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Broad-scale genetic assessment of Southern Ground-Hornbills (*Bucorvus leadbeateri)* to inform population management

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A R T I C L E I N F O

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A B S T R A C T

The Southern Ground-hornbill (SGH) (*Bucorvus leadbeateri*) is considered an umbrella species for biodiversity conservation in savannah biomes since they require large territories and significant protection measures that help to conserve a wide range of biodiversity with similar savanna and grassland requirements. Declines of the species are attributed to low reproductive rates coupled with multiple anthropogenic threats, including secondary poisoning, and persecution. Little is known about connectivity and population structure of SGH populations in Africa, south of the equator. Knowledge of population differentiation is needed to ensure that targeted conservation management plans can be implemented to slow population declines and ensure survival of the species. To inform a long-term conservation strategy, we investigated the broad-scale population structure of Southern Ground-hornbill across their sub-equatorial range. Our study based on 16 microsatellite loci identified moderate variation (average of 5.889 alleles per locus and a mean observed heterozygosity of 0.546) similar to other long-lived avian species. In contrast, mitochondrial DNA sequences analysis identified low diversity (Hd = 0.3313, $\pi = 0.0015$). A Bayesian assignment approach, principal component analysis, analysis of molecular variance and phylogenetic analysis identified weak to moderate population structuring across long distances and mitochondrial data showed a shallow phylogeny. Restriction to long-distance dispersal was detected that could not be attributed to isolation by distance, suggesting that other factors, such as their dispersal biology, are shaping the observed genetic differentiation. Although our study does not support the designation of populations as independent conservation units, we advocate that population management should continue to follow the Precautionary Principle (mixing founders from the same range state, rather than allowing mixing of founders from the extremes of the range) until there is scientific certainty. Following further research, if no independent

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conservation units are detected, then the global captive population can contribute to reintroductions across the range. In the wild, populations at the edge of the species range may need additional management strategies and gene flow should be promoted between neighbouring populations.

