Population genetic structure of the thick-tailed bushbaby (*Otolemur crassicaudatus)* from the Soutpansberg mountain range, northern South Africa, based on four mitochondrial DNA regions

Metlholo Andries Phukuntsi[1,2], Morne Du Plessis[1,3], Desiré Lee Dalton[1,4], Raymond Jansen[2], Frank P. Cuozzo[5], Michelle L. Sauther[6] and Antoinette Kotze[1,7]

[1]*South African National Biodiversity Institute, P.O. Box 754, Pretoria, 0001, South Africa*
[2]*Department of Environment, Water and Earth Sciences, Tshwane University of Technology, Private Bag X360, Pretoria, 0001, South Africa*
[3]*Department of Biotechnology, University of Western Cape, Robert Sobukwe Rd, Bellville, Cape Town, 7535*
[4]*Department of Zoology, University of Venda, Thohoyandou, South Africa*
[5]*Lajuma Research Centre, P.O. Box 522, Louis Trichardt (Makhado) 0920, South Africa*
[6]*Department of Anthropology, University of Colorado, Campus Box 0233, Boulder, Colorado, 80309-0233, USA*
[7]*Department of Genetics, University of the Free State, P.O. Box 339, Bloemfontein, 9300, South Africa*

Corresponding author: d.dalton@sanbi.org.za

ORCiD ID:
Metlholo Andries Phukuntsi 0000-0003-4482-184X
Morne Du Plessis: 0000-0003-4154-7830
Desiré Lee Dalton: 0000-0001-5975-6425
Raymond Jansen 0000-0003-4741-3307
Frank P. Cuozzo 0000-0002-8870-5173
Michelle L. Sauther 0000-0003-4747-4417
Antoinette Kotze: 0000-0001-5975-6425

Abstract

The aim of our study was to investigate the genetic variation in a thick-tailed bushbaby, *Otolemur crassicaudatus,* population in the Soutpansberg mountain range, Limpopo Province, South Africa. Four mitochondrial regions, ranging from highly conserved to highly variable, were sequenced from 47 individuals. The sequences were aligned and genetic diversity, structure, as well as demographic analyses were performed. Low genetic diversity and sub-structuring was observed with two divergent haplogroups being identified. This suggests the population may have experienced fixation of mitochondrial haplotypes due to limited female immigration, which is consistent with philopatric species or that alternative haplotypes are not native to this population, and that there may be male mobility from adjacent populations. This study provides the first detailed insights into the mitochondrial genetic diversity of a continental African strepsirrhine primate and demonstrates the utility of mitochondrial DNA in intraspecific genetic population analyses of these primates.

Keywords: bushbaby, DNA, genetic variation, *Otolemur crassicaudatus*, heterogeneity, mitochondria

Introduction

Among continental Africa's endemic primates, galagos are the most successful of the strepsirrhine primates in terms of species diversity and geographic range (Nekaris and Bearder, 2011). However, they are a relatively understudied group of non-human strepsirrhine primates, especially as compared to the abundant research on Malagasy lemurs (e.g. Gould and Sauther, 2006; Sauther et al., 2015). Much of our knowledge of galago behaviour, sociality, and biology stems from data collected in the 1970s and 1980s (e.g. Bearder, 1974; Bearder and Doyle, 1974; Charles-Dominique, 1975; Harcourt, 1980; Nash, 1983; Masters *et al*., 1988; Harcourt and Bearder, 1989). More recent research has generally focused on heterothermy in small bodied galagos (e.g. Nowack *et al*., 2013). Current knowledge of within-species diversity in African galagids has been described based on acoustic, morphological and geographic distributional data (Anderson *et al*., 2000; Masters and Bragg, 2000; Ambrose, 2003; Karlsson, 2006; Bearder, 2007; Butynski *et al*., 2006; Butynski *et al*., 2013; Masters and Génin, 2016a; 2016b; Masters *et al.,* 2017).

Genetic variation is seen as a foundational tool to evaluate both biodiversity and the ability of species to cope with environmental change (Toro and Caballero, 2005). Analyses of

mitochondrial and nuclear sequence data has previously been applied to supraspecific analyses of the galagid and lorisoid genera (Delpero *et al*., 2000; Masters *et al*., 2007; Pozzi *et al*., 2014a)., but no intraspecific analyses have been applied for the population structure of a single species. The latest reviews on bushbabies call for inclusion of a genetic assessment, with emphasis on assessing populations due to fragmented habitats, encroachment as well as anthropogenic activities (Masters and Couette*;* 2015; Masters and Génin, 2016a; Masters and Génin, 2016b). Such data are critical to determine their conservation status, as basic genetic diversity data can assist stakeholders to make decisions regarding the viability of populations, as well as to clarify the taxonomy and gene flow between closely related species and subspecies (Perry *et al*., 2012; Osada, 2015). Thus, the importance of the data presented herein.

