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ORIGINAL ARTICLE

Population and genetic structure of a male-dispersing strepsirrhine, *Galago moholi* (Primates, Galagidae), from northern South Africa, inferred from mitochondrial DNA

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Abstract

The habitats of *Galago moholi* are suspected to be largely fragmented, while the species is thought to be expanding further into the southernmost fringe of its range, as well as into human settlements. To date, no intraspecific molecular genetic studies have been published on *G. moholi*. Here we estimate the genetic diversity and connectivity of populations of *G. moholi* using two mitochondrial gene regions, the cytochrome C oxidase subunit I gene (*COI*) and the displacement loop of the control region (D-loop). Samples from five localities in northern South Africa were obtained from archived collections. The two mitochondrial DNA gene regions were amplified and sequenced to provide population summary statistics, differentiation [proportion of the total genetic variation in a population relative to the total genetic variance of all the populations (F_{ST}), differentiation within populations among regions (Φ_{ST})], genetic distance and structure. There was discernible genetic structure among the individuals, with two *COI* and six D-loop haplotypes belonging to two genetically different groups. There was population differentiation among regions ($F_{ST} = 0.670$; $\Phi_{ST} = 0.783$; $P < 0.01$). However, there were low levels of differentiation among populations, as haplotypes were shared between distant populations. Adjacent populations were as divergent from each other as from distant populations. The results suggest that genetic introgression, most likely due to past migrations or recent unintentional translocations that include the animal trade, may have led to connectivity among populations.

Keywords *Galago moholi* · Population genetics · Strepsirrhini · Genetic diversity · Bushbaby

Introduction

Galago moholi (Smith 1834) is a galagid primate with two subspecies recognized, *Galago moholi moholi* and *Galago moholi bradifieldi* (Grubb et al. 2003; Masters and Génin 2016a). *G. moholi bradifieldi* is distributed in the western parts of the species' distribution, from western Botswana into Angola, while *G. moholi moholi* is distributed in the eastern parts, from northern South Africa into Tanzania (Pullen and Bearder 2013; Masters and Génin 2016a). While distributional data have been largely used to describe and delimit the two subspecies, prior studies have failed to provide evidence of morphological and acoustic disconnectivity in the subspecies across their eastern and western distributions (Anderson et al. 2000; Masters and Bragg 2000; Pullen and Bearder 2013; Génin et al. 2016). The two subspecies form hybrid zones within their respective western and eastern distribution ranges in two countries, Botswana and Zambia (Pullen and Bearder 2013). In South Africa, only *G.*

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moholi moholi has been reported; it occurs in the Limpopo and North West provinces, as well as the in northern and eastern parts of Gauteng and Mpumalanga provinces (Masters and Génin 2016a). Northern South Africa represents the southernmost fringe of the species' distribution.

Various agricultural and socioeconomic activities affect the natural habitats of bushbabies and other arboreal primates in South Africa and may contribute heavily to population and habitat fragmentation (Bergl et al. 2008; Mbora and McPeek 2010; Marsh et al. 2013; Masters and Génin 2016a, 2016b). Fragmentation has previously led to reduction in genetic diversity and connectivity in species (Brenneman et al. 2011; Stevens et al. 2018; van der Valk et al. 2018). Currently, the habitats of *G. moholi* are suspected to be largely fragmented (Masters and Génin 2016a). The latest review of *G. moholi* suggested that the genetic monitoring of bushbaby populations in South Africa should be undertaken for conservation purposes (Masters and Génin 2016a).

Genetic monitoring includes the assessment of both genetic connectivity and diversity of individuals within and between geographical regions (Mbora and McPeek 2010; Brenneman et al. 2011; Allendorf et al. 2013; Dalton et al. 2015; Madisha 2015). Despite its glaring drawbacks (Rubinoff and Holland 2005; Leite 2012), mitochondrial DNA (mtDNA) has been used extensively in assessing the population genetics and biogeography of many species, including lorisids and other African primates (Heckman et al. 2006; Wirdateti et al. 2006; Pan et al. 2007; Pozzi et al. 2014). By studying their genomes, it has been possible to elucidate various genetic indices in different species, including historical and contemporary gene flow, genetic diversity, temporal and geographical changes in genetic composition and evolutionary patterns in populations (Rubinoff and Holland 2005; Allendorf et al. 2013; DeSalle et al. 2017). For example, high mobility rates between populations may lead to overall low levels of mitochondrial heterogeneity, while the reverse is true for populations with restricted mobility, where large variations are expected between them (Mbora and McPeek 2010; Madisha 2015). Primates that practise female philopatry provide good examples of these differences, where a highly defined mitochondrial genetic structure is expected between populations, while mitochondrial homogeneity is expected within populations or regions. Deviations from these expectations provide information regarding genetic connectivity or evolutionary history of the populations.