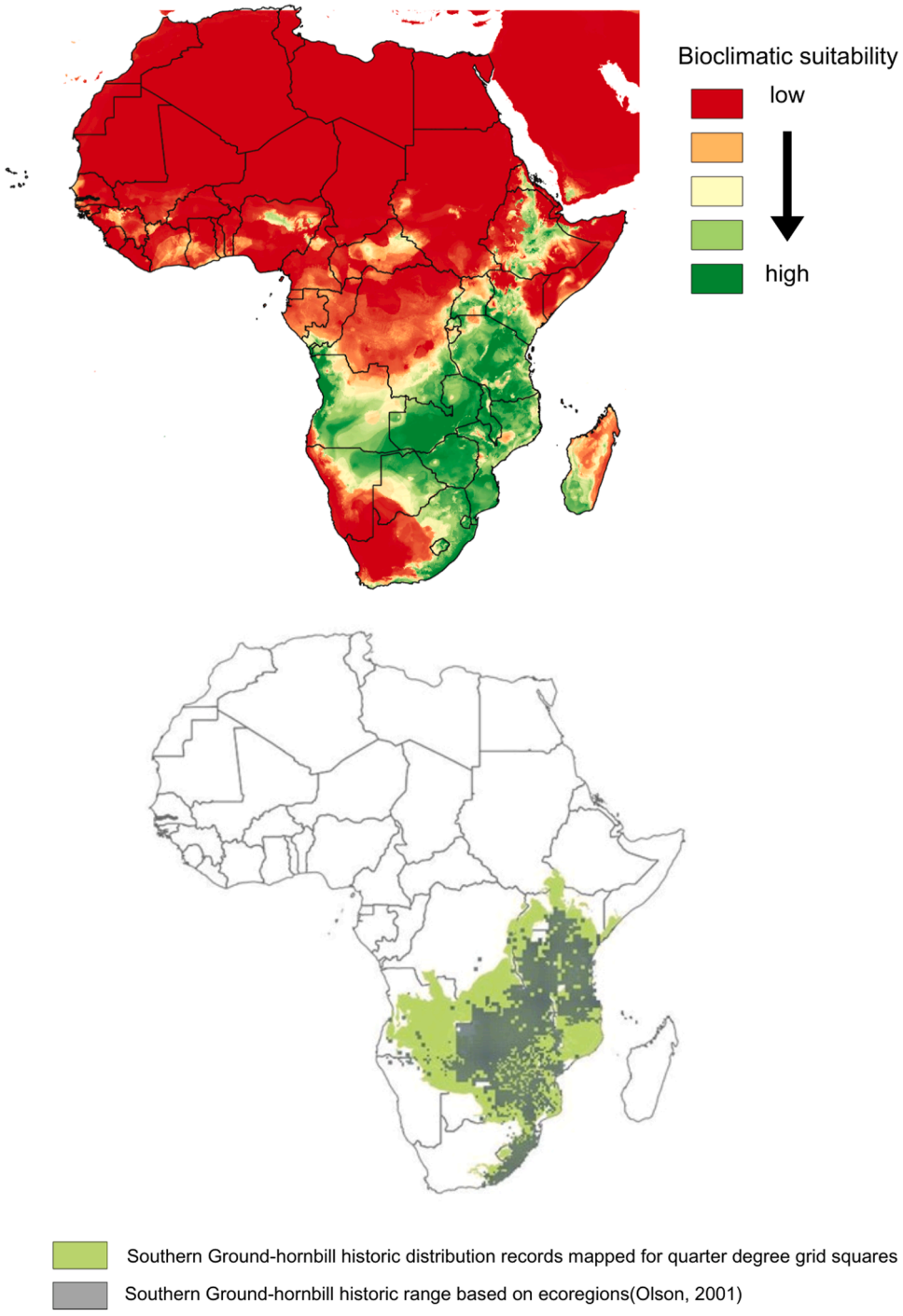

Fig. 1. The distribution of the Southern Ground-Hornbill in sub-Saharan Africa with areas with high bioclimatic suitability and thus probability of occurrence.

1. Introduction

Migration is a primary mechanism facilitating gene flow between natural populations (Campbell et al., 2018). Several factors affect the dispersal behaviour of a species such as geographic barriers, selection, dispersal capacity, demography, social system, genetic drift or interactions with other species and these have a direct consequence on the genetic structure (Wright, 1978; Storfer et al., 2007). In general, birds are highly mobile and are more likely to be panmictic, either due to gene flow or random mating between populations (Goldberg et al., 2011). For example, the wandering albatross (*Diomedea exulans*) is a highly mobile seabird with low levels of genetic population structuring (Milot et al., 2008). These authors attributed this finding to ongoing gene flow, recurrent long-distance dispersal and source-sink dynamics. However, certain species of birds may have populations that are naturally genetically differentiated due to elaborate social systems or high breeding site fidelity (Joosten and Couwenberg, 2001; Morinha et al., 2017). The snail kite *(Rostrhamus sociabilis*) is a highly mobile species that exhibits population structure due to natal site philopatry for breeding (Fletcher et al., 2015). In addition, continued fragmentation may occur due to anthropogenic impacts or by demographic or environmental stochasticity, which can lead to populations that are separated into sub-populations with strongly reduced gene flow, resulting in a higher probability of local extinction (Ceia-Hasse et al., 2018; Cayuela et al., 2021).