The thick-tailed greater galago or bushbaby (*Otolemur crassicaudatus* Geoffroy, 1812) is one of three bushbaby species described in South Africa, the other two being the southern lesser galago (*Galago moholi*) and Mozambique dwarf galago (*Galagoides granti*) (Grubb *et al*., 2003; Groves, 2005; Butynski *et al*., 2013; Génin *et al*., 2016). Breeding is promiscuous, with a multiple-female and multiple-male mating system (Bearder and Svodoba, 2013). The species is considered as of “least concern” under the International Union for Conservation of Nature (IUCN) Red List of endangered species (Bearder, 2008; Bearder *et al*., 2008; Masters and Génin, 2016a; 2016b).

Thus far, there is no population and intra-specific-level genetic data on *O. crassicaudatus.* Investigating populations within a “restricted geographic locality and time horizon” as suggested by Plavcan and Cope (2001, cited in Cuozzo *et al*., 2013), as well as Hague and Routman (2016), may provide valuable information with regard to population genetics and dynamics. This study was undertaken to assess genetic variation within an *O. crassicaudatus* population in the Soutpansberg Mountains in and around the Lajuma Research Centre, Limpopo Province, South Africa. The population in Lajuma is wild, and there is an ongoing project with this population concentrating on different aspect of this species’ biology, ecology and conservation. Studying genetics of this population will provide a window into the population dynamics of this species, as well as help to inform the population variation and, ultimately, the biogeography of South African bushbabies. This is a bottom up approach to understand population genetic structure of the species in a uniform environment, and will inform future research with regard to sampling strategies. Therefore, the data in this study will provide key missing information for strepsirrhine

primates and population genetics, while also providing valuable data for future research on this taxon. The mitochondrial diversity of the population was explored by assessing sequence variation among individuals using gene regions representing diversity at a conserved region (*16S*), two relatively variable regions (*COI* and *Cyt b*) and a highly variable region (*D-loop*) (Hwang and Kim, 1999). A high-level of mtDNA homogeneity is expected in the population, as *O crassicaudatus* females are highly philopatric (Mbora and McPeek, 2010). However, a higher than expected level of nucleotide and haplotype diversity would indicate parameters such as migration and selective pressures (Tajima, 1989; Luikart and Allendorf, 1996; Fu, 1997; Cornuet and Luikart, 1996; Mbora and McPeek, 2010; Osada, 2015). Furthermore, gene flow can be estimated from a single deme (Ray *et al*., 2003). As such, analyses of this population would provide the first look into the genetic variation and demographic processes in the Soutpansberg populations.

Materials and methods

Study site and sampling

The study site encompasses a portion of the 10,000 hectares (ha) Lajuma Research Centre which forms part of the Luvhondo Nature Reserve on the Soutpansberg within the UNESCO Vhembe Biosphere Reserve in Limpopo Province, South Africa (Figure 1). The Soutpansberg has montane grassland to woodland, thicket and mistbelt forest and a remarkable diversity of plants and animals, including a large number of endemic, rare and endangered species (Willems, 2007; Mostert *et al*., 2008; Hahn, 2017). The sampling area is roughly 3 km^2, constitutes a continuous landscape of various forest types, and includes an elevation gain of 300 m from south-east (SE) to north-west (NW). All individuals were captured along a roughly 3 km transect running SE to NW, with traps set along this transect up to 500 m north or south of this transect (the primary dirt track that runs through the research centre). It is imperative to note that this sampling area only represents the capture area, and not the total area used by this population. A number of the capture traps on the edges of the sampling area occur on cliff boundaries and other topographic areas which cannot be traversed by humans to set traps, but are easily moved through by *O. Crassicaudatus* (Sauther and Cuozzo personal observation). In addition, individuals can move as far as one or two km in a single night. Thus the samples represent the “molecular signature” of an area far greater than the 3km^2 capture grid.