In this study, two mitochondrial fragments, the coding cytochrome C oxidase subunit I gene (*COI*) and the non-coding displacement loop of the control region (D-loop), were used to assess the genetic diversity and connectivity in *G. moholi* populations in northern South Africa. One of the greatest concerns regarding species in the fringes of their range include isolation of subpopulations, which may lead to local extinctions (i.e. extirpations). This study could therefore help identify potential conservation management units, as well as determine if there are population isolates (Van Vuuren et al. 2017). To date, only one study of population genetic diversity monitoring of a galagid species (*Otolemur crassicaudatus*) has been published (Phukuntsi et al. 2020). The D-loop region was the fragment of choice as it is routinely utilized in population genetics studies of mammals, including primates (Whittaker et al. 2007; Adenyo et al. 2013; Badhan et al. 2015). The *COI* gene fragment was added to assess potential intraspecific taxonomic variation, as the geographic limits of the two subspecies of *G. moholi* are ill-defined in the southwestern limits of their range, i.e. northern South Africa (Pullen and Bearder 2013; Génin et al. 2016).

Materials and methods

Study areas and sampling

The samples in this study were obtained from the Biobank at the National Zoological Garden of South Africa (NZG), South African National Biodiversity Institute (SANBI). The samples originated from five locations, in two provinces in northern South Africa, namely Limpopo and Gauteng (Fig. 1). The sample composition was 15 from Limpopo (11 from the Mokopane Biodiversity Conservation Centre and four from the Lajuma Research Centre in the Soutpansberg Mountain Range, both within Limpopo) and 24 from Gauteng (19 from Pretoria Central in the vicinity of the NZG, four from Pretoria North East in the Roodeplaat Nature Reserve, and a single sample from Pretoria East in the vicinity of Lynnwood, all in Gauteng). Mokopane, Lajuma and Pretoria are each separated by more than 100 km. Pretoria Central and Roodeplaat are separated by a geographical barrier, the Magaliesberg Mountain (Long 2017), while Lynnwood and Pretoria Central are separated by an urban sprawl with a 10-km radius. Thus, we treat individuals from different localities as members of different populations. The Lynnwood sample was muscle tissue obtained from roadkill donated to the Biobank. The remaining samples were obtained as blood on FTA paper (Whatman) from the archives of the Biobank of the NZG. All samples originated from individuals in wild populations, including those within the vicinity of the NZG. The project was registered and approved as P17/07 by the Research and Ethics Committee (RESC) of NZG, SANBI. The samples had previously been archived as part of other projects (P13/04 and P16/17, NZG RESC; Long 2017) as well as part of the Biobank sample collection. The list of samples included in this study is indicated in Supplementary Table S1.

