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Localized population divergence of vervet monkeys (*Chlorocebus* spp.) in South Africa: evidence from mtDNA

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Abstract

Objectives—Vervet monkeys are common in most tree-rich areas of South Africa, but their absence from grassland and semi-desert areas of the country suggest potentially restricted and mosaic local population patterns that may have relevance to local phenotype patterns and selection. A portion of the mtDNA control region was sequenced to study patterns of genetic differentiation.

Materials and Methods—DNA was extracted and mtDNA sequences were obtained from 101 vervet monkeys at 15 localities which represent both an extensive (widely across the distribution range) and intensive (more than one troop at most of the localities) sampling strategy. Analyses utilized Arlequin 3.1, MEGA 6, BEAST v1.5.2 and Network V3.6.1

Results—The dataset contained 26 distinct haplotypes, with six populations fixed for single haplotypes. Pairwise P-distance among population pairs showed significant differentiation among most population pairs, but with non-significant differences among populations within some regions. Populations were grouped into three broad clusters in a maximum likelihood phylogenetic tree and a haplotype network. These clusters correspond to (i) north-western, northern and north-eastern parts of the distribution range as well as the northern coastal belt; (ii) central areas of the country; and (iii) southern part of the Indian Ocean coastal belt, and adjacent inland areas.

Discussion—Apparent patterns of genetic structure correspond to current and past distribution of suitable habitat, geographic barriers to gene flow, geographic distance and female philopatry. However, further work on nuclear markers and other genomic data is necessary to confirm these results.

Keywords

mtDNA; vervet; South Africa; microevolution; population genetics

INTRODUCTION

Widely distributed groups of interbreeding organisms that exhibit local phenotypic variation provide a special problem for taxonomists and evolutionary biologists. These widely ranging taxa have traditionally highlighted the importance of and difficulties inherent to understanding macro-level phylogenetics (e.g., at what level of variation, or according to what traits, do we draw the line between taxonomic units; Grubb 2006, Irwin et al. 2001), but of equal importance is regional variation and microevolutionary patterns that offer the first signs of local population divergence. This level of understanding is of especial interest within the Order Primates, as there are several taxa with expansive ranges and high local phenotypic diversity that often defy traditional attempts at classification. These taxa also often have very deep phenotyping, defined as a comprehensive analysis of variation in individual phenotypic components (Robinson, 2012), in multiple dispersed local populations (e.g., via long-term behavioral or morphological field studies), necessitating a more nuanced understanding of how regional variation may fit in with, or be distinct from, larger patterns of variation.

Vervet monkeys (*Chlorocebus sensu lato*), found throughout sub-Saharan Africa and in a human-mediated radiation in the Caribbean, occupy a wide array of ecological zones while exhibiting extensive phenotypic variation. Like the better-studied and also widely distributed baboons (*Papio*) and macaques (*Macaca*), the taxonomy of vervets is a matter of contention, and these disputes occur at regional as well as at more global levels. While Groves (2001) recognized vervets as belonging to six separate species based on visible phenotypic variation, Grubb *et al.* (2003) defined all vervets as belonging to a single species. More recent analyses of variation in vervet monkeys have focused on comparing individuals from the entirety of their range across sub-Saharan Africa. Cardini *et al.* (2007) found that the 3D geometric morphometrics of skull shape and size scaled clinally across the wider population, with distinct variations in size and certain skull shapes more prominent in certain geographic regions. Although geography and spatially-structured environmental variables – such as rainfall and seasonality – explained some of this localized variation, 60–80% remained unexplained. Further study by this group found localized variation within vervets to be of particular interest because of evident plasticity of vervets leading to rapid expansion into previous underutilized areas that could lead to localized adaptive response to microclimates; the authors characterized such local variation as ‘incipient stages in a process of evolutionary radiation’ (Elton *et al.* 2010). Large-scale genetic studies have also attempted to explain taxon-wide variation. Haus *et al.* (2013), using mitochondrial DNA (mtDNA) sequenced at the *cytochrome b* locus, found distinguishing differences between two East African and one South African *pygerythrus* populations (identified as *C. p. hilgerti*, *C. p. rufoviridis*, and *C. p. pygerythrus*, respectively), but did not sample sufficiently in southern Africa to look at finer-grained population distinctions in that region. Despite these more recent large scale studies in which South African vervet monkeys are grouped together into

one unit, researchers historically have noted many distinguishing characteristics that may separate South African vervet monkeys into phenotypically distinct evolutionary units (see Tables 1 and 2). The historic phenotypic distinctions are, however, not adequate for understanding the biological underpinning of population distinction.