A total of 47 wild *O. crassicaudatus* individuals were caught and sampled between 2013 and 2017 (Supplementary S1). Ethics approval was obtained from the Research Ethics and Scientific Committee (RESC) at the National Zoological Garden, South African National Biodiversity Institute (NZG, SANBI) with the project number P13/04. Havahart™ live traps were baited with a mixture of bananas and honey and/or peanut butter. Once secured in a trap, the individuals were anesthetised by a certified wildlife veterinarian, via intramuscular injection of a tiletamine/zolazepam (Zoletil®, Virbac, South Africa) combination at an estimated dose of 4.8 – 7.5 mg/kg. Subsequent anesthesia was administered using inhaled Isoflurane™ in doses between 2-4% mixtures with air as prescribed in Larsen *et al*. (2011). The sex and age grade (e.g., subadult vs. adult) of each individual were noted. Overall health evaluations were conducted, and somatic and dental variation and health data were collected as part of an on-going study. Individuals were microchiped (ID100 Trovan), as part of the long term study of this population, so that repeat individuals were not included in the data set. As part of an overall assessment of galago health, a small amount of blood was placed on filter paper (Whatman FTA "Elute" Microcards, GE Healthcare), part of which was subsequently used for these genetic analyses.

DNA processing and analyses

The Zymo Research Tissue Mini Prep (Zymo Research, USA) was used to isolate DNA from the FTA paper blood samples according to the manufacturer's protocol. Thereafter, DNA was amplified and sequenced according to materials and methods specified in Phukuntsi *et al.* (2016). Sequences were edited manually using MEGA6 (Tamura *et al*., 2013) and each sequence was queried on GenBank (Benson *et al*., 2005) using the BLAST plugin on MEGA6. The GenBank sequence with the accession number KJ434961.1 (Pozzi *et al*., 2014b) was added to each dataset as a reference, as it consistently yielded a 'top match' with all sequence queries. This sequence was also used to align codons of each coding region. An *Otolemur garnetti* sequence from the GenBank database was added to each dataset, as an outgroup. To test other hypotheses, a single individual from Mokopane (Limpopo Province; Supplementary S1) was alternatively added to the dataset for reference. The sequences were aligned using the ClustalW (Larkin *et al*., 2007) plugin in MEGA6.

A haplotype network for each dataset was generated by implementing the median-joining method (Bandelt *et al*., 1999) and drawing the haplotype networks on Popart v1.7 (http://popart.otago.ac.nz). Haplotype networks that included the Mokopane individual and other

reference sequences were also drawn to test the relationship of the haplotypes in the Soutpansberg with other regions. Population summary statistics, as well as diversity measures, were also calculated using MEGA6 and DnaSP (Rozas *et al.,* 2017). Divergence from expectations of neutral evolution was calculated using the Tajima's test of neutrality, as well as both Fu and Li's D* and F* and Fu's Fs (Fu, 1997). Demographic events were inferred by implementing coalescent simulations in DnaSP and Arlequin (Excoffier and Lischer, 2010). These included tests for population substructuring, neutral evolution, as well as tests for demographic and spatial expansion. While both Arlequin and DnaSP may be used to calculate many similar demographic estimators, they each have extra unique features that make hypothesis testing easier.

Results

DNA analyses

Sequences were successfully obtained for *16S* (45/47 individuals), *COI* (42/47 individuals), *Cyt b* (38/47 individuals) and *D-loop* (42/47 individuals), as shown in Supplementary S1, with summary statistics for each region provided in Table 1. The remaining sequences could not be obtained due to failed amplification and limited sample availability. The sequences had at least one parsimonious information site, varying from one in *Cyt b* to thirteen in *D-loop*.

In the *16S* and *Cyt b*, two haplogroups, H1 and H2 (for haplotype 1 and haplotype 2, respectively), were observed (Figure 2). The H1 was observed at a higher frequency than H2 in both gene trees. In the *COI* gene tree, the H1 cluster split into two haplotypes, H1A and H1B (Figure 2). These haplotypes were observed at a more or less equal frequency. The H2 retained its integrity, still at a lower frequency than either of H1A and H1B. In the *D-loop* gene tree, the subgroups H1A and H1B were observed, while H2 also split into two subgroups, H2A and H2B (Figure 2). However, these two groups (H2A and H2B) seemed to be divided on the basis of an indel. The motif is a six/seven A/T repeat, which would increase the chances of an insertion or deletion in the non-coding region. Supplementary S2 shows the genetic distance between the observed haplotypes in each region, based on pairwise differences. While *16S* and *Cyt b* indicated only a few mutational steps between haplotypes, the *COI* and *D-loop* networks showed several mutational steps between the haplotypes, suggesting that some haplotypes may have been lost over time. Figure 3 illustrates the distributions of the haplotypes among the Lajuma population. The individual from Mokopane also possessed the H2 haplotype

(Supplementary 3), indicating that the H2 haplotype group was shared with the individuals from outside the Lajuma population.