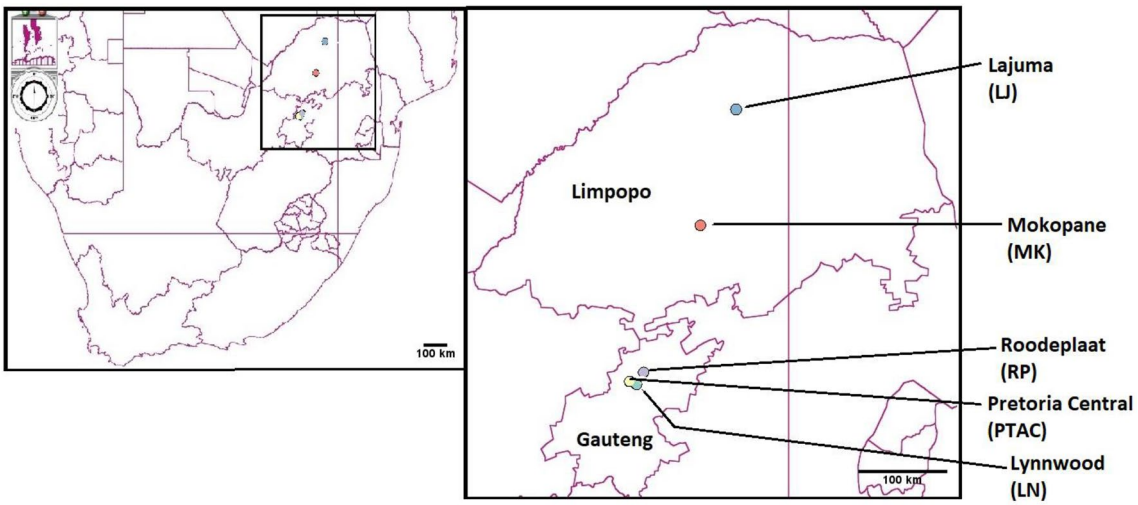

Fig. 1 *Left* Sample localities (*inset*) of 40 *Galago moholi* individuals in northern South Africa. *Right* The enlargement of the inset shows the provinces with the five sample localities, which are indicated by labels. Locality abbreviations are given *in parentheses*

DNA processing and analyses

DNA was isolated using the Zymo Tissue Mini Prep kit (Zymo Research, CA) following the manufacturer's instructions. Two mitochondrial regions, D-loop and *COI*, were amplified and sequenced as described in Phukuntsi et al. (2016). However, the annealing and extension times were reduced to 30 and 50 s, respectively, for D-loop. The sequences were manually edited using the program Molecular Evolutionary Genetics Analysis v10 (MEGAX) (Kumar et al. 2018). Each sequence was queried on the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov) using the Basic Alignment Search Tool plugin (Altschul et al. 1990) on MEGAX. The sequence with the GenBank accession number KC757396 (Finstermeier et al. 2013) was added to each alignment dataset as a reference sequence. *Galago senegalensis* and *Otolemur crassicaudatus* sequences were downloaded and were used as a sister taxon and outgroup, respectively, as these are the closest taxa to *G. moholi* (Pozzi et al. 2014). Each sequence dataset was aligned using the ClustalW (Larkin et al. 2007) plugin on MEGAX. The best models by log-likelihood analysis, using Akaike's information criterion (Akaike 1974), were determined to be the Kimura 2-parameter model for *COI* and the Hasegawa–Kishino–Yaho model (HKY) (Hasegawa et al. 1985), γ-distributed (HKY + G), for D-loop.

The phylogenetic structure among the individuals was explored by reconstructing phylogenetic trees using three methods, namely, distance, Bayesian and maximum likelihood, implemented in MEGAX, BEAST 2 (Bouckaert et al. 2014) and PHYML (Guindon et al. 2009), respectively. Haplotype networks were inferred by implementing the median-joining method (Bandelt et al. 1999) in Popart v1.7 (Leigh and Bryant 2015; http://popart.otago.ac.nz). Geographic distribution of haplotypes was drawn on a map using QGIS (https://www.qgis.org/en/site/). Population parameters were estimated from the D-loop dataset in Arlequin v3.5 (Excoffier and Lischer 2010), DNAsp v6 (Rozas et al. 2017), MEGAX and PopArt v1.7 to estimate levels of within- and between-population differentiation and genetic diversity. For population differentiation, only four of the five populations were used, with the exclusion of the lone sample from the Lynnwood locality. Genetic diversity estimates included pairwise distances, haplotype number and diversity. Measures of population differentiation included estimations of the proportion of the total genetic variation in a population relative to the total genetic variance of all the populations (F_{ST}), as well as analysis of molecular variance (Excoffier et al. 1992). A subset of data excluding males was also analysed to directly assess mtDNA gene flow through maternal lineages.