Although studies of *Chlorocebus* have historically been conducted in the East African *aethiops* morph (e.g., Turner et al., 1997; Isbell et al., 2009; Seyfarth et al., 1980), a recent renaissance of interest in the taxon (for example, see Jasinska, et al., 2013) as a model for numerous traits of biomedical and evolutionary interest to humans – such as social learning (van de Waal & Whiten, 2012), immunodeficiency virus resistance (Ma et al., 2013; Ma et al., 2014), parasite ecology (Gaetano et al., 2013), sensory ecology (Cramer et al., 2013; 2014), sexual selection and genital allometry (Rodriguez et al., 2015, Rodriguez et al., *in press*), life history and morphology (Turner et al., 2014), thermoregulation (McFarland et al., 2014), and ethnoprimateology (Loudon et al., 2014) – has firmly relocated that nexus of interest to research groups focused on the *pygerythrus* morph in South Africa. Despite work having been done in this region for quite some time (e.g., Henzi & Lucas, 1980), and the ongoing deep phenotyping being conducted within the area, very little is yet known about local population processes. Although recent extensive taxonomic sampling has been able to compare East African to South African extensions of the *pygerythrus* range (Haus et al., 2013, Elton et al., 2010) they did not sample sufficiently in South Africa to address local population divergences with any resolution. Such finer scale work has been done in the East African *aethiops* morph, or grivets, in which evidence was found of mtDNA substructuring despite widespread monotypy in phenotype, perhaps as the result of demographic shifts due to climatic change (Shimada, 2000; Shimada et al. 2002). Earlier work by our group on SIV viral divergence that sampled intensively across South Africa gestured toward a complex phylogeographic history in the region (Ma et al., 2013), that warrants closer investigation of vervet genetic divergence in the area that has, until now, been sorely lacking. Knowledge of these microevolutionary processes are of increasing importance so that researchers may make evolutionary sense of – and in some cases control for – localized patterns of phenotypic divergence that are becoming increasingly apparent as deep phenotyping continues within this critically important area of the *Chlorocebus* radiation.

Here we present a first step in the process of evaluating population differentiation in vervet monkeys in South Africa by determining the level of genetic differentiation and patterns of connectivity within and between populations from a significant portion of the distribution range across the country. Our goal here is not to define taxonomic units *per se*, but rather to expand on and augment previous work on the population genetics of the taxon as a whole by elucidating localized population processes in an area of critical research value.

MATERIALS AND METHODS

Populations and samples

Results presented here are based on tissue samples collected from 101 vervet monkeys at 15 localities in South Africa (Fig. 1). These samples were collected as part of a larger collaborative project by a subset of the authors as well as the International Vervet Research Consortium. The goals of the Consortium were designed to elucidate aspects of the

population genetics, morphology and behaviour of vervet monkeys and establish repositories of biological material for future research projects (Jasinska, et al., 2013; Lorenz, et al., 2005). These samples have already been used in several studies of South African vervets cited above (Ma et al., 2013; Cramer et al., 2013; Gaetano et al., 2014; Loudon et al., 2014; Turner, et al., 2014). Our goal was to sample both extensively (widely across the distribution range) and intensively (more than one troop at most of the 15 localities).

South Africa is topographically divided between lowland coastal regions and an elevated interior. The coastal regions are warmer and more humid while some of the elevated regions have much colder temperatures. The following broad areas of the distribution range in South Africa were sampled: north (N), north-west (NW), north-east (NE), central (C), southern coastal belt with adjoining interior (SC) and northern coastal belt (NC). Sample sites, with indication of altitude, vegetation type (biome) and specific locality are detailed in Table 3. Localities sampled represented both lowland (lowveld) and highland (highveld) ecosystems. All maps used were created using the SANBI (South African National Biodiversity Institute) South African Biome map as template. Figure 1 also shows the location of the Drakensberg, a potential barrier to gene flow in vervets; and the Orange River system, whose riverine vegetation provides possible routes for dispersal through otherwise non-suitable habitat in the dry regions of South Africa.

Animals were trapped as described by Grobler and Turner (2010) and sedated. An ear punch or biopsy of approximately 5×3 mm was taken from each individual and then stored in 90% ethanol. As indicated above, we used this opportunity to collect materials that could be analyzed for other biological traits including morphological, serological, endocrinological and other biological variables. After sampling, the animals were placed in a recovery area where they were protected from predators as well as conspecific rivals.

All research protocols were reviewed and approved by the Institutional Care and Use Committee (IACUC) of the University of Wisconsin – Milwaukee (2000–2010) and the Inter-Faculty Animal Ethics Committee of the University of the Free State, South Africa (2006–2010). Permissions to conduct this research were granted by the South African Department of Environmental Affairs and the Provincial Environmental Affairs departments of the Free State, Mpumalanga, Limpopo, KwaZulu Natal, Eastern Cape and Gauteng Provinces. This project adhered to all South African legal requirements. The research adhered to the American Association of Physical Anthropologists Code of Ethics and the American Society of Primatologists Principles of Ethical Treatment of Non Human Primates.

Molecular marker

We used a 460 bp fragment of the mitochondrial D-loop (or CR), which included part of the hyper-variable region I (HVI) and the central conserved region of hyper-variable region II (HVII). This fragment overlaps approximately with positions 16303–16,569 in the *Homo sapiens* complete mitochondrion genome (GenBank no. ref NC012920.1). Fragment lengths of this size in this genomic region are consistently used to investigate genetic differentiation in populations of relatively recent divergence sampled across similar geographic scales (e.g.,

Lagothrix: Botero et al. *in press*; *Hylobates*: Whittaker et al., 2007). All sequences generated in this study are available in GenBank with accession numbers KP231259- KP231284.

Two DNA isolation kits, the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics) and the Qiagen QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) were used interchangeably. We followed the manufacturer's instructions, except that initial digestion was carried out overnight. The quality and quantity of extracted DNA was determined using a Nanodrop spectrophotometer.