The Southern Ground-hornbill (*Bucorvus leadbeateri; hereafter SGH)*, is one of only two species in the genus *Bucorvus*, family Bucorvidae, within the avian order Bucerotiformes, and shows a contiguous occurrence throughout its sub-equatorial range. It is both a culturally and ecologically important species and by each group permanently occupying a large territory, is thus both a flagship and umbrella species for the conservation of savannah and grassland biomes (Le Roux, 2002). SGHs occur from the East African biogeographical suture zone in southern Kenya and Uganda, in line with Lake Victoria, south through savanna and grassland habitats to the Eastern Cape Province, South Africa (Fig. 1). Northern (or Abyssinian) Ground-hornbill *B. abyssinicus* occur in savannas north of and *B. leadbeateri* south of the equator, likely caused by the separation of savannas through the expansion of equatorial rainforest (as suggested by Kemp and Crowe, 1988). The only area of overlap in their ranges is small (Kemp, 1995) and falls within a multi-species east African suture zone (Lorenzen et al., 2012). SGHs are territorial and exhibit wide habitat preferences. However, little is known about their population structuring and there are few geographical or bioclimatic barriers for SGH that fall within a southern African savanna distribution (Voelker et al., 2012). Extremes of wet (forests) and dry (desert and semi-desert) habitats are limiting factors to the wider distribution of these birds. The SGH is globally both the largest hornbill and the largest avian obligate cooperative breeder (Kemp, 1995). Individuals live in socially complex groups, comprising an alpha breeding pair, with predominantly male offspring as non-breeding helpers (Kemp and Kemp, 1980; Kemp, 1988; Kemp, 1995). They occur naturally at low densities in South Africa; one group per 50–250 km^2 (Kemp, 1988; Knight, 1990; Wyness, 2011; Theron et al., 2013; Combrink et al., 2017). Each group is highly territorial, with low dispersal rates between groups (Kemp, 1995; Wilson and Hockey, 2013; Carstens, 2017). Males in the Greater Kruger National Park disperse on average at 45 months of age and move just one or two territories from their natal groups, while females disperse on average at 11 months of age and settle up to four territories from their natal territory (Carstens et al., 2019). Southern Ground-hornbills, although widespread, are listed as globally Vulnerable (BirdLife International, 2016), and regionally Endangered within the southern extent of their range by the International Union for Conservation of Nature (IUCN) (Simmons et al., 2015; Taylor and Kemp, 2015; Kemp et al., 2020). Bucorvidae face greater extinction risks simply by being a largely tropical species (Reif and Štěpánková 2016). Both ground-hornbill species populations have experienced population decline due to loss and fragmentation of suitable habitat, with habitat becoming increasingly transformed by the expansion of human settlements, subsistence farming, large-scale monoculture, and charcoal production, and degraded by bush encroachment and afforestation (BirdLife International, 2014). As the species is mostly resident in established territories, local losses due to anthropogenic threats create gaps in an otherwise contiguous population. These threats include persecution for window-breaking, being trapped for the aviculture trade or for traditional cultural rituals and medicines (Coetzee et al., 2014), poisoning by bait meant for 'pest' species such as rats, jackals, feral dogs, leopards and hyenas, electrocution on transformer boxes (Jordan, 2011) and lead toxicosis from spent lead ammunition (Koeppel and Kemp, 2015). These deterministic threats are the primary reason for the declines but are coupled by stochastic threats such as demographic challenges (sex-biased populations, reduction in dispersal potential, reduction in viable group size, density-dependent allele effects, frequency of sub-population extinction and recolonization events) (Wilson and Hockey, 2013). Genetic factors are poorly understood but have an equally significant role to play in population persistence (Allendorf et al., 2013).

To date, few genetic studies have focused solely on SGH. Delport et al. (2002) sequenced the mitochondrial control regions of six *Bucerotiformes* species, including the SGH and indicated that this region is useful for phylogenetic and population studies. Further, Von Stephan and Prinzinger (2003) identified a distinct population structure based on mitochondrial mtDNA, cytochrome b (CYTB) and reported two sub-populations in captive birds with unknown origins. An assessment of the genetic structure and differentiation of SGH populations has thus far not been reported. It is well known that the degree of dispersal between groups and the frequency of extinction and recolonisation events have direct consequences on the long-term persistence of such metapopulations (Brown and Koderic-Brown, 1979). Indeed, different patterns in metapopulation structures may have different genetic and evolutionary consequences (McCauley, 1991) and this calls for diverse conservation strategies. Thus, an accurate understanding of the population structure of SGH populations, is key to establishing a baseline for the development of more effective protection and conservation management strategies. In this context, we applied mitochondrial and nuclear markers to investigate genetic diversity, broad-scale population structure and demographic history of wild SGH populations across their distribution range. We hypothesise that because SGHs are habitat-generalists, there would be few barriers to dispersal, thus the populations would exhibit high gene flow with an absence of genetic structure. However, low dispersal distances across geographic scales may be a potential barrier to gene flow leading to genetic differences between populations, especially at the extremes of their range.

