy, 1812), mostly distinguished by overall size, chromosome rearrangements, relative cranial and jaw proportions, size of ears and pelage coloration (Olson, 1979; Masters *et al.*, 1987; Masters & Lubinsky, 1988; Groves, 2001b; Grubb *et al.*, 2003; Nekaris, 2013). While *O. crassicaudatus* is widely distributed throughout southern and eastern Africa, *O. garnettii* is endemic to easternmost east Africa, ranging from the lowland coastal forests in southern Somalia to Tanzania, including the Zanzibar Archipelago (Nekaris, 2013; De Jong *et al.*, 2019). Early genetic work on this taxon used allozymes and karyotypes focusing on species-level distinction that later led to the designation of *Otolemur* Coquerel, 1859 as a separate genus and subsequent separation between *O. garnettii* and *O. crassicaudatus* (Chu & Bender, 1961; De Boer, 1973; Masters *et al.*, 1987), which was also confirmed by studies on morphology, behaviour, reproduction and physiology (Olson, 1979; Masters & Dunn, 1988; Masters & Lubinsky, 1988). However, at the subspecies level, the criteria used to justify the geographic and phenotypic segmentation of the intraspecific variation are less clear. Like many nocturnal taxa, subspecies of *Otolemur garnettii* were proposed based on poorly characterized morphological variation and mostly guided by untested geographic barriers (see details in Taxonomic overview, below). Moreover, the presumed role of certain geographic barriers – such as the Zanzibar Channel – in the phenotypic and genetic differentiation of populations has not yet been formally tested. Resolving these standing issues will benefit from characterizing

genetic variation in the small-eared greater galago, allowing a test of whether spatial genetic structure matches the current subspecific taxonomic arrangement.

The concept and application of subspecies in vertebrates have been actively discussed (Padial & De la Riva, 2021). Most currently recognized subspecies were proposed decades ago based on phenotypic traits, largely without consideration for evolutionary relationships. However, in the past decades, some authors have argued that subspecies should also correspond to distinct evolutionary lineages. Recent proposals have promoted the view of subspecies as 'incompletely separated lineages' within a more inclusive lineage, the species (De Queiroz, 2020; Hillis, 2020; Reydon & Kunz, 2021). Under this proposal, subspecies correspond to early diverging lineages still connected by some level of gene flow. Although the idea of subspecies as incipient or emerging species goes back to Darwin (1859), genetic data have allowed researchers to test hypotheses of recent or ongoing divergence more directly. In some cases, reassessments of subspecies in the light of genetic data have revealed ancient population isolation and thus led to the elevation of subspecies to species (Carlen *et al.*, 2017). In other cases, genetic studies have found no correspondence between subspecies designations and genetic groups, revealing that certain traits thought to indicate population divergence correspond to polymorphisms (Balakirev *et al.*, 2019) or genetically connected metapopulations (Humeau *et al.*, 2020). These studies have clarified the links between phenotypic variation, geographic barriers and evolutionary divergence in an increasing number of organisms. However, few traditional subspecies schemes have been re-evaluated under this framework. This is the case of most nocturnal primates, including the greater galagos.

In the present study, we incorporate genetic information to assess if currently recognized subspecies in the greater galago *Otolemur garnettii*, proposed based on coat colour pattern and geographic distributions (see below), represent coherent evolutionary lineages. We designed our study on the premise that geographically structured morphological variation, as is thought to occur in *O. garnettii*, might be indicative of evolutionary divergence. To test this hypothesis, we combined phylogeographic and population genetic analyses, characterized genetic diversity and the distribution of mitochondrial DNA haplotypes across the species range, and assessed the level of congruence among the subspecies using molecular species-delimitation methods. Our results have implications for current taxonomic classifications, while providing insights into the role of historical landscape shifts in the evolutionary history of this elusive group of nocturnal primates.

MATERIAL AND METHODS

Taxonomic overview of the subspecies of *Otolemur garnettii*

Four subspecies are currently recognized in *O. garnettii* (Fig. 1A): *O. g. garnettii* (Zanzibar small-eared galago), restricted to the islands of the Zanzibar Archipelago, Tanzania; *O. g. panganiensis* Matschie, 1905 (Pangani small-eared galago), ranging from southern Kenya to northern Mozambique; *O. g. lasiotis* Peters, 1876 (white-tailed small-eared galago), distributed from southern Somalia, southwards along the Kenya coast to northern Tanzania; and *O. g. kikuyuensis* Lönnberg, 1912 (Kikuyu small-eared galago), endemic to the Central Kenya Highlands east of the eastern Rift Valley (Fig. 1B). These four subspecies were proposed based on geographically structured differences in coat colour pattern, fur texture and a few dental and cranial proportions (Olson, 1979; Groves, 2001). Namely, the insular *O. g. garnettii* has slightly greenish tones in its red-brown fur, the underside is yellow and the last-half of its tail is black. *Otolemur. g. lasiotis* is greyer than *O. g. garnettii* with a white-tipped tail. The largest of all subspecies, *O. g. panganiensis*, lacks green tones in its pelage and only the last-quarter of its tail is black. Finally, *O. g. kikuyuensis* exhibits iron-grey fur, a yellow-white underside and, usually, a light-brown tail with the terminal-quarter black (Rowe *et al.*, 1996; Groves, 2001). In cranial traits, all subspecies have consistent levels of similarity, and the most significant cranial differences occur between the ones in the extreme east–west range of the distribution of the species (*O. g. kikuyuensis* and *O.g garnettii*; Olson, 1979: 302). More recent work suggests that these subspecies can also be differentiated in their long-distance advertising calls (i.e. patterns of trailing and clustered squawk calls; Bettridge *et al.*, 2019).

Despite the presumed distinction in the external morphology of the four subspecies, careful inspection of museum samples and recent long-term surveys across sites in eastern Africa showed that most variation in biometric and morphological features of *O. garnettii* have high levels of local individual variation in body size, dentition and coat colour (especially dorsoventrally and the distal part of the tail). This phenomenon was already noticed by Olson (1979), who conducted the most extensive systematic study of museum (and all type) specimens of greater galagos, to include body measurements, cranial and tegumentary traits. By the time of its publication, all recognized taxa were classified as subspecies of