Primer sequences were designed in collaboration with Inqaba Biotec and included Vervet-HVR-Fw (5'-CGT GCA TTA CTG CTA GC-3') and Vervet-HVR-Rev (5'-GTG TTG TGG GTT GGT TG-3'), which amplify a target of approximately 700 bp. PCR amplification was performed in 25 µl reaction with 25–100ng DNA, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1.5 U Super Therm GOLD Taq polymerase, 5 µM of each primer and 9.3 µl dH₂O. The cycle parameters consisted of 10 minutes at 95°C for initial denaturation, amplification for 40 cycles of 30 seconds at 94°C, 30 seconds annealing at 55–63°C (the annealing temperature for both primers was 57°C) and 1 minute extension at 72°C, with a final extension step of 10 minutes at 72°C, and with a 4°C incubation step to stop the reaction. Amplified products were viewed with a known size-standard ladder on a 1% agarose gel stained with GelRed™ (Biotium Inc., Hayward, CA, USA). PCR products of adequate quality were purified using the BioSpin PCR Purification Kit (BioFlux, Tokyo, Japan). Sequencing reactions were carried out using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Division, Perkin-Elmer, Foster, CA, USA). Sequencing reaction mixtures consisted of 2.5 µl BigDye Terminator v3.1 sequencing buffer (X5), 0.5 µl BigDye® Terminator mix, 1 µl of 2 mM primer, 4 µl dH₂O and 2 µl DNA template. Sequencing reactions were performed as follows: 2 minutes at 94°C for initial denaturation, amplification for 35 cycles of 15 seconds at 94°C, 10 seconds annealing at 53°C and 3 minutes at 60 min, with a 4°C incubation step to stop the reaction. Sequencing products were purified using the ZR DNA Sequencing Clean-up™ Kit (Zymo Research, Orange, CA, USA) and analyzed with an ABI 3130 Genetic Analyzer.

Analyses

Raw gene sequences were viewed and aligned using the software Geneious Pro 5.4 (Drummond et al., 2011). The sequences were trimmed to 460 bp to ensure uniform sequence lengths during analysis. DNA SP software (Rozas et al., 2003) was used to screen for duplicate haplotypes, and MEGA 6 software (Tamura et al., 2013) was used to find the model of nucleotide substitution that best fits the available data. The haplotype frequency distribution among samples was calculated using Arlequin v3.1 (Excoffier et al. 2005). Intrapopulation nucleotide diversity analysis was also determined for each sampled population, using the Arlequin software. Genetic differentiation among all pairs of populations was determined as P-distance, using Arlequin v3.1.

A phylogenetic tree was constructed using maximum likelihood procedures in MEGA 6, to identify the relationships between the sites sampled, after determining the model of nucleotide substitution that best describes the current data. Phylogenetic testing was done using the bootstrap method, with 1,000 bootstrap replications. To root trees during

phylogenetic analysis, we used sequences for *Chlorocebus tantalus* (GenBank accession number NC009748.1), *C. sabaesus* (JQ256913.1), *C. cynosuros* (JQ256915.1), *C. aethiops* (AY863426.1; NC007009.1). In addition, sequences of *C. pygerythrus* (NC009747.1; EF597501.1) were included to validate our results.

BEAST v1.5.2 (Drummond & Rambaut, 2007) software was used to infer a time-measured phylogeny and obtain estimates of divergence times for regional populations of vervet monkeys. Infiles for BEAST was created using BEAUti v1.5.2 software (part of the BEAST package). The model was calibrated using an estimated divergence time of 1.42MYA between *C. pygerythrus* and *C. tantalus*, from Guschanski et al. (2013). The 26 sequences of *C. pygerythrus* were aligned with the corresponding region on sequence NC_009748.1 version gi:156471205 of *C. tantalus* from GenBank, and all 24 haplotypes used for subsequent molecular clock based analyses. Parameters for this analysis were: an HKY model of substitution with heterogeneity among sites and gamma-distributed rate, an uncorrelated lognormal relaxed molecular clock, a chain length of 10,000,000 MCMC iterations and with trees thinned every 1,000 steps. The divergence date of 1.42MYA between *C. pygerythrus* and *C. tantalus* set as prior, with allowance for a standard deviation of 5%. Tracer v1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>) software was used to analyze of the MCMC molecular clock runs, to ensure that run of chains were long enough. Trees were inferred using maximum clade credibility and with mean node heights, using TreeAnnotator v1.7.3. Final phylogenetic trees were viewed using the FigTree v1.3.1 drawing tool (<http://tree.bio.ed.ac.uk/software/figtree/>).

A phylogenetic network was constructed with the software Network v4.6.1 (Fluxus Engineering, 2012), using the haplotype data obtained (polymorphic sites only). A median joining approach (Bandelt *et al.*, 1999) was used, and the number of mutational steps which most probably might have occurred between the linked haplotypes was calculated.

RESULTS

Results from DNA SP show 26 distinct mtDNA haplotypes in the 101 individuals sampled across 15 populations. Haplotype frequencies, haplotype diversities and nucleotide diversities are indicated in Table 4. Many populations had relatively small sample sizes, and six out of 15 populations were fixed for single haplotypes, which may introduce bias into diversity estimates. Of the populations that showed polymorphism, most haplotype diversity was observed in the C1 and C2 populations (0.978–0.733), followed by the NW2, C3, NE1 and NE2 populations (0.500–0.667) and finally, populations N1 and SC3 (0.250–0.400). Trends from nucleotide diversity suggested most diversity in NW2, NE1 and C1 (0.005–0.012), followed by the remaining polymorphic populations (0.001–0.003).