Results

The sequence labels for the successful amplifications are shown in Supplementary S1. Some sequences could not be successfully generated due to stochasticity and/or low DNA volume and quality. Thirty-seven *COI* sequences were successfully generated, and these were subsequently deposited in Genbank (accession numbers MW300617–MW300653). Alignment fragments for *COI* were 552 bp in length and included two variable sites and two parsimonious informative sites. Two haplotypes were observed based on the *COI* region (Fig. 2). The first haplotype (HapA) was observed in

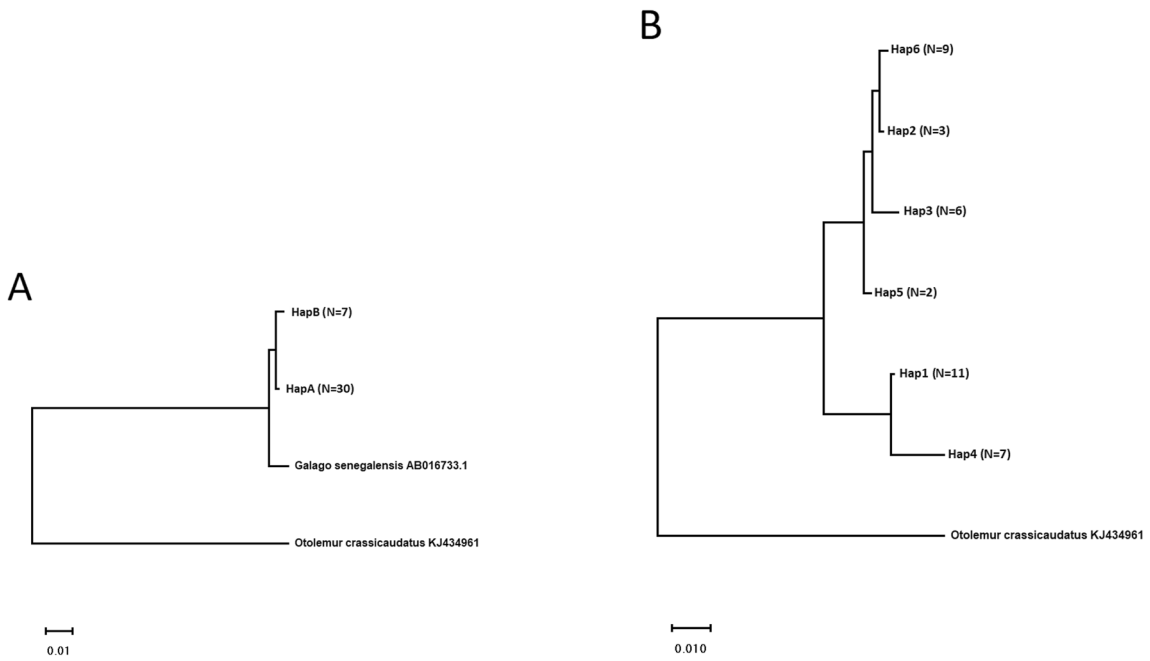

Fig. 2 Neighbour-joining trees showing genetic clusters of *G. moholi* from five localities in northern South Africa, based on two mitochondrial DNA (mtDNA) regions, the cytochrome oxidase subunit I gene (*COI*) (**a**) and displacement loop of the control region (D-loop) (**b**). The model of evolution used to infer each tree was Kimura 2-parameter for *COI* and Hasegawa-Kishino-Yano for D-loop. Two *COI* haplotypes, namely, HapA and HapB, and six D-loop haplotypes, namely, Hap1, Hap1, Hap3, Hap4, Hap5 and Hap6, are indicated. *N* Number of individuals

individuals from Mokopane, Lajuma and Pretoria (except for the Roodeplaat locality) while the second haplotype (HapB) was observed in individuals from Roodeplaat and Pretoria Central. The nucleotide diversity in the *COI* gene region was observed at 0.00193, while haplotype diversity was observed at 0.315 (Table 1). The pairwise difference between the two genetic groups in the *COI* region was 0.006 (0.6%).