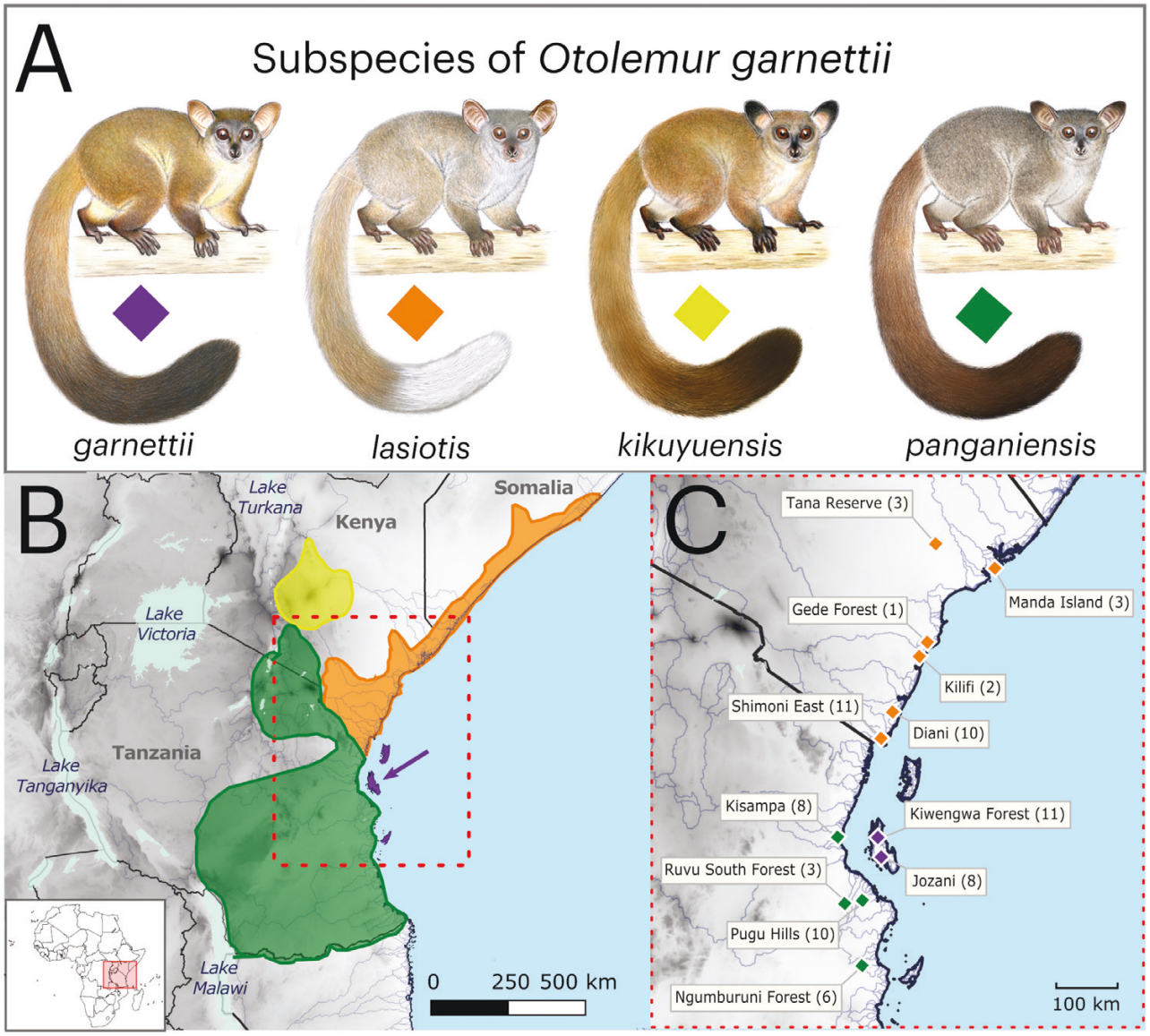

Figure 1. Illustrations of the recently recognized *Otolemur garnettii* subspecies (A), their geographic range according to the IUCN red list (B), and localities sampled in this study (C). It is worth noting that illustrations represent the extremes of variation found in the subspecies and the overall range of distribution should be interpreted as areas in which they might be expected to occur. *Otolemur g. lasiotis*, in particular, is known to occur in highly discontinuous distributions.

O. crassicaudatus. For instance, the coloration of the tail tip used to be considered the main diagnostic trait to distinguish subspecies of *O. garnettii*, until Olson declared this trait to be 'individually too variable to be taxonomically useful' (Olson, 1979: 276). This observation was confirmed recently by De Jong & Butynski (2009) who carefully evaluated over 50 living specimens in East Africa and concluded that tail tips show a poorly structured gradient from white to greyish and black, with different morphs often co-occurring in the same locality. Moreover, moulting and scent-marking during the reproductive season is known to lead to changes in fur density and coloration in various galago species. More specifically, freshly moulted adults tend to have more yellowish lower bodies, and the adults lose chest fur after repeated rubbing of ventral glands when scent-marking (Olson, 1979: 269, 382), a behaviour that, when performed in certain species of trees (e.g. neem, *Azadirachta indica* A.Juss.), creates a distinct orange-yellowish tint in the face, neck, throat, chest, upper-legs and genitals (De Jong & Butynski, 2011: 6). However, neem trees are not equally distributed in the landscape and, as reported by De Jong and Butynski (2011), individuals

of *O. g. lasiotis* observed within a 20-km range showed such yellowish-orange tints only in the presence of this species of tree. Altogether, these observations seem to dispute the supposed coherence and distinction of the four subspecies in external morphology.

In this study, we used the currently accepted geographic range of small greater galago subspecies published in the 2019 IUCN list of threatened species assessment (De Jong and Butynski, 2018; Fig. 1). If the geographic separation between subspecies is the major factor preventing the populations assigned to different subspecies from exchanging genes, the proposed geographic distribution of subspecies can be used to delineate taxa assignment. Therefore, subspecies assignment was conditional on the provenance of the sample, and confirmed upon a visual inspection of fur traits in the field. This approach also allowed us to test the role of proposed geographic separation to the observed patterns of structure in genetic variation.

Sampling, DNA amplification and sequencing and molecular datasets

Our dataset included 76 specimens of small-eared greater galago, *Otolemur garnettii* (Primates: Galagidae). Samples were collected between 2010 and 2012 from 12 localities distributed throughout the coast of Kenya (6) and Tanzania (6), including the Zanzibar Archipelago (Fig. 1C; Supporting Information, Table S1). The number of specimens sampled per population ranged from one to 11 (average ~6.3 specimens per population). Animals were trapped using either Tomahawk live traps or Chardonneret box traps (Pozzi *et al.*, 2014a, 2019). A maximum of 20 traps were placed at night between ground level and 3 m of height on trees and checked four to five times during the night. Traps were baited with fruit, insect larvae and palm wine following standard protocols for galagos (Ambrose, 2003; Pozzi *et al.*, 2019). Tissue samples were obtained by ear-clipping, using disposable biopsy punches and preserved in RNAlater buffer (Ambion Inc.). Hair samples were obtained by pulling hairs from the base of the tail of each animal. Permissions for fieldwork and sample collection were provided by the Ministry of Education, Science and Technology in Kenya and the Tanzania Wildlife Research Institute in Tanzania. CITES export permits were obtained from both Kenya and Tanzania. Sample collection was approved by the University Animal Welfare Committee at New York University (IACUC animal care protocol 10-1334) and adhered to the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates (see https://www.asp.org/2021/04/20/principles-for-the-ethical-treatment-of-non-human-primates/). No animals were harmed or sacrificed for this study. Genetic data generated in this study are available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/ (accession numbers OP329354-OP329364; Supporting Information, Table S1).