Differentiation between populations, expressed as pairwise P-distance among population pairs, is presented in Table 5. The majority of values (and associated p values) were indicative of significant ($p < 0.05$) differentiation among population pairs. Exceptions were between pairwise combinations of the populations from the central regions (C1 / C2 / C3) and between SC2 and SC3 in the southern coastal belt.

The pattern of substitution in the dataset was best described by a Hasegawa-Kishino-Yano (HKY+G) model of nucleotide substitution, with the Kimura 2-parameter model the next most likely. A maximum likelihood tree, based on the HKY+G model, is presented in Figure 2. In the HKY-ML tree, populations are grouped into three broad clusters. Haplotypes of vervet monkeys from the north-western, northern and north-eastern parts of the country, including NW1, NW2, N1, NE1, NE2 and C4; as well as the coastal NC1 population from northern coastal belt, form a distinct cluster (designated Cluster I), with 96% bootstrap support. A second cluster (Cluster II) contains a tight group of all the vervet monkeys from C1, C2 and C3 in the central area, with 100% bootstrap support. A third cluster (Cluster III) contains the populations from the southern coastal belt (SC1–SC5) and the most southern population from the northern coastal belt (NC2), with 100% support.

Clustering in a phylogenetic tree created using Bayesian evolutionary analysis (BEAST) mirrored the results from MEGA 6 (Fig. 3). Analyses of molecular clock runs to verify posterior estimates showed that all ESS (effective sample size) values were above 300. Divergence times between the main clusters of vervet monkeys (with 95% highest posterior density - HPD) were: 1.099 MYA (0.612–1.463) between Cluster III and Cluster I / Cluster II; and 0.815 MYA (0.400–1.211) between the latter two clusters.

A minimum spanning network, showing relationships among haplotypes, is presented in Figure 4. The network is superimposed on a map of South Africa, with nodes arranged to represent the best fit between genetic clusters, sampling localities and landscape features. The number of mutational steps separating haplotypes are indicated on the network. Results broadly mirror the outcome of the phylogenetic tree. Populations from the north-western, northern and north-eastern localities cluster together, as well as those from NC1. A second group contains animals from the central region exclusively. A third group, that branches from the NW haplotypes, contains all individuals from the southern coastal belt, as well as the NC2 population.

DISCUSSION

Patterns of connectivity dictated by landscape features

Phylogenetic tree building, haplotype network and pairwise differences consistently divided the 15 sample localities into three broad clusters, containing (I) individuals from the north-western, northern, north-eastern regions and the northern-coastal region; (II) individuals from the central area, and (III) individuals from the southern coastal regions and adjoining areas. In keeping with previous arguments related to microevolutionary patterns in vervets, we attempt to explain these local divergences using current and historic landscape features and long term climatic perturbations in combination with behavioural factors such as habitat preference to construct the most likely model of historical connectivity among the localities. Cooke (1964), Eeley *et al.* (1999), Lawes (1990) Lawes *et al.* (2007) and Sithaldeen *et al.* (2009) have postulated a broad spatial and temporal scale model of dispersal of mammal species into Southern Africa which recognizes migration along a north-east/south-west axis from East Africa into Southern Africa. Using this model of colonization in a north to south direction, we postulated that suitable habitats, including vegetation along river courses and homogeneous stretches of Savannah and Thicket biomes, were features that could promote

connectivity (see Mucina and Rutherford, 2006 for a discussion of South African biomes). Areas of unsuitable habitat including open grassland (without river courses) and major mountain ranges, as well as absolute geographical distance, were regarded as potential barriers to gene flow. Vervet monkeys are probably not affected by most physical landscape features, and their presence now in mountainous Drakensberg region of South Africa suggest that they can find ways to traverse some of the mountain ranges, especially those at lower elevations and with suitable corridors. Mountains with higher elevations associated with lower temperatures would however be more difficult to cross because of unfavourable environmental conditions and a lack of resources.

For the past two million years, southern Africa has experienced approximately twenty glacial/interglacial cycles each lasting about 100,000 years (Chase and Meadows, 2007; Lawes, 1990; Eeley et al., 1999; Faith, 2013). These cycles are characterized by large scale environmental change. During periods of glacial maxima the climate is cooler and more arid leading to a reduction of forests and expansion of grasslands. The opposite is true of hyperthermal periods between periods of ice sheet expansion (Eeley et al., 1999). This expansion and contraction of the forests and the grassland has had profound effects on the distribution of animal populations, including early hominins (Segalen and Lee-Thorpe, 2007). De Menocal (1995) hypothesized that the development of periodically cooler and drier African conditions after 2.8 MYA, and their subsequent intensification after 1.7 and 1.0 MYA, may have resulted in expanded grasslands and led to ecological fragmentation and genetic isolation in a variety of species. Meadows and Linder (1993) and Faith (2013) also suggest that these extensive grasslands are ancient and predate permanent occupation by people and human agency. Although, the grasslands are maintained by burning, Meadows and Linder (1993) believe that this is natural burning and the grasslands are not solely the result of anthropogenic forces. Lawes (1990) and Lawes et al., (2007) postulated that extreme changes in climate and vegetation during the last 100,000 years especially are responsible for the current distribution of samango monkeys, blue and red duiker, giant rats, nyala and other mammals found in forest environments. Lawes (1990) and Lawes et al., (2007) describe the distribution of *Cercopithecus mitis* and relate subspeciation in this species to expansion and contraction of forests. Sithaldeen et al. (2009) suggest that the influence of glacial climate change extends further back than the last glacial maxima, and that multiple cycles of climatically mediated speciation molded current patterns of diversity in the chacma baboon. We propose that the development of climate-driven areas of unsuitable habitat may have isolated vervet monkeys in the southern coastal belt from the closest conspecific populations in South Africa, starting long before the most recent glacial period. Furthermore, Scott (2002) found that areas with a notable C4 component (which equates to potentially suitable vervet habitat), characterized by summer rainfall, high temperatures, growth during warmer period, did not expand southwards towards the Cape winter-rain region during the last glacial maximum. An area of unsuitable habitat for vervet monkeys could thus have persisted despite temperature and vegetation fluctuations under glacial and interglacial conditions in southern Africa, resulting in an extended barrier to gene flow.