Diversity measures (Table 2) were also inconsistent among the gene regions. The *D-loop* region exhibited the highest level genetic diversity [nucleotide diversity (π)=0.0127], followed by *COI* which was three times less diverse (π=0.0038). Both *16S* (π=0.0010) and *Cyt b* (π=0.0007) were at least three times as conserved as *COI.* The haplotype diversity (*h*) of the *D-loop* and *COI* were more or less equal at 0.577 and 0.616, respectively. The *16S* region had the lowest haplotype diversity in the Lajuma population at 0.162, followed by *Cyt b* at 0.229. It is worth noting, however, that the sample size of *Cyt b* sequences was slightly smaller than *16S*. However, *COI* was more diverse than *16S*, despite the analysis having more *16S* sequences.

Tajima's D, as well as Fu and Li's D* and F* were not significantly different from a model of neutral evolution in all mitochondrial regions (P>0.10; Table 2). On the other hand, Fu's Fs was positive and significant in the *D-loop* region at the 95% CI (6.538) while positive but not significant for the rest of the regions (Table 2). The sum of squared deviations and the raggedness index *r*, which are small for a population that has undergone sudden population or spatial expansion (Rogers and Harpeding, 1992; Ramos-Onsins and Rozas, 2002) were large in both the *COI* region and the *D-loop* region. Pairwise mismatch distribution were multimodal (Figure 4). The simulations indicated that the polymorphism observed in the two regions was significantly different from a population that has undergone sudden population but not significantly different from a population that has experienced sudden spatial expansion. There was not enough polymorphism to perform coalescent simulations for both *Cyt b* and *COI*.

Discussion

Genetic diversity measures

The overall mitochondrial diversity of the Lajuma population was found to be lower than the mitochondrial diversity of populations of other primates, including other non-human primate species such as the African samango monkey (*Cercopithecus mitis*; π<0.001 and *h*<0.5 in *Cyt b* and *16S* per population), Asian lorisoids such as the slow lorises (*Nycticebus*; π<0.001 and *h*>0.7 in *12S* in some populations), as well as endangered species such as the Neotropical northern muriqui (*Brachyteles hypoxanthus*; π<0.01 and *h*<0.6 in *D-loop* per population) (Wirdateti *et al*., 2006; Pan *et al*., 2007; Chaves *et al*., 2011; Kawamoto *et al*., 2013; Dalton *et al*., 2015). Our study is the first to estimate mitochondrial haplotype diversity in a continental

African strepsirrhine population, excluding Malagasy lemurs from the island of Madagascar. Thus, comparison with another member of the galagid family could not be made. The Soutpansberg population is near the southernmost edge of this species' distribution in southern Africa. Such areas are characterized by fringe populations with low genetic diversity across domains of life, as their preferred habitats tend to be fragmented and are mostly pioneer populations with signatures of founder events (Hallatschek *et al*., 2007; van der Valk, 2018). As such, we would expect low genetic diversity in this population compared to others. Furthermore, isolated populations show an extremely high level of mitochondrial homogeneity due to fixation of haplotypes, especially in slower-evolving gene regions (Lacy, 1987; Luikart *et al*., 1998; Carr and Dudash, 2003; Blanquart *et al*., 2012; Ang *et al*., 2016). However, in this population, there were two divergent haplogroups that most likely led to a higher than expected heterogeneity.