We extracted total DNA from tissue samples using the QIAamp DNA Micro Kit (Qiagen Inc.) following the manufacturer's protocol. To ensure the amplification of mitochondrial DNA (instead of non-functional segments of mtDNA that have been inserted into the nuclear genome), we performed long-range polymerase chain reaction (PCR) targeting a ~7000 bp fragment that far exceeds our region of interest. The long-range PCR was performed using Platinum Taq DNA Polymerase High Fidelity with reaction volumes of 25 µL. The components of the reaction mix for each PCR tube included 2.5 µL of High-Fidelity PCR buffer (10×), 1 µL of magnesium sulfate (50 mmol/L), 0.5 µL of each primer and dNTPs (10 mmol/L), 0.1 µL of Taq DNA Polymerase High Fidelity (5 U/µL). The primers used for the long-range PCR were 5′-GAACCAAACA GAACGATTAAACGCAG-3′ (forward) and 5′-AGAA AATGTAGCCCATTTCTTCCCAC-3′ (reverse). PCRs were carried out with the following thermal cycler settings: 94 °C for 2 min, followed by the first round of 35 cycles denaturing at 94 °C for 15 s, annealing starting at 60 °C (and decreasing 0.1 °C after cycle 5 to eventually decrease to 57 °C) and extension at 68 °C for 7 min. We successfully amplified long fragments for 28 samples. Remaining samples were subjected to amplification of a fragment of approximately 3000 bp. We performed PCR amplification using AmpliTaq Gold 360 Master Mix, with reaction volumes of 25 µL. The components of the reaction mix for each PCR tube included 12.5 µL of AmpliTaq Gold 360 Master Mix and 0.5 µL of the forward and reverse primers. The primers used were 5′-TGACATGAAAAATCACCGTTGTAA TTC-3′ (forward) and 5′-GCATAGTGGGGTATCTAA-TCCCAG-3′ (reverse). PCRs were carried with the following thermal cycler settings: 95 °C for 10 min, followed by the first round of 35 cycles denaturing at 95 °C for 15 s, annealing for 30 s starting at 55 °C (and decreasing 0.1 °C after cycle 15 to eventually decrease to 53 °C), and extension at 72 °C for 3 min. The 35 cycles were followed by a final extension at 72 °C for 7 min. All PCR products were then analysed on 1% agarose gels and those samples that produced clear, single bands of the predicted length were selected for sequencing. The PCR products were delivered for magnetic bead purification and Sanger sequencing at the DNA Sequencing Facility at the University of Texas at Austin.

Consensus sequences for each specimen were generated by assembling forward and reverse sequences using GENEIOUS v.11.1.4 (Biomatters). All consensus sequences were aligned using MUSCLE (Edgar, 2004). Due to differences in amplification

success (see Results), we analysed two subsets of sequences. Dataset 1 included only partial cytochrome b data (402 bp, 76 samples) and dataset 2 included the full cytochrome *b* and the hypervariable regions 1 and 2 of the D-loop (2061 bp, 56 samples).

Phylogenetic and population genetic analyses

For each dataset, we estimated mitochondrial genetic diversity, including the total number of haplotypes, haplotype diversity and nucleotide diversity, using PopART v.1.7 (Leigh & Bryant, 2015). We used the same software to create haplotype median joining networks for both datasets (Bandelt *et al.*, 1999). Finally, to test whether the variance observed in both datasets is better explained by differentiation between or within subspecies (i.e. among populations), we performed an analysis of molecular variance (AMOVA) with 1000 permutations in PopART (Leigh & Bryant, 2015).

To estimate the phylogenetic relationship between samples, we analysed only the cytochrome *b* data using both maximum likelihood (RAxML v.8.2.12; Stamatakis, 2014) and Bayesian phylogenetic analyses (BEAST v.1.10.4; Drummond *et al.*, 2012). Phylogenetic analyses were run on a subset of the cytochrome *b* data to include only one sequence per haplotype and 37 galagid sequences retrieved from GenBank. These additional sequences included eight *Otolemur* samples, five of which were of *O. garnettii* from unknown localities and three *O. crassicaudatus* É.Geoffroy, 1812, the only other species in the genus *Otolemur*. Two lorisid species, *Nycticebus coucang* (Boddaert, 1785) and *Loris tardigradus* (Linnaeus, 1758), were used as outgroup taxa. All the sequences used in this study, including location, collector and GenBank accession numbers, are provided in the Supporting Information, Table S1.

We used PartitionFinder 2 (Lanfear *et al.*, 2017) to identify the optimal partitioning scheme for our dataset and the best-fit nucleotide substitution model for each partition. All PartitionFinder analyses used the greedy search algorithm (Lanfear *et al.*, 2012), linked branch lengths and the Bayesian information criterion (BIC) for selecting the best-fit partitioning schemes. Maximum-likelihood phylogenetic analyses were run using RAxML (Stamatakis, 2014). Support for each internal node was assessed with bootstrap support (BS) analyses using the boot-stopping criteria autoMRE (majority rule criterion). Simultaneous Bayesian phylogenetic inference and divergence-time estimation were performed using BEAST. We conducted four replicate runs with four Markov chain Monte Carlo (MCMC) chains, sampling every 10 000 generations for 100 million generations each (with 25% as burn-in). Based on two fossil stem galagids, *Wadilemur elegans* Simons, 1997 and *Saharagalago mirrensis* Seiffert *et al.*, 2003, dated around 35–37 Mya (Seiffert *et al.*, 2003, 2005), we calibrated the divergence between Lorisidae and Galagidae (crown Lorisiformes) using a normal distribution with mean 40 Mya (SD = 2.0; 95% range: 36.08–43.92) following Pozzi *et al.* (2014b, 2015) and Pozzi & Penna (2022). Convergence of all parameters was visually assessed using TRACER v.1.7.1, and all BEAST analyses were run to achieve an effective sample size of at least 200 for all estimated parameters once burn-in was removed.

To characterize genetic structure among our samples and to evaluate if the current subspecies represent distinct evolutionary lineages, we used five different species-delimitation methods: the single-threshold generalized mixed Yule coalescent (sGMYC; Pons *et al.*, 2006; Fujisawa & Barraclough, 2013), the multiple-threshold GMYC (mGMYC; Monaghan *et al.*, 2009), the multi-rate Poisson tree processes (mPTP; Kapli *et al.*, 2017), the automatic barcode gap discovery (ABGD; Puillandre *et al.*, 2012) and the assemble species by automatic partitioning (ASAP; Puillandre *et al.*, 2021). Comparing the consistency of results arising from different methodological approaches allowed us to assess the existence of independent lineages at the molecular level. We performed species-delimitation analyses using both datasets 1 and 2. Since identical sequences should generally be removed when using species-delimitation methods, we used results from haplotype diversity to filter our molecular data. The final dataset 1 included a total of 11 unique sequences (or haplotypes), while dataset 2 included 25 haplotypes.

GMYC models (Pons *et al.*, 2006; Fujisawa & Barraclough, 2013) were run on an ultrametric tree estimated using BEAST. We used the R package 'splits' (Ezard *et al.*, 2009) to fit both the single- (sGMYC) and multiple-threshold (mGMYC) models to the data. mPTP analyses were run using the non-ultrametric tree generated using RAxML. This method accommodates different rates of coalescence within clades (Kapli *et al.*, 2017). We performed an MCMC analysis of five runs, each of 100 million steps, sampling every 10 000 steps and ignoring the first two million steps as burn-in. Analyses were run with different starting delimitations: null model, maximum likelihood and random. We used the *–multi* option to incorporate differences in rates of coalescence among species and used a minimum branch length of 0.0001. Finally, we ran two distance-base methods for species delimitation, the ABGD (Puillandre *et al.*, 2012) and the ASAP (Puillandre *et al.*, 2021). ABGD was run using default settings for the prior range for maximum intraspecific divergence (0.001, 0.1). Results were compared using

both JC69 and K80 corrected distances and minimum slope increase (*X*) of 1.0 and 1.5. ASAP analyses were run using the ASAPweb server (https://bioinfo.mnhn.fr/abi/public/asap). The Kimura K2P (Kimura, 1980) was selected as nucleotide-substitution model and all the other parameters were left as default. ASAP delimitation results were defined evaluating both the partitions with first and the second-best ASAP-score according to Puillandre *et al.* (2021).