2. Materials and methods

2.1. Sample collection and ethical approval

The number of samples collected per country were as follows: Kenya (n=8), Democratic Republic of Congo (n=2), Tanzania (n=15), Angola (n=1), Zambia (n=3), Zimbabwe (n=4), Botswana (n=3), Mozambique (n=2), Namibia (n=6), and South Africa (Limpopo Province n=8, Kruger National Park that includes Limpopo and Mpumalanga Provinces n=28, KwaZulu-Natal Province n=10, and additional samples in Eastern Cape Province n=6) (Fig. 2). Although care was taken to include wild individuals putatively unrelated based on field observations, evaluation of microsatellite data using the software Friends and Family Version 22 (de Jager et al., 2017) identified highly related individuals. We therefore excluded individuals based on a relatedness cut-off value of 0.25, leaving a sample size of 83 individuals (Kenya (n=6), Democratic Republic of Congo (n=2), Tanzania (n=13), Angola (n=1), Zambia (n=3), Zimbabwe (n=4), Botswana (n=3), Mozambique (n=1), Namibia (n=5) and South Africa (Eastern Cape Province n=6, Kruger National Park n=23, KwaZulu-Natal Province n=9, Limpopo Province n=7). Close relatives were removed as related individuals can affect the ability of Bayesian clustering algorithms such as STRUCTURE, to accurately identify the number of subpopulations (Rodríguez-Ramilo and Wang, 2012).

Different sample types were collected from birds with known origin and included blood and tissue. Blood samples were collected from individuals trapped in the wild and from captive populations. A small *ex-situ* population is managed as a Pan-African Association of Zoos and Aquaria (PAAZA) African Preservation Programme, with a studbook, thus the provenance of captive individuals is known and recorded. To prevent pseudo-replication just one or two wild individuals (preferably the alpha pair to represent two natal groups assuming inbreeding avoidance) were targeted for capture and no offspring of reintroduced individuals were included. Birds were bled from the brachial vein with a 21-gauge needle and 5 ml syringe and stored in EDTA preservative (0.5 ml) or on filter paper. Toe pad tissue samples (3 ×3 mm) were sourced from archival museums specimens and stored dry until DNA extraction could take place. Given the longevity of the species the museum samples were at most two generations old and as such would not have any bearing on the genetic differentiation results. Ethical clearance for the research was obtained from the University of the Free State Ethics Committee