Genetic structure, neutrality and demography

The haplotype networks and genetic distance data in this study indicate that there is clear genetic sub-structuring in the Lajuma population. The Lajuma population is heterogenous in the relatively slower-evolving regions of the mitochondrion, especially the *16S* region. The haplotypes in the H1 groups are very closely related to each other than to both H2 haplotypes and are reciprocally monophyletic to the H2 group. Coalescent simulations indicated that the observed polymorphism in this population was significantly different from a population that has undergone sudden population expansion. On the other hand, a higher than expected genetic diversity and mitochondrial heterogeneity in a population may also be a result of episodic colonization and/ or introgression of mitochondrial haplotypes from allopatric populations (Slatkin and Excoffier, 2012; Morgan-Richards *et al*., 2017). The two divergent haplotypes suggest that there are two ancestral maternal lineages in the population. The first maternal lineage, H1 is found at a disproportionately larger frequency than H2. Furthermore, all of the individuals possessing the H2 haplotypes are males. Mitochondrial DNA can only be passed on maternally in mammals, and as such it is highly unlikely that the origin of the H2 haplotype is from within the Lajuma population, given the apparent lack of H2 females. Lastly, the H2 haplotypes are more related to reference individuals from outside the Soutpansberg than the H1, further indicating that the two observed haplogroups have different origins. Unfortunately, this latter evidence should be interpreted with caution as the sample number of individuals outside the population in the Soutpansberg was very low, and the locality of only one of those is known.

Thus, it is likely that the H1 and H2 haplotypes did not originate from the same locality, or population. Coalescent simulations do indicate that the polymorphism observed in this population is not statistically different from the one expected in a population that has undergone sudden spatial expansion, while pairwise mismatch analyses showed multimodal distribution of pairwise differences. Such a pattern can be seen in a population with some limited contact between adjacent populations (Ray *et al*., 2003). In addition to the apparent absence of female H2 haplotypes in the population, our analyses suggest that the mitochondrial heterogeneity in the Lajuma population is a result of mitochondrial introgression from dispersing males.

Therefore, our data indicates that there is male-biased gene flow into the Lajuma population. This suggests immigration of individuals from more than one direction into the Lajuma area, a strong possibility given the multiple river drainages (and thus migration corridors) throughout the Soutpansberg, at least prior to recent habitat fragmentation. While this may check effects of inbreeding, it does not do anything for the mitochondrial diversity of the population, as the observed mitochondrial heterogeneity is superficial. Loss of habitat and fragmentation of populations in this species could lead to total mitochondrial homogeneity and fixation in local populations as females are extremely philopatric.

This study provides the first insights into the mitochondrial genetic structure and diversity of a bushbaby population. There is clear mitochondrial heterogeneity and genetic structure within the Lajuma population and it is possible that this heterogeneity is not due to neutral evolution. The presence of non-native haplotypes in the males of this population, as well as absence of alternate haplotypes in the females, indicates that there are selective barriers into and out of the Lajuma population. It is likely that gene flow in this population is influenced by dispersal dynamics. This study further indicates that *COI* and *D-loop* capture adequate haplotype diversity within populations of *O. crassicaudatus* for use in population genetic analyses. Further analyses should include adjacent populations, as well as incorporate paternally-inherited Y-chromosome, nuclear and microsatellite data to further investigate sex-biased gene flow in the species, and to provide insight into their fine-scale genetic structure and large scale phylogenetics.

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Declaration of interest statement

No potential conflict of interest was reported by the authors.

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Tables:

Table 1: Summary and statistics of four mitochondrial sequences obtained from 47 *Otolemur crassicaudatus* individuals from a region of the Soutpansberg mountain range, South Africa.

	16S	***COI***	***Cyt b***	***D-loop***
Model of evolution	K2	K2	HKY	HKY+G
Length	469	526	405	383
R	0	0	0	0
Conserved	466	518	404	369
Variable	3	8	1	14
Parsimonious-informative	3	5	1	13
Thiamine	24.2	28.3	26.7	29.7
Cytosine	24.7	28.5	32.1	26.8
Adenine	31.7	26.6	28.1	30.7
Guanine	19.4	16.6	13.1	12.9

Table 2: Summary population and diversity statistics of four mitochondrial sequences obtained from 47 *Otolemur crassicaudatus* individuals from a region on the Soutpansberg mountain range, South Africa. S.E indicates standard error values.