RESULTS

Molecular datasets and genetic diversity analyses

We successfully obtained sequences for all 76 sampled specimens of *Otolemur garnettii*. These samples included three of the four subspecies currently recognized within this taxon (Fig. 1): *O. g. lasiotis* (30 samples), *O. g. garnettii* (19 samples) and *O. g. panganiensis* (27 samples). For 56 out of the 76 specimens, we obtained a fragment of mitochondrial DNA (2061 bp) encompassing the full cytochrome *b* (*Cytb*) and part of the control region, including both the hypervariable region 1 and 2 of the D-loop (HVR1 and HVR2). For the remaining 20 specimens, only the partial *Cytb* was available (402 bp).

For the dataset 1 (402 bp of partial *Cytb*, 76 samples), we identified 11 unique mtDNA haplotypes (Fig. 2). We identified six haplotypes exclusive to *O. g. lasiotis* (I, II, III, IV, V and VI), two exclusive to *O. g. garnettii* (VIII and X) and two to *O. g. panganiensis* (IX and XI). Only one haplotype (VII) was shared among all three putative subspecies. The frequency distribution of the mtDNA haplotypes was relatively skewed, ranging from haplotypes represented by only one individual (I) to others being common (16 individuals with haplotype IV and 32 with haplotype VII). However, the grouping of these exclusive haplotypes, as inferred by the median-joining network, did not correspond to the *O. garnettii* subspecies as currently recognized (Fig. 3). For instance, samples from Zanzibar Island, which correspond to *O. g. garnettii* and are represented by two populations (Kiwengwa Forest and Jozani Chwaka Bay National Park), did not form an exclusive group. Instead, they clustered together with other mainland samples assigned to a different subspecies, *O. g. lasiotis* (haplotype X) or *O. g. panganiensis* (haplotypes X and VIII). Therefore, no clear geographic patterns could be identified based on dataset 1. The exception is a northern group composed of samples from Manda Island

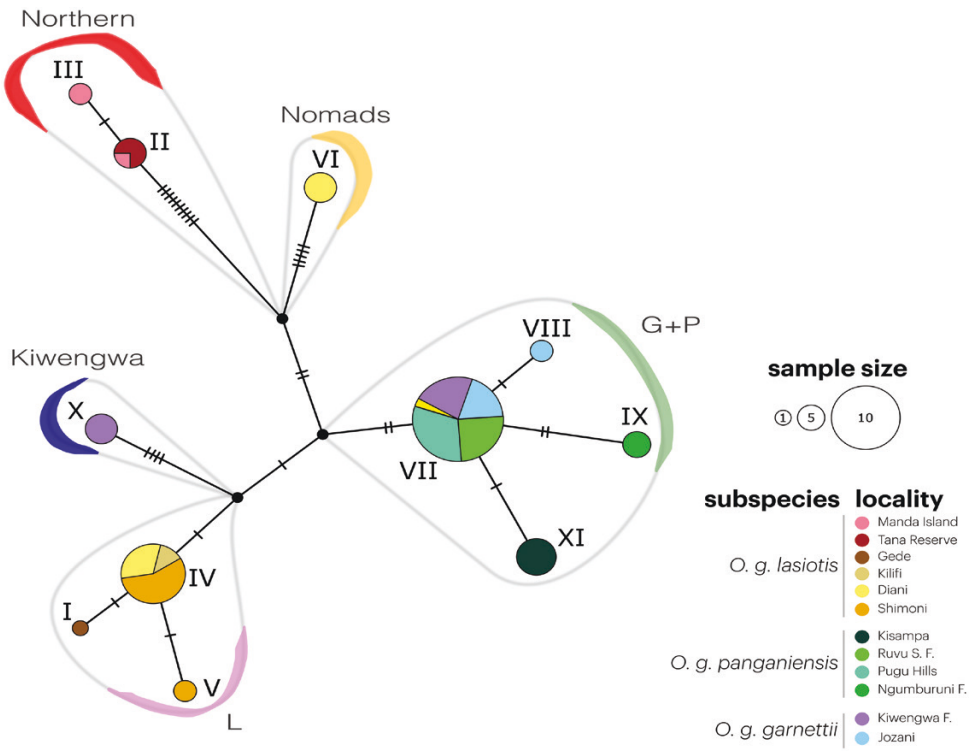

Figure 2. Haplotype median joining network estimated from dataset 1, comprising 76 samples of partial cytochrome *b* (402 bp).

and Tana River Primate Reserve (haplotypes II and III), which represented the most divergent haplotypic group in the network (separated by ten mutational changes; Fig. 2, Supporting Information, Table S1, S2). Out of 412 bp of the partial *Cytb* sequence, 29 were polymorphic and 28 were parsimony-informative. The overall nucleotide diversity was estimated at 0.0127 and the overall haplotype diversity at 0.7701 (Table 1).

For the dataset 2 (2061 bp of the full *Cytb* and D-loop, with 56 samples), we identified 25 unique mtDNA haplotypes (Supporting Information, Fig. S1). None of these haplotypes was shared between the three putative subspecies: 15 haplotypes were exclusive to *O. g. lasiotis* (I, II-a, II-b, II-c, III-a, III-b, IV-a, IV-b, IV-c, IV-d, IV-e, IV-f, V, VI and VII-g), four to *O. g. garnettii* (VII-a, VII-b, VII-c and VIII) and six to *O. g. panganiensis* (VII-d, VII-e, VII-f, VII-h, VII-i and VII-j). The frequency distribution of these haplotypes ranged from being present in a single individual to a maximum of eight individuals (for haplotype VII-h). Except for haplotype VII-g, represented by a single individual from the Diani Forest, all samples of *O. g. lasiotis* formed a separate group. In contrast, haplotypes corresponding to *O. g. garnettii* and *O. g. panganiensis* clustered together. Once again, the most distinctive group (with 76 mutational changes) grouped haplotypes present in individuals from a northern group (haplotypes II-a, II-b, II-c, III-a and III-b). The second most distinctive group (haplotype VI, with 27 mutational changes) was represented by four individuals from the Diani Forest, which we refer to as the 'Nomads group' (Supporting Information, Fig. S1). Out of the 2061 bp in the dataset 2, a total of 174 positions were polymorphic and 160 were parsimony-informative. The overall nucleotide diversity was estimated at 0.0214, and the overall haplotype diversity was estimated at 0.9513 (Table 1).

Finally, we performed an analysis of molecular variance (AMOVA) based on both molecular datasets. For dataset 1 (partial *Cytb*), the AMOVA inferred that only 3.37% of the total genetic variation is explained by differentiation between subspecies. Most of the variation was explained by the differentiation among sampling localities (77.69%; $P < 0.001$). Last, variation within localities explained the remaining 18.94% of the total genetic variation (Table 2). Similarly, for the dataset 2, the AMOVA inferred that differentiation among localities explained 91.1% of the total genetic variation ($P < 0.001$), while variation within populations explained 10.61% of the total variation. The differentiation among subspecies corresponded to 1.71%, but that was not statistically significant (Supporting Information, Table S3).