The mtDNA results indicate that Cluster I appears to form a distinct group that follows the current Savannah biome of South Africa, and includes vervet monkeys from the NW1, NW2, N1, NE1, NE2 and C4 localities, as well as the coastal NC1. This same pattern, which includes a northern coastal population with an otherwise inland group, has also been described in impala (*Aepyceros melampus*) by Schwab et al. (2012) and other South African mammals (Lawes et al., 2007). Cooke (1962), Van Zinderen Bakker (1978) and Scott (2002) suggested that evergreen forest may have stretched from the northern region (NE1/NE2) into KwaZulu-Natal (NC1) before and after the last glacial maxima, 16,000–18,000 years before present. Such a forested landscape would provide multiple routes for migration along the Lowveld and coastal belt on the eastern side of the country. These migration routes, however, might have much greater time depth. However, the presence of the Orange River system within the area defined by Cluster 1 provides a plausible mechanism to explain patterns of connectivity in the north-western areas of the country in an area that spans three biomes (Savannah, Grassland and Nama Karoo – Fig. 1). Areas of suitable habitat along the course of the Orange River may provide a suitable avenue for gene flow among the NW1 and NW2 populations, stretching to the C4 locality further east. The most likely avenue for gene flow between these north-western populations (NW1 and NW2) and the clusters furthest to the north (N1, NE 1 & 2) is unclear from the current sample set, but the avenue of connectivity most likely follows the Savannah areas of north-western South Africa and through Botswana, including the banks of the Limpopo River system, which divides South Africa from Botswana and Zimbabwe.

In Cluster II, populations from the central area are separated from those sampled further north by a 31 bp difference in the mtDNA control region, which is high relative to other inter-cluster distances observed in this study. Many of the populations in Cluster I and all populations in Cluster II are located in similar high lying areas of the central plateau of South Africa (1,260 – 1,470 m above sea level). Avenues for gene flow between northern and central subpopulations were probably restricted to pockets of suitable habitat in an otherwise sparsely covered grassland biome. Areas of suitable habitat that occur at irregular intervals only could explain the high level of divergence between these two groups. This relatively large sequence divergence argues for relatively low contemporary gene flow. The populations from C1, C2 and C3 are also among the only ones studied that do not show significant differentiation from each other (as seen in P-distances - Table 1), which suggests close identity between these three populations from the grassland biome.

In the southern part of the distribution range, along the southern coastal belt, populations form a distinct cluster (Cluster III) and there is evidence to suggest gene flow between populations from SC1 to SC4 as well as NC2. Such an exchange of animals is feasible considering that all these localities share and are linked by a similar combination of coastal belt and Thicket biomes, interspersed with grassland. Expansion to the north might be limited by the Tugela River system. We note that the distribution of this genetically relatively distinct cluster of vervet monkeys from the southern coastal belt also mirrors the proposed distribution of the subspecies *C. pygerythrus cloeti*, described by various authors as distinct from *C. pygerythrus pygerythrus* found in the rest of the country (Table 1). In relation to other identified groupings, the southern coastal belt lineage is most closely

associated with the NW1-2 cluster centred on the Orange River system. A 32 bp difference separates the latter monkeys from those on the southern coastal belt and estimated divergence time between the two clusters is approximately 1.213 MYA. Both the divergence time and number of base pair differences are high in the context of the current results, but does not appear extensive enough relative to other larger-scale studies to warrant taxonomic distinction. Indeed, such a distance mirrors the typical variation seen within rather than between the larger mitochondrial haplotype clusters delineated by Haus *et al.* (2013) when comparing across the genus (with the notable exception of their C1 and C2 haplotype clusters, which show a 16 bp difference between *C. tantalus* and *C. aethiops* clusters); however, the substitution rates between the *cyt b* sequence used in Haus *et al.* (2013) and the HVI/HVII sequence used here may vary (e.g., Wakely 1993) and so such direct comparisons are limited in their utility. Genetic differentiation between central South Africa and the southern coastal belt region (and adjoining areas) was recently also described for haplotypes of the simian immunodeficiency virus (SIV) carried by vervet monkeys (Ma *et al.*, 2013). The latter study was based on a subset of the animals used in the current study, and these authors calculated a divergence date of 0.5 MYA years based on diversity in the *Env* gene in the virus. Given the margin of error intrinsic to molecular divergence time estimates, these two values are broadly comparable and any of the values (0.5–1 MYA) support the hypothesis of extended isolation.