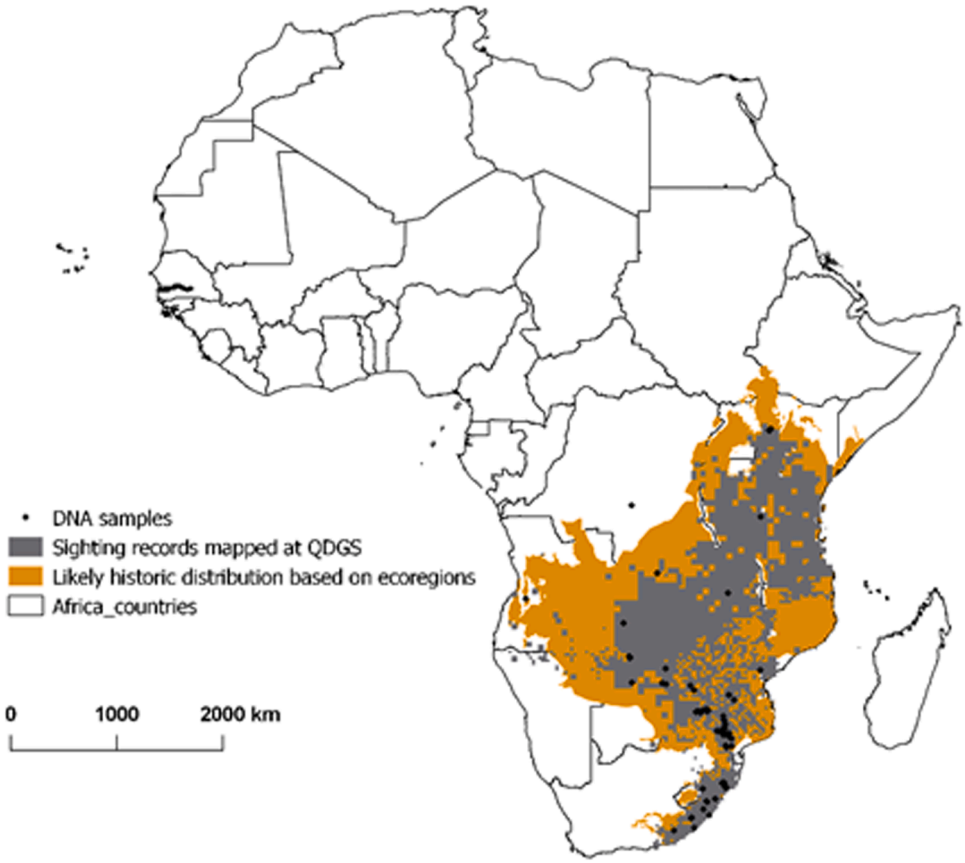

Fig. 2. Spatial distribution of samples (recent and museum, indicated as black circles) used in this study overlayed on actual distribution records for contemporary Southern Ground-Hornbill populations mapped against likely historic range based on suitable ecoregions. The outlier in the Democratic Republic of Congo is accurate as this is a museum sample with locality data, with the historic distribution model limited by available sighting records.

(ref: 06/2015) and the South African National Biodiversity Institute (SANBI) Research Ethics and Scientific Committee (ref: P06/08). Required permits in terms of Section 20 of the Animal Diseases Act, 1984 (ref: 12/11/1/1/9), CITES import permit (ref: 125641), veterinary permits for the transport and import of genetic samples, both locally and internationally (ref: 13/1/1/30/2/0–2016/04/006045 and −2016/05/001507) and capture permits (provincial nature conservation permits: Eastern Cape (ref: 09453), KwaZulu-Natal (ref: OP 4232/2015) and Limpopo (ref: 28241), Namibia (ref: 1957/2014) and Mozambique (PNG/ DSCI/C6/2015) were obtained.

2.2. Microsatellite genotyping and analysis

DNA from all the collected biomaterials was extracted using the Zymo Research Tissue Mini Prep (Zymo Research, USA) following the manufacturer's protocol. All samples were genotyped at 16 species-specific microsatellite loci with six markers (GHB21, GHB19, GHB15, GHB14, GHB20, and GHB26) previously published by the Molecular Ecology Resources Primer Development Consortium et al., (2011). In addition, ten markers were included (Table S1, clone FZ03KKT04H1ODH, South Africa) as developed through high-throughput sequencing technology where 200 bp-paired end sequencing was performed on the Illumina platform (Illumina, USA) at the Agricultural Research Council (ARC, South Africa). Additional markers were developed as described previously (Molecular Ecology Resources Primer Development Consortium et al., 2011). The Polymerase Chain Reaction was performed using Kapa 2 G robust hot start ready-mix (Lasec, South Africa) according to the manufacturer's protocol as follows: 4 μL (1X) Kapa 2 G robust hot start ready-mix; 2 μL (10 pmol) primers (forward and reverse); 4 μL double distilled water (ddH_2O) and 2 μL template DNA (20 ng). The cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 40 min. All reactions were performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA). Negative controls were included to ensure absence of contamination. Primers that shared similar annealing temperatures and compatible dye sets were pooled in one reaction to form a multiplex. In total, six multiplexes were set up, each containing 2–4 markers. Marker GHB 20 was genotyped separately. PCR products were run against a GeneScan™ 500 Liz® internal size standard (Life Technologies) on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Inc., USA). The ABI PRISM® uses the matrix standard dye set (DS-33) to analyse amplicons amplified with microsatellite markers labelled with fluorescent dyes (6-FAM, PET, NED and VIC). Microsatellite loci were detected on the genetic analyser by mixing 0.4 μL GeneScan™500 Liz® with 8.6 μL Hi-Di™ Formamide (Life Technologies and 1 μL of PCR product to make a total reaction volume of 10 μL. GeneMapper V. 4.0 software (Applied Biosystems, Inc., USA) was used to visualize and score alleles for each marker. Possible genotyping errors, allele dropout and non-amplified alleles (null alleles) were detected using MICRO-CHECKER (van Oosterhout et al., 2004). Deviations from Hardy-Weinberg Equilibrium (HWE) and gametic disequilibrium were calculated using Arlequin v 3.5.2.2 (Excoffier and Lischer, 2010). Genetic diversity estimates: mean observed heterozygosity (H_o), mean expected heterozygosity (H_e), unbiased expected heterozygosity ($_uHz$) and the mean number of alleles was determined using the GenAlEx software. In addition, average number of alleles and the inbreeding co-efficient (F_{IS}) was calculated using GenAlEx. Private alleles were identified using GenAlEx (Peakall and Smouse, 2006).