Thirty-nine D-loop sequences were successfully generated and subsequently deposited in Genbank (accession numbers MW307832–MW307870). The length of the alignment fragments for D-loop was 358 bp and included 19 variable sites and 18 parsimonious informative sites. Six haplotypes were observed based on the D-loop region (Fig. 3). These D-loop haplotypes were subsequently labelled Hap1 to Hap6. Five of the six haplotypes (Hap1 to Hap5) were observed in the Pretoria Central locality while three haplotypes were observed in Mokopane (Hap2, Hap3 and Hap6). Only one haplotype each was observed in Lajuma (Hap6), Lynnwood (Hap5) and Roodeplaat (Hap4) localities, respectively. Thus, multiple haplotypes were shared among

Table 1 Polymorphic data on the displacement loop of the control region (*D-loop*) and cytochrome oxidase subunit I (*COI*) mitochondrial DNA (mtDNA) regions of *Galago moholi* from four populations in northern South Africa

	D-loop						*COI*					
	PTAC	MK	RP	LJ	LN	Total	PTAC	MK	RP	LJ	LN	Total
Number of samples per population	20	11	3	4	1	*39*	19	10	2	5	1	*37*
Number of sequences	20	11	3	4	1	*39*	19	11	2	5	1	*37*
Number of polymorphic sites	18	3	0	0	NA	*18*	2	0	0	0	NA	*2*
Total number of mutations	18	3	0	0	NA	*18*	2	0	0	0	NA	*2*
Average number of nucleotide differences	5.663	1.636	0	0	NA	*7.919*	0.818	0	0	0	NA	*0.631*
Nucleotide diversity	0.0239	0.0069	0	0	NA	*0.0334*	0.0025	0	0	0	NA	*0.0019*
Number of haplotypes	5	3	1	1	NA	*6*	2	1	1	1	NA	*2*
Haplotype diversity	0.653	0.636	0	0	NA	*0.814*	0.409	0	0	0	NA	*0.315*

PTAC Pretoria Central; *MK* Mokopane; *RP* Roodeplaat; *LJ* Lajuma, Soutpansberg; *LN* Lynnwood

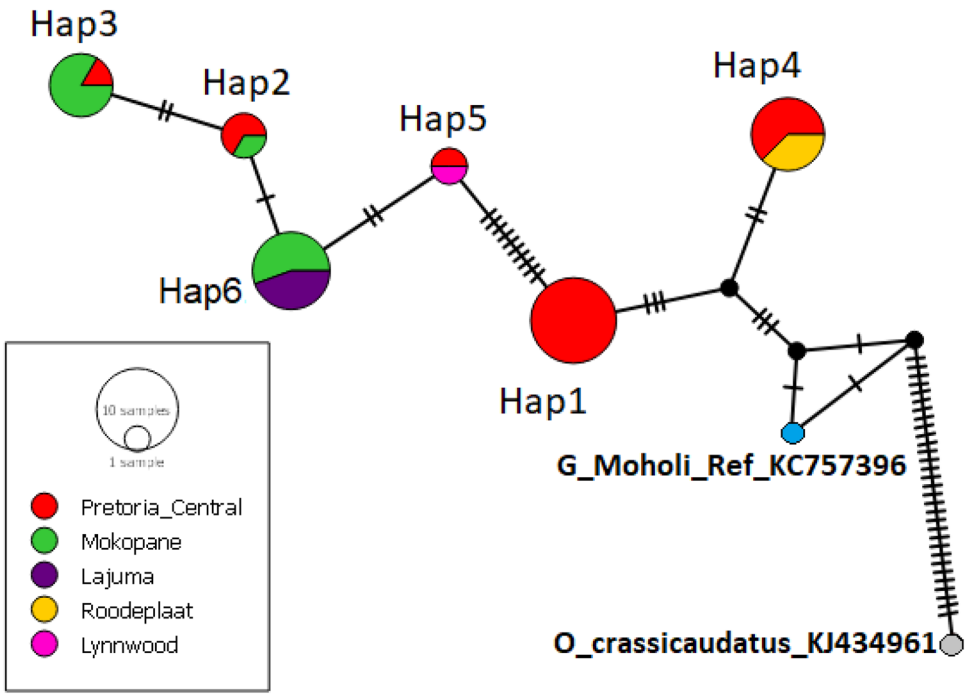

Fig. 3 Haplotype networks of the control region of the mtDNA (D-loop) in individuals from five localities in northern South Africa. *Colour-filled circles* show the frequency of the haplotypes, while the number of *strokes* across the branches indicates the number of mutational steps between haplotypes. *Black dots* denote missing haplotypes

different localities and provinces. Notably, the Pretoria Central population shared haplotypes with three localities. The geographic distribution of haplotypes is shown in Fig. 4. When only females were considered, haplotypes were still shared across populations (Supplementary S2).