Phylogenetic and population genetic analyses

Maximum likelihood (RAxML) and Bayesian (BEAST) analyses yielded nearly identical phylogenetic relationships among the sampled specimens. All six genera in the family Galagidae were recovered as monophyletic. The needle-clawed galagos (*Euoticus* Gray, 1863) was inferred as sister to the remaining genera, followed by the western dwarf galagos (*Galagoides* Smith, 1833). In turn, the lesser (*Galago* É. Geoffroy, 1796) and eastern dwarf galagos (*Paragalago* Masters *et al.*, 2017) were recovered as sister-taxa. The squirrel galagos (*Sciurocheirus* Gray, 1872) was inferred as the sister of *Otolemur*. All these relationships were strongly supported (PP > 90% and BS > 0.95; Fig. 3).

Within *Otolemur garnettii*, samples corresponding to the northern haplotypic group (Manda Island and Tana River Primate Reserve) formed a clade, which was sister to the remaining samples of this taxon. As in the haplotype network results, none of the three putative subspecies

Table 1. Haplotype statistics for dataset 1 (partial cytochrome *b*) and dataset 2 (full cytochrome *b* and the D-loop)

Dataset	Sequence length (bp)	Sample size	Number of Haplotypes	Nucleotide diversity	Haplotype diversity	Segregating sites	Parsimony-informative sites
1 (Partial *Cytb*)	402	76	11	0.0127	0.7701	29	28
2 (*Cytb* + D-loop)	2061	56	25	0.0214	0.9513	174	160

Table 2. Summary of AMOVA results for dataset 1 (partial cytochrome *b*)

Source of variation	df	SSD	σ2	% Variation	Fixation index	*P* value
Between subspecies	2	421.17	0.92	3.37	ΦCT = 0.03367	0.13
Among populations within subspecies	9	1114.99	21.20	77.69	ΦSC = 0.80395	< 0.001
Within populations	64	330.93	5.17	18.94	ΦST = 0.81055	< 0.001
Total	75	1867.09	27.29			

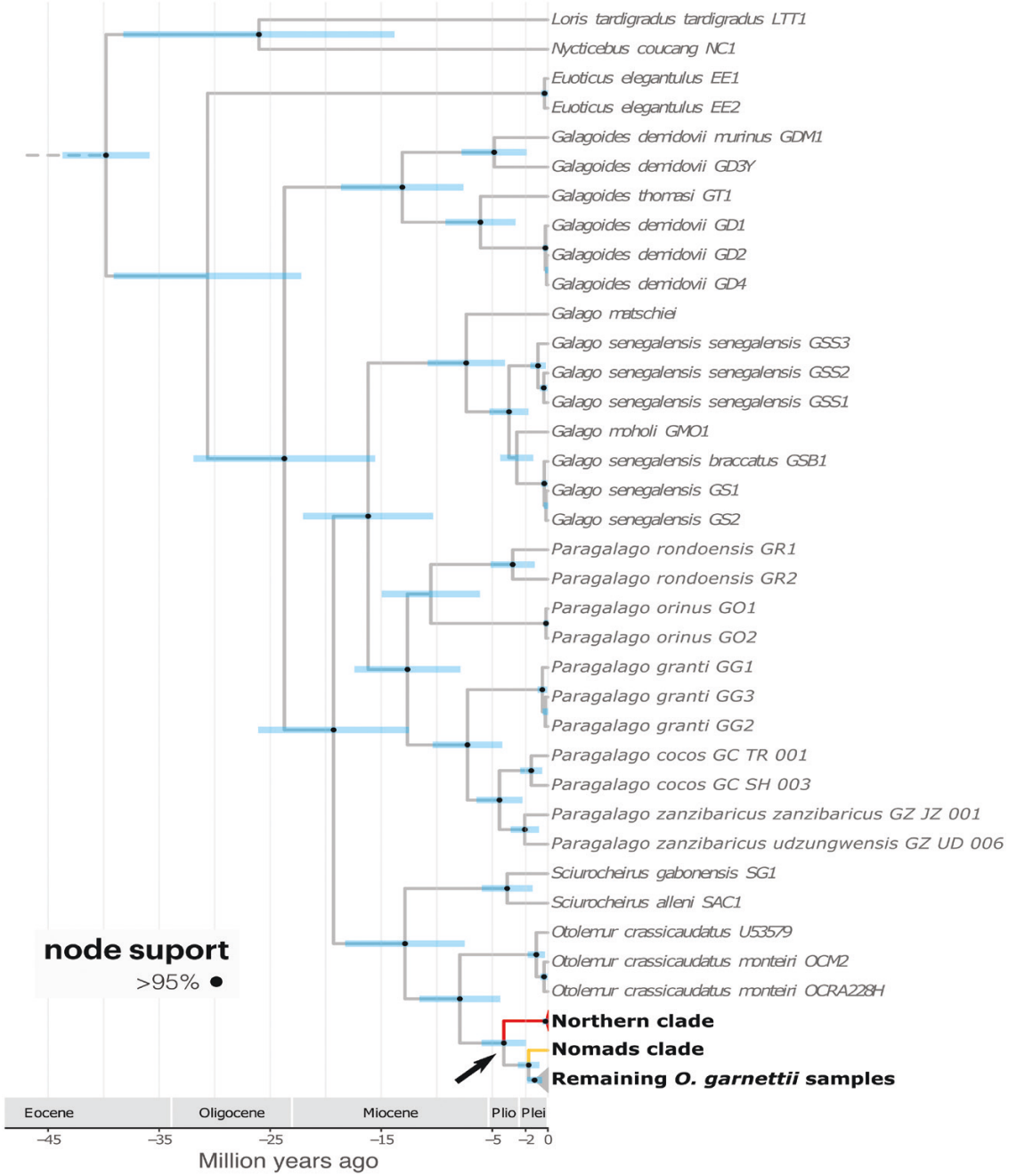

Figure 3. Phylogenetic tree estimated using BEAST from dataset 1 (cytochrome *b*) and node-calibrated using the fossil record.

constituted a monophyletic group. For instance, samples from Zanzibar Island, which correspond to the subspecies *O. g. garnettii*, did not cluster together. Instead, they grouped with samples from mainland Tanzania, which are classified as *O. g. panganiensis*. As in the haplotype networks, one sample of *O. g. lasiotis* from the Diani Forest clustered together with samples from Tanzania that correspond to *O. g. garnettii* and *O. g. panganiensis* (Supporting Information, Fig. S2).

Molecular dating analyses using BEAST inferred the divergence between the two greater galago species (*O. crassicaudatus* and *O. garnettii*) to have occurred approximately 7.92 Mya [95% highest posterior density (HPD): 4.73–12.00 Mya]. The most recent common ancestor of all *O. garnettii* samples was dated at approximately 3.96 Mya (95% HPD: 2.25–6.29 Mya). The Northern clade was recovered as sister to all other *O. garnettii* samples, and the Nomads clade

was recovered as sister to the other remaining samples (~1.73 Mya; Fig. 3).