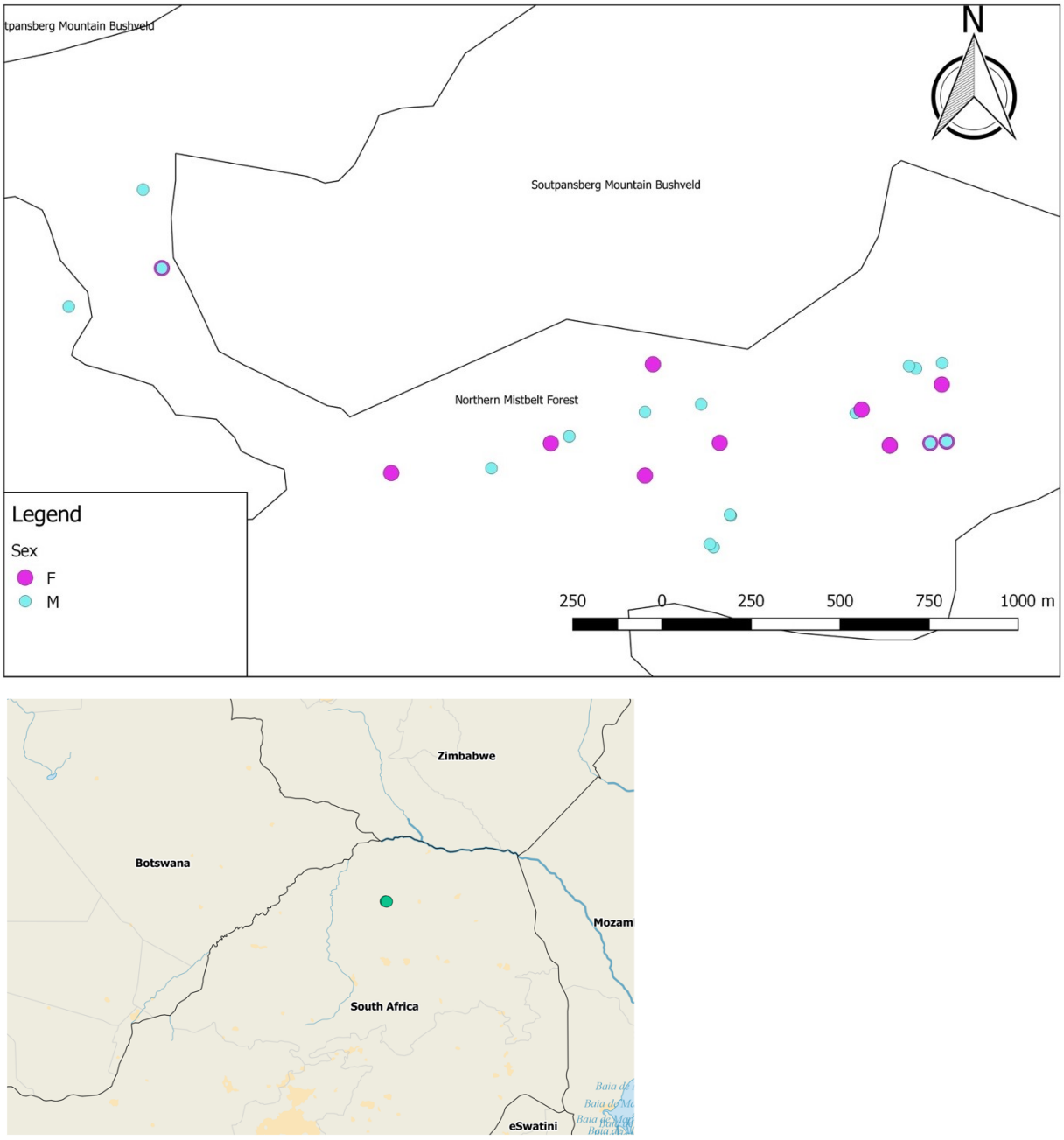
	16S	*COI*	*Cyt b*	*D-loop*
Number of sequences	45	43	38	42
Number of polymorphic sites	2	5	1	12
Total No of mutations	2	5	1	13
Average no. of nucleotide differences	0.331	1.562	0.235	3.815
Nucleotide diversity	0.001	0.00377	0.00075	0.0122
Number of Haplotypes	2	3	2	4
haplotype diversity	0.166	0.592	0.235	0.584
Tajima's D (P-value)	-0.505^{0} (P>0.10)	0.879^{0} (P.0.10)	-0.020^{0} (P>0.10)	0.814^{0} (P>0.10)
Fu and Li's D* (P-value)	0.758^{0} (P>0.10)	1.110^{0} (P>0.10)	0.569^{0} (P>0.10)	1.440^{0} (P>0.05)
Fu and Li's F* (P-value)	0.425^{0} (P>0.10)	1.121^{0} (P>0.10)	0.465^{0} (P>0.10)	1.363^{0} (P>0.10)
Fu's Fs	1.079^{0}	3.475^{0}	0.455^{0}	6.538^{1}
R2	0.0828	0.156^{0}	0.1174	0.159^{0}
SSD (population expansion)		0.114^{0} (P=0.06)		0.210 (P=0.004)
SSD (spatial expansion)		0.073 (P=0.216)		0.109 (P=0.173)
Raggedness *r* (population expansion)		0.406 (P=0.039)		0.479 (P=0.005)
Raggedness *r* (spatial expansion)		0.406 (P=0.278)		0.479 (0.310)

Figures:

Figure 1: A. Sample localities of a wild population of *Otolemur crassicaudatus* in an area at the Soutpansberg mountain range. B. Map of Limpopo Province indicating the study site.