Phylogenetic trees were drawn to assess the relationship between the D-loop haplotypes. The three trees yielded similar topology, so only the neighbour-joining tree is shown (Fig. 2). The Hap1 and Hap4 haplotypes nested together while the other four haplotypes formed a monophyletic group (Fig. 2). Individuals with the HapA *COI* haplotype had Hap1, Hap2, Hap3, Hap5 and Hap6, while individuals with the HapB *COI* haplotype had Hap4. Thus, the HapA *COI* genetic group observed was not reciprocally

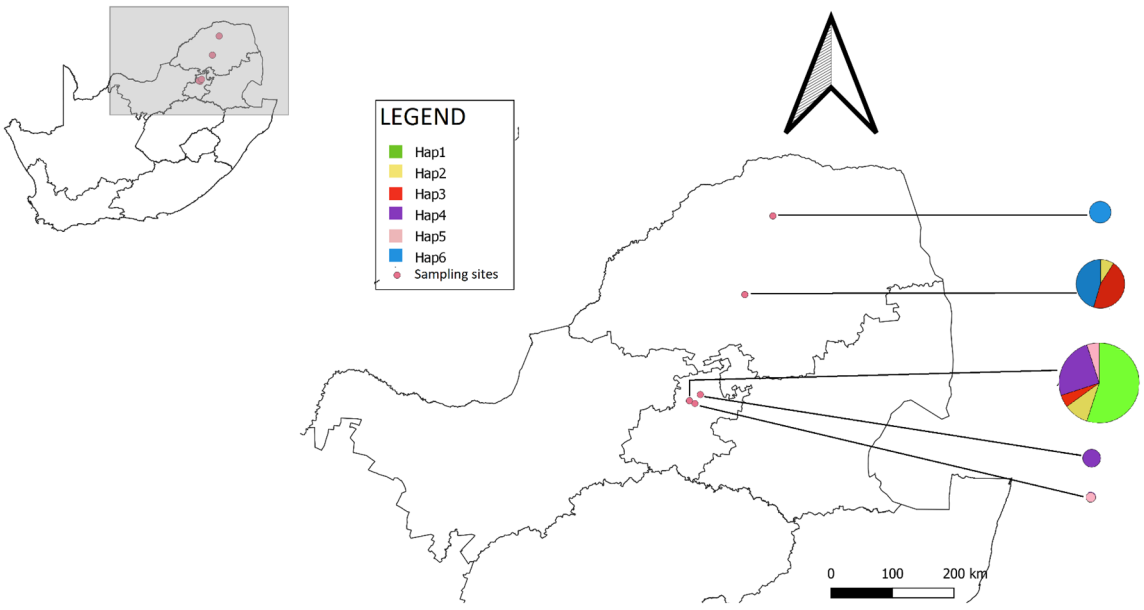

Fig. 4 Geographic distribution of haplotypes in five *G. moholi* populations in northern South Africa. Haplotypes were inferred from the D-loop region of the mtDNA

monophyletic with the HapB *COI* genetic group in the D-loop data. As a result, we considered the individuals as belonging to a single genetic unit.

Genetic diversity was determined within and between localities and haplotypes. The overall nucleotide diversity was 0.0334, while haplotype diversity was observed at $h = 0.841$ (Table 1). The Pretoria Central population had the higher number of segregating sites (18) and average number of differences (5.663) compared to Mokopane (3 and 1.636, respectively) (Table 1). Nucleotide diversity was 3.5 times higher in Pretoria Central (0.0239) than in the Mokopane population (0.0069). However, the haplotype diversity for the two populations was comparable (0.63 vs. 0.653). The individuals from the Lajuma and Roodeplaat localities did not exhibit any polymorphism, which may be attributed to low sample size ($n = 5$ from Lajuma and $n = 3$ from Roodeplaat) and/or short fragment length used in this study.