Molecular species-delimitation analyses, based on the two molecular datasets and five different algorithms, yielded largely congruent results, but there were some minor differences among analyses. In all cases, patterns of genetic grouping did not match the presumed composition of the three subspecies of *O. garnettii* and their proposed geographic distributions. For the dataset 1, the number of lineages identified ranged from one (using mPTP) to five (ASAP, sGMYC and mGMYC). For the dataset 2, the number of lineages ranged from two (ABGD) to four (mPTP, ASAP, sGMYC and mGMYC). The higher variability in the dataset 1 might be related to a lower number of unique sequences included in the corresponding analyses (11 haplotypes). None of the species-delimitation analyses matched the subspecies scheme as currently recognized. However, the samples corresponding to the northern clade and corresponding haplogroup (i.e. from Manda Island and Tana River Primate Reserve, with haplotypes II and III) were consistently inferred as a distinct group from the remaining *O. garnettii* samples (in nine out of the ten analyses). Another group that was consistently recovered as distinct (in seven out of the ten analyses) was composed of samples from the Diani Forest (corresponding to haplotype VI), which we refer to as the Nomads clade. A summary of the results from the species-delimitation analyses for the dataset 1 is shown in Fig. 4 (for dataset 2, see Supporting Information, Fig. S3).

DISCUSSION

Nocturnal mammals are often highly cryptic at the morphological level, showing little variation in diagnostic traits that can be used to identify species and subspecies (Bickford *et al.*, 2007; Ceballos & Ehrlich, 2009). The use of molecular data has provided alternative and powerful ways to investigate species diversity within highly cryptic mammalian taxa. Many new cryptic species of shrew moles (e.g. He *et al.*, 2017), rodents (e.g. Rivera *et al.*, 2018; Suárez-Villota *et al.*, 2018) and bats (e.g. Taylor *et al.*, 2018), among others, have been identified using molecular data. The recognition of many new cryptic species has also affected the systematics of nocturnal primates in the last two decades, leading to the description of several new species of mouse, dwarf and sportive lemurs (Craul *et al.*, 2007; Thiele *et al.*, 2013; Hotaling *et al.*, 2016), galagos (Svensson *et al.*, 2017), lorises (Munds *et al.*, 2013) and tarsiers (Shekelle *et al.*, 2017). These recent descriptions demonstrate that the diversity within nocturnal primates is much higher than originally thought, based on morphological data alone. Among those, galagids are some of the most cryptic of all primates. Aside from the decade-long research conducted at the Lajuma Research Centre in the Soutpansberg Mountain Range, North of South Africa with *Otolemur crassicaudatus* and *Galago moholi* A.Smith, 1836, no other long-term studies are being conducted on any galagid species, and most of our knowledge is based on surveys or short-term studies in a few locations (Ellison *et al.*, 2021). Their taxonomy has been long hampered by the lack of genetic data describing within- and between-group variation. More recently, galagid systematics has substantially benefitted from the incorporation of acoustic data (Bearder *et al.*, 1995; Svensson *et al.*, 2017; Pozzi *et al.*, 2019; Génin, 2021). Moreover, molecular studies have suggested that acoustic data overall reflect genetic diversity within galagos (Pozzi *et al.*, 2019), but that species diversity in galagids as a whole is probably still misestimated (Svensson *et al.*, 2017; Pozzi *et al.*, 2020).

In this study, we investigated the genetic diversity within small-eared greater galagos, *Otolemur garnettii* (Primates: Galagidae), a group with limited genetic data available to date, and analysed the patterns of genetic structure considering the biogeographical history of eastern Africa. Mitochondrial DNA data have been widely employed as a proxy for evolutionary divergence at species level, which is useful to answer biogeographic questions and can be a starting point for future taxonomic studies (Groves, 2001). Inferring evolutionary histories from single-locus datasets has limitations when compared to multilocus approaches due to potential discordances between different gene trees (Knowles & Carstens 2007; Padial *et al.*, 2010, Hailer *et al.*, 2012; Carstens *et al.*, 2013). Still, our single-locus approach is the first population-level survey of the patterns of genetic diversity in the small-eared greater galago and provides novel evidence towards a better understanding of the genetic diversity and evolutionary history of the species. In particular, we found little support for the traditional classification of three different subspecies in the coastal region of Kenya (*O. g. lasiotis*), mainland Tanzania (*O. g. panganiensis*) and the Zanzibar Archipelago (*O. g. garnettii*). We discuss in detail our findings relative to each subspecies below.

THE NORTHERN CLADE: A PUTATIVE UNDESCRIBED CRYPTIC LINEAGE?

Of the three putative subspecies, *O. g. lasiotis* was the only one that showed some support as distinct. Although not recovered as monophyletic in any of the analyses we performed, the specimens from six sites