Figure 2: Median-joining haplotype networks of four mitochondrial regions (*16S*, *COI*, *Cyt b* and *D-loop*) in a population of *Otolemur crassicaudatus* in an area of the Soutpansberg mountain range. The coloured circles indicate the frequency of the haplotypes while the bars across the branches indicate the number of mutational steps between haplotypes. Black circles represent missing haplotypes.

Figure 3: Distribution of the two maternal mitochondrial lineages across the wild population of *Otolemur crassicaudatus* in an area of the Soutpansberg mountain range, showing the lack of H2 haplotype in the females of the population.



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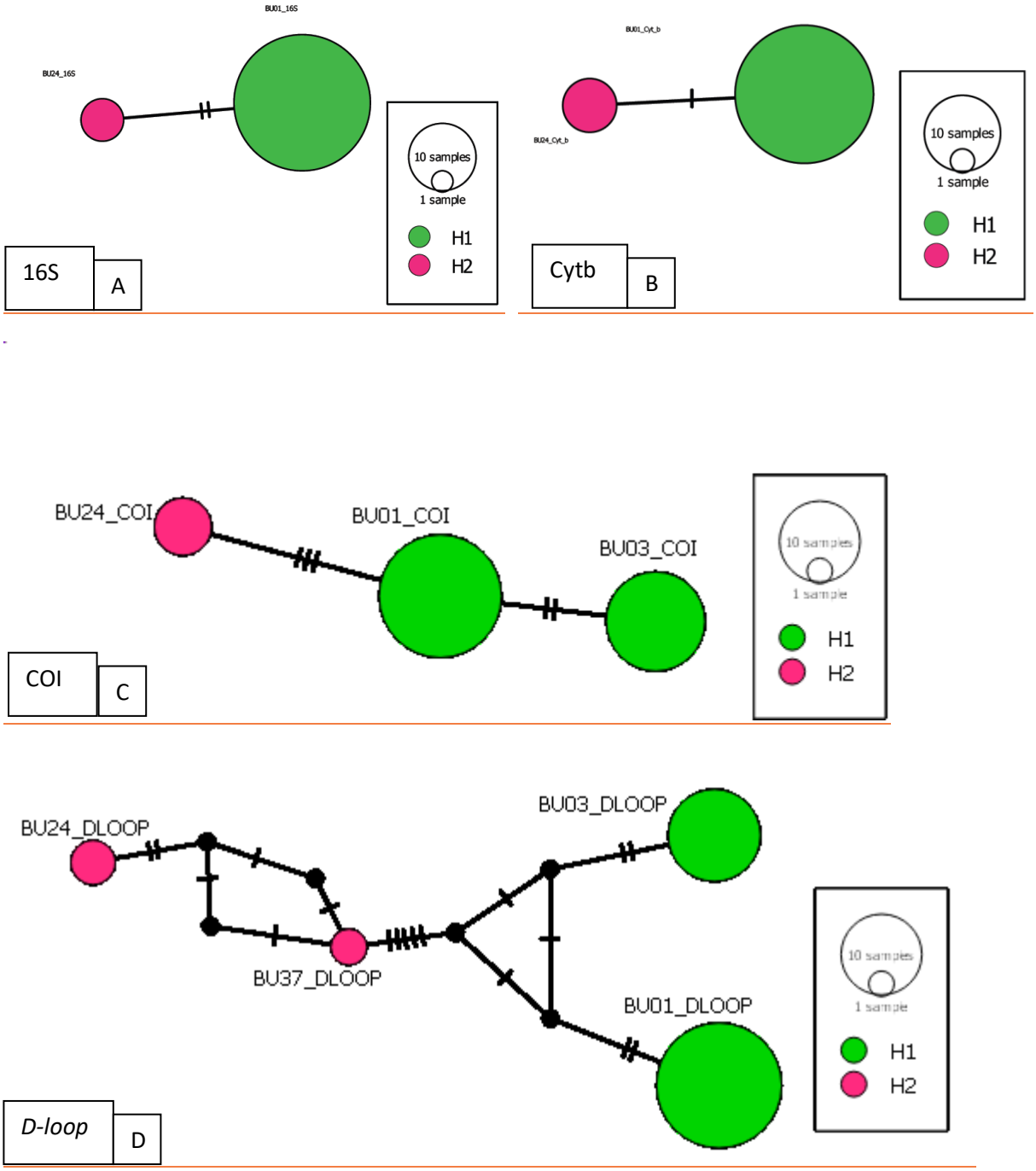