Genetic differentiation summary statistics among the populations are shown in Table 2. When the individuals were divided according to two regions (Limpopo and Gauteng provinces), a significant level of genetic differentiation among the regions (0.783; $P < 0.01$) and within populations (0.382; $P < 0.05$) was observed, while differentiation among populations was not significant ($P > 0.10$). The highest percentage of variation was due to differences among the groups; however, the proportion of variation among the populations was smaller than the variation within the populations. When only females were considered, only the differentiation between the regions was significant ($P < 0.001$), while the proportion of variation among populations was greater than within populations. On the other hand, variation within males among populations was higher than among populations, although differentiation among regions was significant ($P < 0.001$). The overall level of genetic differentiation was relatively high ($F_{ST} = 0.826$; Table 3). The lowest pairwise population differentiation was between Mokopane and Lajuma ($F_{ST} = 0.438$), followed by Pretoria Central and Roodeplaat ($F_{ST} = 0.520$). The highest was between Lajuma and Roodeplaat ($F_{ST} = 1.000$), followed by Mokopane and Roodeplaat ($F_{ST} = 0.950$) (Table 3). Differentiation between Gauteng populations was not significant, nor was differentiation between Limpopo populations; however, differentiation between any of the Limpopo and any of the Gauteng populations was significant (Table 3).

Table 3 Gene flow estimates (F_{ST}) among individuals of populations from two localities in Limpopo (MK and LJ) and two in Gauteng (PTAC and RP)

Population	RP	PTAC	LJ	MK
RP		0.08 (0.009)	0.04 (0.005)	0.03 (0.02)
PTAC	0.520		0.001 (0.001)	0.000 (0.000)
LJ	1.000*	0.747*		0.173 (0.011)
MK	0.950*	0.661*	0.438	

Proportion of the total genetic variance in a population relative to the total genetic variance of all the populations (F_{ST}) is shown *below the diagonal*. *P*-values are shown *above the diagonal* with SEs *in parentheses*

For other abbreviations, see Table 1

* $P < 0.05$

Discussion

Genetic divergence of 0.6% or less has been observed in the *COI* and/or *Cytb* regions between mammal subspecies and described evolutionary significant units (Ball Jr and Avise 1992; Luikart and Allendorf 1996; Tolley et al. 2006, 2008; Koh et al. 2012). On the other hand, genetic divergence in a South African bushbaby subspecies (*Otolemur crassicaudatus crassicaudatus*) was observed at over 1% in the *COI* region (Phukuntsi et al. 2020). Furthermore, in the present study, the two haplotypes observed in the *COI* trees are not reciprocally monophyletic in the D-loop trees (Fig. 2). Thus, it is likely that the observed sequence divergence in this study only reflects mitochondrial variation within subspecies and that the individuals in this study represent a metapopulation of a single subspecies. Nevertheless, intraspecific genetic variation between the two subspecies of *G. moholi* has not yet been investigated. Furthermore, our sampling

Table 2 Estimates of variation among groups, as well as between and among populations of *G. moholi* from five localities in northern South Africa, based on analysis of molecular variance of mtDNA D-loop haplotypes

Grouping	% Variation among groups	% Variation among populations	% Variation within populations	Total	Φ_{ST} (*P*-value)	Φ_{SC} (*P*-value)	Φ_{CT} (*P*-value)
Between regions (males and females)	64.90	13.40	21.70	100	0.783 ($P < 0.01$)	0.382 ($P < 0.05$)	0.649 ($P > 0.05$)
Between regions (females)	62.74	19.23	18.02	100	0.819 ($P < 0.001$)	0.516 ($P > 0.05$)	0.627 ($P > 0.05$)
Between provinces (males)	67.66	2.27	30.07	100	0.699 ($P < 0.001$)	0.070 ($P > 0.05$)	0.677 ($P < 0.001$)

Φ_{ST} Differentiation within populations among regions, Φ_{SC} differentiation between populations within regions, Φ_{CT} differentiation among regions

area is limited to the eastern limits of the species' distribution in South Africa. This highlights the importance of subspecies delineation, geographically and genetically, in population genetics studies, as suggested by other authors (Ball Jr and Avise 1992; Hopken et al. 2015). This also warrants further genetic investigation of *G. moholi* across the eastern (Mpumalanga province and Mozambique) and western (North West province and Botswana) distribution regions to determine the distribution of the divergent haplotypes and confirm that there is no other evolutionary significant unit and therefore no hybrid zone in South Africa.