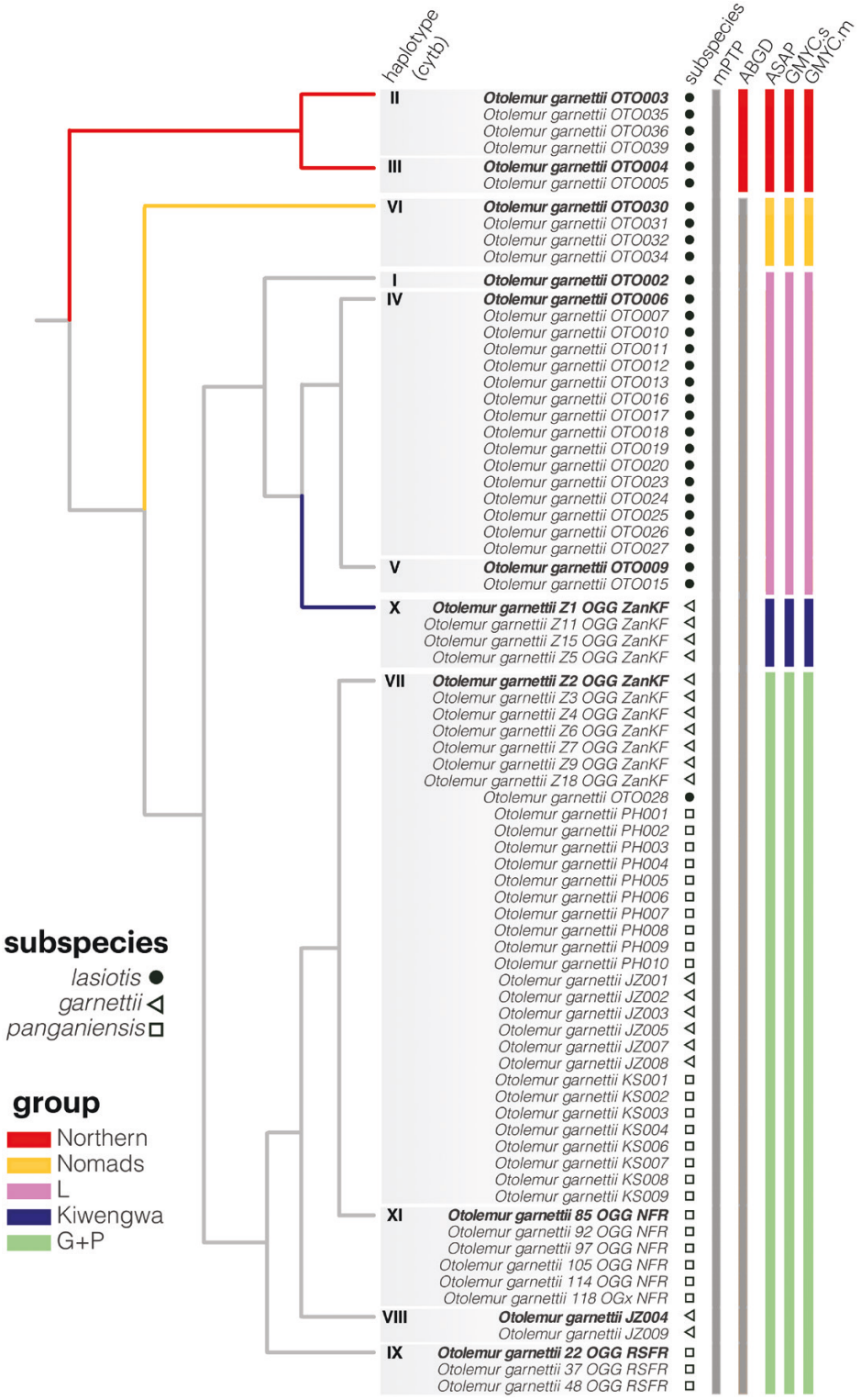

Figure 4. Summary of results from five different species-delimitation analyses for dataset 1, comprising 76 samples of partial cytochrome *b* (402 bp).

in Kenya formed at least three well-defined clusters (Fig. 4). Surprisingly, one sample (OTO028 from Diani Forest) clustered closer to specimens in Tanzania. All the other samples of *O. g. lasiotis* consistently clustered in a distinct group in all analyses. A thorough analysis of the OTO028 sequence did

not show any clear evidence of sequencing errors or contamination, but we cannot exclude this possibility.

One striking result from our study is the consistent finding of a 'northern clade' composed of six samples collected from the north-eastern to the lower Tana River – the longest river of Kenya. This clade is composed of samples assigned to *O. g. lasiotis* collected on Manda Island (Lamu Archipelago) and the Tana River Primate Reserve. These samples clearly constitute a monophyletic group and the most divergent group in the haplotype network analyses. Similarly, these samples were the only ones consistently recovered as a different cluster in nine out of ten species-delimitation analyses conducted in the study. According to our dated phylogeny, the divergence of this group from all remaining samples is remarkably old, dating approximately 4 Mya (Fig. 3; Supporting Information, Fig. S2). Surprisingly, such old divergence is in line with the split between many galagid sister-species, including members of the *Paragalago zanzibaricus* (Matschie, 1893) complex recently analysed with similar methodologies (Pozzi *et al.*, 2019, 2020; Fig. 3). This northern lineage could potentially represent an incipient species, but there is not yet enough evidence to justify taxonomic change for this unique mitochondrial group. Recent studies conducted on the sister-species *Otolemur crassicaudatus* report little intrapopulation variation in mtDNA of specimens within in a 3-km^2 range (Phukuntsi *et al.*, 2020). Results from our study also have not recovered highly divergent haplotypes occurring in a same area, which might suggest that this northern clade is not an artefact due to differences in sex dispersal. However, our research encompasses a much broader geographic scale, and the results tentatively indicate high levels of variation between the northern clade and the rest of the specimens analysed. The remaining forest fragments around the Tana River Basin are among the areas with the highest number of endemics in the eastern African coastal forests (Burgess *et al.*, 2008), potentially representing forest refugia where species persisted and diversified (Barratt *et al.*, 2018; Jenkins *et al.*, 2021). Interestingly, the East Africa coastal forests are disrupted in the north by the Tana River Basin, where steppe and bushland vegetation are more predominant (Burgess *et al.*, 1998). Moreover, the forests around the Tana River are home to two endemic primates: the eastern red colobus [*Piliocolobus rufomitratus* (Peters, 1879)] and the Tana River mangabey (*Cercocebus galeritus* Peters, 1879), both listed as critically endangered by the IUCN Red List but with unsettled taxonomic status. No known morphological, behavioural or acoustical differences have yet been reported between the southern and northern populations of *O. g. lasiotis* in Kenya. Although the distributional range of *O. g. lasiotis* expands to North Kenya and Somalia, as far north as the Juba River, the northern-most range of its distribution is uncertain (De Jong *et al.*, 2019). Unfortunately, no genetic samples are currently available from this region. Moreover, none of the comprehensive morphological studies completed to date accounted for the area occupied by populations belonging to this northern clade, as no specimens from this region are found in museum collections. While our study provides preliminary evidence that this northern clade represents a distinct phylogenetic lineage, nuclear markers and other non-genetic data (e.g. acoustic, behavioural, morphological and ecological) should be analysed to justify any taxonomic revisions of these populations (Padial *et al.*, 2010; Hillis, 2020). Future studies should aim at expanding the geographical sampling for *O. g. lasiotis* to confirm the genetic, morphological and acoustic distinctiveness of this northern clade.

Understanding the colonization of the Zanzibar Archipelago

Another interesting result from our study was the lack of evidence of a separation between populations from mainland Tanzania and Zanzibar Island. More specifically, our genetic analyses indicate that the two subspecies, *O. g. panganiensis* and *O. g. garnettii*, do not represent distinct evolutionary lineages. The 19 samples from two different localities in Zanzibar [Jozani Chwaka Bay National Park (N = 8) and Kiwengwa Forest (N = 11)] included in this study were not recovered as a monophyletic group. Instead, these *O. g. garnettii* samples clustered together with specimens from mainland Tanzania, suggesting that populations on Zanzibar might not be genetically distinct. Olson has already argued that the insular specimens do not differ appreciably in their morphology from those collected in coastal Tanzania (Olson, 1979: 312), but later populations from all the three islands of the Zanzibar Archipelago were assigned their own subspecific category (Groves, 2001; Grubb *et al.*, 2003). Our investigation used only a single mitochondrial marker, which can provide robust phylogenetic and phylogeographic reconstructions. However, in the absence of independent data (molecular or morphological) we were unable to test the hypothesis of introgression due to past hybridization. Interestingly, our result is in line with previous studies conducted on the Zanzibar dwarf galago (*Paragalago zanzibaricus*), a species sympatric with *O. garnettii*. Once described as two distinct subspecies, one on mainland Tanzania (*P. z. udzungwensis*) and one in Zanzibar (*P. z. zanzibaricus*), these populations show no significant differences in either bioacoustical (Pozzi *et al.*, 2019) or molecular data (Pozzi *et al.*, 2019, 2020). The fact that the same pattern was observed in two sympatric species of galagos suggests that the separation between mainland and island populations is relatively recent, and not enough differences have accumulated

over time to separate these two populations at the genetic level. Therefore, even if today they are separated by the Zanzibar Channel, our study suggests that populations of *O. g. panganiensis* and *O. g. garnetti* correspond to incompletely separated lineages. This result is also in accordance with the geological history of the archipelago. The separation of Zanzibar from the mainland is geologically recent, and the island was probably connected to the mainland until around 9–8 kya (Prendergast *et al.*, 2016). The only genetic structure recovered in our analyses of mtDNA diversity in *O. g. garnettii* was a clade represented by samples from Kiwengwa Forest (north Zanzibar Island). Recovered in the haplotype network analyses and in three out of the five species-delimitation analyses (Figs 3, 4), this clade diverged from remaining mainland populations around 1 Mya (but node had support < 95%; Supporting Information, Fig. S2).