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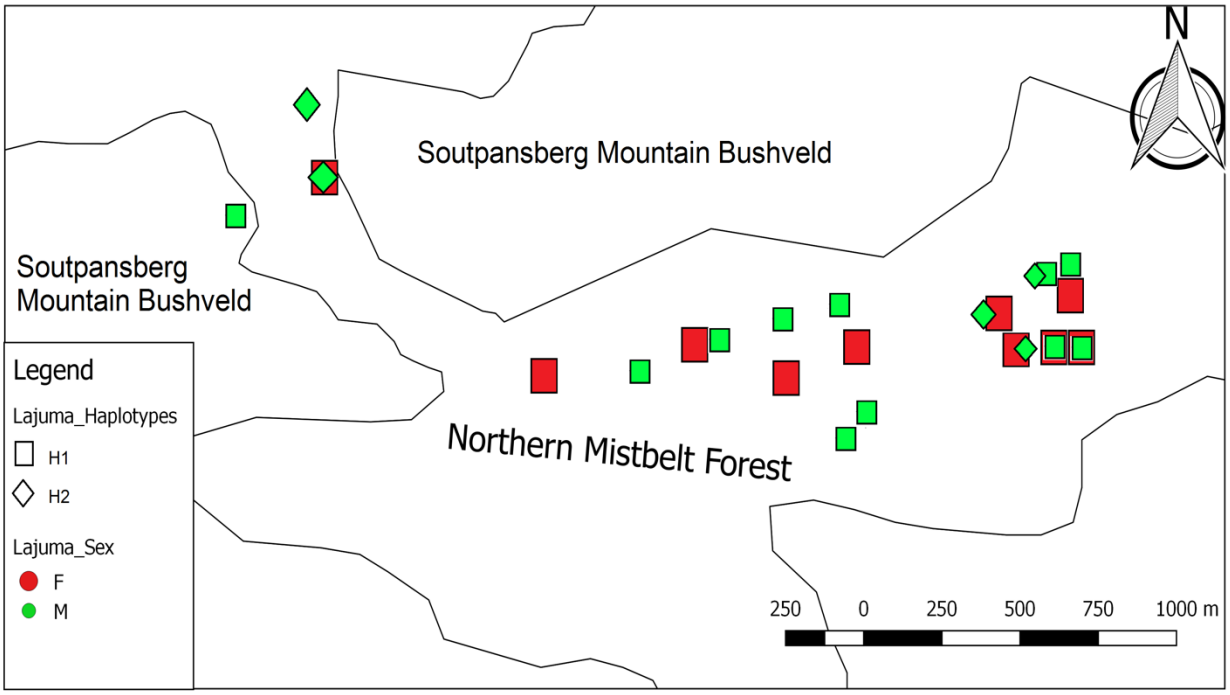

Figure 3: Distribution of the two maternal mitochondrial lineages across the wild population of *Otolemur crassicaudatus* in an area of the Soutpansberg mountain range, showing the lack of H2 haplotype in the females of the population.

Supplemental online material:

Supplementary S1: List of *Otolemur crassicaudatus* samples obtained in an area of the Soutpansberg Mountain Range in South Africa.

Supplementary S2: Median-joining haplotype networks inferred from two mitochondrial regions (*COI* and *D-loop*) of *Otolemur crassicaudatus* in Limpopo, South Africa. The coloured circles indicate the frequency of the haplotypes while the bars across the branches indicate the number of mutational steps between haplotypes. Black circles represent missing haplotypes.

Supplementary S3: Distance matrix of haplotypes observed in an *Otolemur crassicaudatus* population in the Lajuma area of the Soutpansberg mountain range, inferred from two mitochondrial regions (*COI* and *D-loop*)