Significant genetic structuring was observed in bushbaby populations in northern South Africa, with two divergent haplotype groups observed among the populations along a northern-southern division. Populations in Limpopo (north) are significantly different from populations in Gauteng (south), with two uniquely southern haplotypes (Hap1 and Hap4). However, a significant portion of the genetic differentiation was found between members of the same population rather than between members of different populations, attesting to limited geographic population structure. This suggests that there is historical genetic connectivity among populations in northern South Africa. The poor phylogeographic signal in the dataset may also be caused by the large number of haplotypes shared by the individuals from the vicinity of Pretoria Central with the other populations. It is highly likely that the high level of differentiation between the provinces was contributed mostly by the individuals with the HapB haplotype, as they were also the most divergent group among the genetic clades, while the populations in Lajuma and Roodeplaat showed complete lack of haplotype sharing. Two populations, one northern (Lajuma) and one southern (Roodeplaat) appear isolated, but this may just be an artefact of inadequate sampling. A previous study of *O. crassicaudatus* indicated that there is male-directed gene flow into the Lajuma region and therefore no barriers for strepsirrhine primates moving into the area (Phukuntsi et al. 2020).

On the other hand, individuals in the southern populations possessed haplotypes also found in the northern populations. We expected local fixation of haplotypes and larger divergences between distant populations because past fragmentation of habitats would have led to isolation of populations and less mitochondrial gene flow between populations due to the philopatric nature of *G. moholi* females (Mbora and McPeek 2010; Pullen and Bearder 2013). The large genetic divergence in Gauteng populations, in conjunction with the observed genetic connectivity between populations in both provinces and the low frequency of northern haplotypes in the southern populations, may be due to introgression of previously divergent lineages into the Gauteng area (Hopken et al. 2015; Morgan-Richards et al. 2017; Lipson et al. 2018). The level of differentiation within populations was significantly reduced when males were removed from the analyses. This may reflect the male-biased dispersal and philopatric nature of females in the species and may allude to dynamic male gene flow between adjacent populations. In a previous study of a population of *O. crassicaudatus*, a strepsirrhine primate with philopatric females, mtDNA heterogeneity was contributed exclusively by males while females where homogeneous (Phukuntsi et al. 2020). However, in this study, there are females sharing haplotypes among populations that are hundreds of kilometres apart or that are separated by geographic and infrastructural barriers. Therefore, it is likely that the observed genetic connectivity between distant populations in this study is a result of past migration events or human-mediated migrations, such as translocations, rather than dynamic gene flow between populations.

Intentional or unintentional translocation of individuals into the more southwestern regions may have facilitated introgression of divergent haplotypes (Sanaei et al. 2016; Ross et al. 2018). Translocation of primates, as well as other animals, may take place, but go undocumented, e.g. through rehabilitation efforts, or through the release/escape of pets and game, which is usually not reported to avoid an unfavourable response from the authorities (Lowe and Gardiner 1975; Moore et al. 2014). *G. moholi* individuals are actively poached for the pet trade, as well as for traditional uses (Masters and Génin 2016a). Populations of *G. moholi* originating from released and escaped individuals have been observed before in the southern regions of Pretoria, and even as far south as Johannesburg (Brulliard 2009; Fairly Wild 2019). However, little else is known or has been published regarding the trade and rehabilitation of South African bushbaby, despite evidence of this in the media, and this is an avenue for future research, especially considering the potential negative aspects of human-mediated migration into new environments.

Conclusion

In this study we aimed to investigate the presence of conservation management units, as well as determine the level of genetic connectivity in *G. moholi* populations in South Africa. We determined that the populations in this region belong to a single intraspecific unit. We observed considerable genetic connectivity among populations. Furthermore, we also observed a clear genetic substructuring contrasted by a weak phylogeographic signal in two mitochondrial gene regions, which contradicts a species that practices female philopatry. The complex genetic structure of populations observed in this study may be a result of past migration events affected by changing environments, as has been observed before in strepsirrhines as well in as other primates. On the other hand, the apparent gene flow between

populations may be better explained by anthropogenically engineered corridors such as unintentional translocations. However, we consider it necessary to revisit the intraspecific taxonomy and distribution of *G. moholi* in southern Africa using genetic tools. More comprehensive geographic sampling and use of autosomal genetic markers in future genetic assessments of strepsirrhine populations may help us to properly identify conservation management units, as well as determine biotic and abiotic factors that influence the current genetic diversity of the populations and will influence it in the future.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10329-021-00912-y.

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