Interestingly, the presence of *O. g. garnettii* has also been recorded in the two other islands of the Zanzibar Archipelago: Mafia to the south and Pemba to the north. The geological history of Mafia is similar to Zanzibar, and its isolation has probably been even more recent and transient than that of Zanzibar (Prendergast *et al.*, 2016). Populations of both *O. g. garnettii* and *P. zanzibaricus* have been recorded in Mafia and, although no genetic data are available today, it is likely that these populations present recent gene-flow with their mainland counterparts. On the other hand, the biogeographical history of Pemba is more complex. The channel separating Pemba from the mainland and from Zanzibar reaches approximately 800 m depth and its separation from the mainland is probably much older – possibly between the Early Pliocene (Kent *et al.*, 1971; Burgess & Clarke, 2000) and the Late Miocene (Moreau & Pakenham, 1940, 1941; Stockley, 1942; Pickford, 2008). While no record exists for *P. zanzibaricus* on Pemba, the presence of *O. g. garnettii* has been reported on the island (De Jong *et al.*, 2019). Geological evidence suggests that the separation between Pemba and the mainland pre-dates the origin of the species *O. garnettii* (~5 Mya according to on our molecular estimates), raising interesting questions regarding the colonization of the island by the greater galagos. However, no genetic samples of *O. garnettii* from Pemba Island are currently available, so we cannot exclude any hypothesis, including the possibility that some galagos were brought to the island by humans. A recent study reported that the trade of live animals is the second most common practice of illegal commercialization of nocturnal galagos (Svensson *et al.*, 2021). Some semi-captive *Otolemurs* are kept as pets, a relatively common practice in touristic areas (De Jong & Butynski, 2009; Svensson *et al.*, 2015, 2021). Surprisingly, our results identified a cluster of four samples, the Nomads clade that diverged from other *O. g. lasiotis* populations around 2 Mya (Figs 2–4). These samples were collected in the forested perimeter of a resort hotel in Diani. This finding raises the possibility that some of these animals are originally from elsewhere but were kept as pets and later released in the forests.

Unfortunately, no samples from the fourth subspecies, *O. g. kikuyuensis*, were available for this study. Endemic to the Central Kenya Highlands East of the eastern Rift Valley (De Jong & Butynski, 2004; Harcourt & Perkin, 2013), this subspecies presents remarkable intraspecific differences in their loud calls when compared to *O. g. panganiensis* (Bettridge *et al.*, 2019). Bettridge and colleagues (2019) have suggested that these acoustic differences were probably the results of geographic isolation caused by vegetational barriers in southern Kenya. To understand how vegetational and climatic changes in East Africa led to the structuration of genetic diversity of *O. garnettii,* future studies should aim at incorporating more population-level samples for all subspecies.

Molecular dating analyses suggest an early split, approximately eight million years ago, between *O. garnettii* and *O. crassicaudatus,* the only two species of greater galago currently recognized. Differently from *O. garnettii*, *O. crassicaudatus* is more widely distributed, ranging from South Africa northwards into Zimbabwe, Mozambique, Malawi, Zambia, Angola, south-eastern Republic of Congo, Burundi, Rwanda and Tanzania (Masters & Bearder, 2019). Only a few samples of *O. crassicaudatus* were included in this study, so no definite conclusions can be drawn about the divergence time and the intraspecific diversity of this species. A better understanding of the time and mode of speciation between the two sister-species will require more extensive sampling across their distribution, especially in regions in which they occur in sympatry.

CONCLUSIONS

Based on mitochondrial DNA sequences from over 70 specimens sampled throughout the coastal forests of eastern Africa, we employed multiple species-delimitation algorithms, haplotype network analyses and divergence-time estimation in a phylogenetic framework. Our results provide insights into patterns of genetic structure in *O. garnettii*, the first analysis of this kind for this nocturnal primate. We found a deep divergence between far-northern populations of *O. g. lasiotis* (northern coastal Kenya, potentially extending into southern Somalia) and populations further south (Kenya). Additionally, we inferred haplotype sharing and paraphyly between insular populations from Zanzibar (assigned to *O. g. garnettii*) and those from coastal mainland Tanzania (assigned to *O. g. panganiensis*). Hence, despite their geographic

separation, and presumed morphological and acoustic differences, these two subspecies do not appear to correspond to distinct genetic pools.

These emerging patterns of mitochondrial diversity indicate that evolutionary lineage diversity within *O. garnettii* may be wrongly estimated, with direct implications for greater galago taxonomy. In the case of *O. g. lasiotis*, the high divergence between northern and more southern parts of the range might be indicative of unrecognized species. By contrast, *O. g. garnettii* and *O. g. panganiensis* do not appear to correspond to distinct phylogenetic lineages. Therefore, island and mainland populations would not be assigned to different taxa under the proposal of subspecies as evolutionary lineages (De Queiroz, 2020).

To further delimit lineages and their distributions in the greater galagos, future analyses should incorporate additional genetic loci, allowing for more direct estimation of genetic introgression, as well as acoustic data, which mediates mate choice. Future efforts will also benefit from reassessments of the morphological attributes presumed to be diagnostic, such as coat pattern and body proportions, under the light of the genetic patterns. Although our geographic sampling is the most comprehensive for *O. garnettii* to date, it will be useful to include samples from additional Zanzibar Archipelago islands (i.e. Pemba and Mafia), as well as savannah and montane habitats in interior Tanzania. Similarly, to further assess whether the northern clade corresponds to an unrecognized species, it will be crucial to sample between the Tana River and Mogadishu in southern Somalia, where *O. garnettii* is known to be patchily distributed (De Jong *et al.*, 2019). Currently, additional sampling is made difficult by political instability in these regions. To overcome this challenge, a promising approach is to incorporate archival DNA from specimens housed in natural history collections (Pozzi & Penna, 2022). Historical DNA has emerged as an important resource to support estimates of genetic diversity, phylogenetic relationships and population trends (Van der Valk *et al.*, 2019; de Abreu-Jr *et al.*, 2020; Roos *et al.*, 2021; Roycroft *et al.*, 2021).

Most primates are threatened by habitat loss and the pet trade. This is also the case for the small-eared greater galago, which has a severely fragmented range in certain regions, such as Zanzibar (Butynski & De Jong, 2019). Besides improving our understanding of galagid evolution, a clear understanding of species diversity is critical to inform conservation priorities and population management.

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DATA AVAILABILITY

Genetic data generated in this study are available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/, and can be accessed with accession numbers OP329354-OP329364 (Supporting Information, Table S1).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1: Detailed information for each sample, including GenBank accession numbers.
Table S2: Haplotype frequency by locality.
Table S3: AMOVA results for dataset #2.
Figure S1: Haplotype median joining network estimated from dataset 2, comprising 2061 bp of the full *Cytb* and D-loop, with 56 samples.
Figure S2: Dated phylogeny expanding the node with subspecies of *Otolemur garnettii*.
Figure S3: Summary of results from five different species delimitation analyses for dataset 2.