The southern coastal belt group contains an unexpected three individuals with the C2 haplotype from much further north. Occasional migration between these localities, along geographic features offering suitable habitat, cannot be excluded completely. However, the association between C3 and the SC populations seems unlikely, given the clustering of the C4 population which is between the two areas. One possible explanation may be that these are translocated animals, potentially released pets.

The preceding descriptions of connectivity suggest that vervet monkeys along the greater Indian Ocean coastal belt may not have a homogeneous origin (with a break between NC1 and NC2), and represent a mix of radiation from the north-east and south-west. This distribution would parallel that found in *C. mitis* (Lawes, 1990), however, *C. mitis* are much more restricted in forest habitat distribution ultimately leading to a greater differentiation among populations (Lawes, 1990; Dalton, unpublished results). The finer distribution of genetic variation along the coastal belt should thus be further investigated.

Genetic diversity within populations

The high level of diversity observed between geographically isolated populations is in many cases not matched by within-population levels of diversity. Six of the 15 regional populations studied were fixed for single haplotypes. This pattern can most likely be explained by the sex-linked nature of migratory patterns found in vervet monkeys, in common with many Old World monkeys (OWM). Cercopithecine primates, including vervet monkeys, are known to show strong female philopatry with dispersal patterns primarily based on the movements of males (Melnick and Pearl, 1987; Pusey and Packer, 1987). Such biased dispersal will result in the relatively even distribution of nuclear DNA throughout the populations, but with mtDNA diversity showing strong homogeneity on the population level

and with well structured inter-population variation. It is thus expected that more distinct clustering patterns will be observed when analysing mtDNA compared to work based on nuclear DNA (Haus et al., 2013). Another possible factor to explain strong patterns of mtDNA-based differentiation is the difference in effective population sizes for nuclear DNA and mtDNA. The haploid nature of mtDNA, as well as it being maternally inherited, leads to an effective population size that is four times smaller compared to autosomal nuclear DNA. The influences of genetic drift and population bottlenecks will thus be more clearly expressed, with large inter-population differentiation, when working with mtDNA (Birky et al., 1983). Finally, the effect of expanding and contracting areas of suitable habitat before and after glacial maxima, and pluvial precipitation, should be considered. It is possible that some extant populations were confined to smaller refuges after a contraction of suitable habitat, leading to drift between populations and the lack of diversity observed within many of the populations studied. Such population history effects may not be addressed with mtDNA, but may be investigated with larger-scale population genomic analyses (Svardal et al., 2014).

Conclusion

The genetic structure of vervet monkeys in South Africa, as determined from mtDNA control region sequences, can be understood through an assessment of the current and historic distribution of suitable habitat, geographic barriers to gene flow, geographic distance and female philopatry. Although some additional data on routes of gene flow may follow from additional work that provide better coverage, there is strong support for the basic premises of a north-western / northern / north-eastern group, a central group and a southern coastal belt group. This pattern partially overlaps with a previous classification of vervet monkeys in South Africa into *pygerythrus* (northern) and *cloeti* (southern coastal belt) populations (Table 1). This work cannot support a phylogenetic distinction between the two groups, but instead indicates that there is some genetic distinction between them. Further work on patterns of genetic variation should consider a number of aspects. (i) The adaptive significance of the observed genetic differences needs to be investigated, and association studies can contribute to a better understanding on the nature of possible adaptive variation. It would be premature to assume that genetic structure based on differences at one specific locus, specifically within the mitochondrial genome, is necessarily correlated to adaptations which will make vervet monkeys able to survive in narrow habitat ranges only. In future, the deep phenotyping currently being conducted in South African vervet monkeys should be linked more explicitly to genomic markers to assess potential adaptive links to population divergence patterns. (ii) The current mtDNA data should be supplemented by nuclear genomic data. It is known that studies based on mtDNA and nuclear DNA markers respectively can result in conflicting outcomes when studying genetic structure (Melnick and Hoelzer, 1992; Shimada, 2000; Tosi *et al.*, 2003). In a recent paper, Zachos *et al.* (2013) warned against the danger of taxonomic inflation, based on assumptions from neutral mtDNA variation. It is thus crucial that the current data from mtDNA should be supplemented by results from nuclear genomic markers to elucidate the complete pattern of genetic structure in vervet monkeys across South Africa. Such whole genome data will soon be available for these populations, and will be used to refine these conclusions (Svardal et al., 2014). (iii) Although sampling for the current study provides

very good coverage of the distribution range of vervet monkeys in South Africa, additional coverage of the area will contribute to a further improved view of historic and contemporary gene flow in these primates. In particular, the potential amalgamation of animals from very different migratory routes in the coastal belt should be investigated using fine-scale sampling between NC1 in the North down to the SC2-4 populations. This work can only be improved by the efforts of current research teams such as the International Vervet Research Consortium (Jasinska, et al., 2013) and other vervet research groups that continue both broad and locally deep sampling.

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Fig. 1.

Distribution of sampling localities in the north-western, northern, north-eastern, central and coastal belt regions of South Africa. Map also shows (i) the major biomes of South Africa; (ii) the Drakensberg mountain range (black dotted line); (iii) the Orange River system, which provide a route for dispersal through otherwise unsuitable habitat (solid black line, central); and (iv) the Tugela River (solid black line, coastal). Map adapted from the SANBI map of South African biomes.

Fig. 2.

Consensus tree showing phylogenetic relationships among 26 haplotypes of 101 vervet monkeys, and seven sequences from GenBank, based on mtDNA control region sequences. Numbers at the branches are the posterior credibility values indicating proportion of trees containing the inferred nodes. Haplotype names include localities where found. Clusters I–III show main clusters identified through ML analysis.

Fig. 4. Minimum-spanning network between 26 haplotypes from 101 vervet monkeys, superimposed on a map of South Africa. Nodes represent haplotypes, with the size of each node proportional to the number of individuals that share that haplotype. Numbers used to denote haplotypes follow Table 4. Numbers next to lines are used to indicate the number of mutations between nodes; where no number is given, only a single mutational step is involved. Red squares (nodes) indicate hypothetical intermediate haplotypes revealed during haplotype construction.

Table 1

Historical taxonomic divisions of vervet Monkeys in Southern Africa.

# samples	Species:	Subspecies:						
		<i>rufoviridis</i> 31	<i>ngamiensis</i> 11	<i>marjoriae</i> 8	<i>helveticus</i> 14	<i>pygerythrus</i> 52	<i>cloeti</i> 9	<i>cynosuroides</i> <i>silaceus</i>
Pocock, 1907	<i>C. pygerythrus</i>	x				x		
Fitzsimmons, 1919	<i>C. pygerythrus</i>	x				x		
Schwarz, 1928	<i>C. aethiops</i>	x				x		
Shortridge, 1934	<i>C. aethiops</i>		x		x	x		x
Roberts, 1951	<i>C. aethiops</i>	x	x	x	x	x	x	
Elleman, 1953	<i>C. aethiops</i>	x	x	x	x	x	x	
Dandelot, 1959	<i>C. pygerythrus</i>	x	x	x	x	x		
Hill, 1966 ²	<i>C. pygerythrus</i>	x	x	x		x	x	
Dandelot, 1971	<i>C. pygerythrus</i>	x	x	x	x	x		
Kingdon, 1971	<i>C. aethiops</i>					x		
Ansell, 1978	<i>C. pygerythrus</i>					x		x
Napier, 1981	<i>C. aethiops</i>	x	x	x	x	x		
Smithers, 1971 ¹ , 1986	<i>C. aethiops</i>	x	x		x	x		
Meester, 1986	<i>C. aethiops</i>	x	x	x	x	x	x	
Groves, 2001	<i>C. pygerythrus</i>				x			
Grubb, 2003	<i>C. aethiops</i>						x	
Skinner & Chimimba, 2005	<i>C. pygerythrus</i>	x	x	x	x	x	x	
Location		N Mozambique	N, NE Botswana	S Botswana	N Namibia, NW Botswana, SW Zambia	Indian Ocean Coastal belt of South Africa	NW and N South Africa, N coastal belt	W. Africa Mozambique probably rufoviridis

¹ Botswana only;

² Superspecies

Diagnostic criteria for separation of vervet monkeys in Southern Africa (Hill, 1966; Groves, 2001)

Table 2

	Whiskers:	Frontal band:	Caudal tuft:	Feet:	Back:	Tail tip:	Face:	Scrotum:	notes
rufoviridis	long with yellow and black	white	rust colored	---	reddish brown speckled with black	black	brownish black	dark blue	
ngamiensis	---	narrow	---	blackish	olive gray with black	black	---	---	similar to helvescens
marjoriae	---	---	---	blackish	paler than cloeti ? If immature	blackish	---	---	
helvescens	---	---	---	---	---	---	---	---	cynosuros to Hill
pygerythrus	Greyish, long	broad white, circles face	no tuft, red	black	Grayish olive	Black	black	bright greenish blue	
cloeti	---	---	---	---	---	---	---	---	larger teeth

Table 3

Distribution of sampling sites in South Africa, with abbreviation, number of troops sampled, altitude (High: 765–1400m; Low: 0–540m), locality and Province and with vegetation zone.

Region:	Site:	n Troops:	Altitude:		Geographic locality:	Biome:
			High:	Low:		
Northern	N1	2	X		Central Limpopo Province (Polokwane)	Savannah
North-West	NW1	2	X		Orange River, Northern Cape	Nama-Karoo
	NW2	2	X		Kimberley, Northern Cape	Grassland / Nama-Karoo
North-East	NE1	2		X	Mpumalanga Lowveld, below escarp	Savannah
	NE2	2	X		Mpumalanga Highveld, above escarp (Blyde River)	Grassland*
Central	C1	1	X		Northern Free State, on Vaal River (Parys)	Grassland*
	C2	?	X		North-Western Free State, on Vaal River (Bloemhof)	Grassland*
	C3	2	X		Central Free State (Soetdoring NR)	Grassland*
	C4	?	X		Southern Free State, Gariep dam area	Grassland*
Southern Coastal belt	SC1	1		X	Oribi (KwaZulu Natal)	Coastal belt
	SC2	?		X	Shamwari (Eastern Cape)	Thicket / Coastal belt
	SC3	2		X	NMMU Campus (Eastern Cape)	Coastal belt
	SC4	2		X	Baviaanskloof (Eastern Cape)	Thicket
	SC5	1	X		Tsolwana NR (Eastern Cape)	Grassland*
Northern Coastal belt	NC1	2		X	St Lucia (KwaZulu Natal)	Coastal belt
	NC2	2		X	Thorny Park / Blythdale (KwaZulu Natal)	Coastal belt

* Regions within the Grassland biome include areas of suitable habitat along river courses, interspersed with areas of open and unsuitable habitat.

Table 4

Populations sampled (see text for abbreviations used), with sample size (n) below population names, haplotype names and GenBank Accession numbers (columns 1–2), haplotype frequencies in each population, haplotype diversity and nucleotide diversity.

Haplotype:	GenBank no.:	NW1 (10)	NW2 (4)	N1 (8)	NE1 (3)	NE2 (6)	C1 (10)	C2 (6)	C3 (4)	C4 (11)	NC1 (10)	NC2 (5)	SC1 (4)	SC2 (6)	SC3 (5)	SC4 (9)
Hap101	KP231259	-	-	0.875	-	-	-	-	-	-	-	-	-	-	-	-
Hap102	KP231260	-	-	0.125	-	-	-	-	-	-	-	-	-	-	-	-
Hap103	KP231261	-	0.750	-	-	-	-	-	-	1.000	-	-	-	-	-	-
Hap104	KP231262	-	0.250	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap105	KP231263	1.000	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap106	KP231264	-	-	-	0.333	-	-	-	-	-	-	-	-	-	-	-
Hap107	KP231265	-	-	-	0.667	-	-	-	-	-	-	-	-	-	-	-
Hap108	KP231266	-	-	-	-	-	0.100	-	-	-	-	-	-	-	-	-
Hap109	KP231267	-	-	-	-	-	0.100	0.500	-	-	-	-	-	-	-	-
Hap110	KP231268	-	-	-	-	-	0.100	-	-	-	-	-	-	-	-	-
Hap111	KP231269	-	-	-	-	-	0.200	0.333	0.750	-	-	-	-	-	-	-
Hap112	KP231270	-	-	-	-	-	0.100	-	-	-	-	-	-	-	-	-
Hap113	KP231271	-	-	-	-	-	0.100	-	-	-	-	-	-	-	-	-
Hap114	KP231272	-	-	-	-	-	0.100	-	-	-	-	-	-	-	-	-
Hap115	KP231273	-	-	-	-	-	0.100	-	0.250	-	-	-	-	-	-	-
Hap116	KP231274	-	-	-	-	-	0.100	-	-	-	-	-	-	-	-	-
Hap117	KP231275	-	-	-	-	-	-	-	-	-	-	-	-	1.000	0.800	-
Hap118	KP231276	-	-	-	-	-	-	-	-	-	-	-	-	-	0.200	-
Hap119	KP231277	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000
Hap120	KP231278	-	-	-	-	-	-	0.167	-	-	-	-	-	-	-	-
Hap121	KP231279	-	-	-	-	-	-	-	-	-	1.000	-	-	-	-	-
Hap122	KP231280	-	-	-	-	-	-	-	-	-	-	0.400	-	-	-	-
Hap123	KP231281	-	-	-	-	-	-	-	-	-	-	0.600	-	-	-	-
Hap124	KP231282	-	-	-	-	-	-	-	-	-	-	-	1.000	-	-	-
Hap125	KP231283	-	-	-	-	0.667	-	-	-	-	-	-	-	-	-	-
Hap126	KP231284	-	-	-	-	0.333	-	-	-	-	-	-	-	-	-	-

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Haplotype:	GenBank no.:	NW1 (10)	NW2 (4)	N1 (8)	NE1 (3)	NE2 (6)	C1 (10)	C2 (6)	C3 (4)	C4 (11)	NC1 (10)	NC2 (5)	SC1 (4)	SC2 (6)	SC3 (5)	SC4 (9)
		0.0	0.500	0.250	0.667	0.533	0.978	0.733	0.500	0.0	0.0	0.600	0.0	0.0	0.400	0.0
		0.0	0.012	0.001	0.009	0.001	0.005	0.003	0.001	0.0	0.0	0.001	0.0	0.0	0.001	0.0

Table 5

Distances among pair-wise combinations of 16 vervet monkey populations, expressed as p-distance. Values in bold indicate a lack of significant differentiation ($p > 0.05$).

	NW1	NW2	N1	NE1	NE2	C1	C2	C3	C4	NC1	NC2	SC1	SC2	SC3
NW2	0.513													
N1	0.992	0.887												
NE1	0.861	0.331	0.930											
NE2	0.983	0.825	0.893	0.882										
C1	0.966	0.912	0.961	0.931	0.949									
C2	0.988	0.922	0.983	0.948	0.974	0.034*								
C3	0.996	0.915	0.991	0.949	0.984	0.077*	0.281*							
C4	1.000	0.271	0.993	0.841	0.986	0.968	0.989	0.997						
NC1	1.000	0.873	0.994	0.941	0.988	0.972	0.990	0.997	1.000					
NC2	0.994	0.922	0.990	0.953	0.984	0.957	0.978	0.987	0.995	0.996				
SC1	1.000	0.926	0.995	0.959	0.991	0.961	0.983	0.994	1.000	1.000	0.905			
SC2	1.000	0.942	0.996	0.970	0.993	0.965	0.986	0.996	1.000	1.000	0.942	1.000		
SC3	0.997	0.929	0.992	0.958	0.987	0.960	0.981	0.990	0.997	0.997	0.896	0.929	0.040*	
SC4	1.000	0.958	0.997	0.979	0.995	0.971	0.989	0.997	1.000	1.000	0.969	1.000	1.000	0.938