A principal component analysis (PCA, Jombart, 2008) was conducted in R v4.0.3 and RStudio v.2023.12.1 (R Core Team 2023) using the ggplot2, ggrepel, ggfortify, ggbiplot, ggforce, and factoextra packages (Horikoshi and Tang, 2016; Tang et al., 2016). The data included seven sites (KEN, NAM, SAE, SAG, SAK, SAL, TAN), which each included a minimum of five representative individuals. In addition, the genetic relationships between populations was inferred via Bayesian clustering analysis using the statistical programme STRUCTURE version 2.2 (Pritchard et al., 2000). With STRUCTURE, a model-based clustering algorithm was used to determine the most probable number of populations, assign individuals to their most likely population of origin and to determine possible admixture in the genetic make-up of individuals. We compared analyses that assumed correlated and independent allele frequencies, both with and without treating sampling locations as a priori information (Pritchard et al., 2000). STRUCTURE was run for 20 replicates from K=1–6, with a run-length of 700,000 repetitions of Markov Chain Monte Carlo, following the burn-in period of 200,000 iterations. The 20 values for the estimated ln(Pr(X|K)) were averaged, from which the posterior probabilities were calculated. The K with the greatest posterior probability (Pr ≈ 1.0000) was identified as the optimum number of subpopulations. STRUCTURE HARVESTER version 0.6.92 (Earl, VonHoldt, 2012) was used to assess and view likelihood values to select the K value that best suits the data. The resulting file generated from an initial run with K=1–6 was imported into the online analysis tool.

Population differentiation expressed as pairwise F_{ST} values and geneflow (Nm) was calculated in Arlequin v 3.5.2.2 using 10000 dememorization steps at a significance value of 0.05. Here, F_{ST} values were assessed among sampling sites ("populations"). Analysis of molecular variation (AMOVA) was used to compare variation among and within individuals where individuals were partitioned as above. Spatial autocorrelation analysis, implemented in GenAlEx was used to determine if dispersal is limited. A significant (probability is less than 0.05) positive correlation (autocorrelation coefficient = r), indicates that individuals within a given distance class are more closely related than would be expected by chance and supports restricted dispersal. Whereas a significant (probability is less than 0.05) negative correlation, indicates that individuals within a given distance class are less closely related than would be expected by chance and supports dispersal. Here, r was plotted as a function of 12 discrete distance classes (0, 211, 422, 633, 844, 1055, 1266, 1477, 1688, 1899, 2110 or 2321 km). In addition to the above autocorrelation analyses, GenAlEx was used to calculate genetic and geographic distance for 41 samples for which locality coordinate data were available. The correlation between the matrices of pairwise genetic and geographic distances was investigated for both sexes separately and together by applying a Mantel test with 999 permutations to test for isolation-by-distance (IBD) between individuals.

2.3. Sequencing and analysis of mitochondrial regions

A random selection of samples from Tanzania (n=5), Kenya (n=1), Zimbabwe (n=3), Botswana (n=1), Mozambique (n=2), Namibia (n=6), South Africa (Limpopo n=12, Kruger National Park n=19, Kwa-Zulu Natal n=6, and the Eastern Cape n=4) were further sequenced. Four mitochondrial genes (NADH dehydrogenase 2 (ND2), cytochrome c oxidase I (COI) and cytochrome b (CYTB) and 12 S ribosomal RNA (rRNA) genes) were sequenced (Hafner et al., 1994; Sorenson et al., 1999; Fain et al., 2007). For the concatenated mtDNA analysis, one published sequence *Bucorvus leadbeateri* (HM640209.1) from Kenya was included, in addition three sequences were used as outgroups including *Bucorvus abyssinicus* (MN356327.1), *Aceros waldeni* (HQ834450.1) and *Buceros rhinoceros silvestris* (MG596878.1). Primers used for 12 S were designed for this study. Following optimization of each primer set, amplification was conducted using DreamTaq Green PCR Master Mix (Thermo Scientific) in a final reaction volume of 25 μL containing 12.5 μL (2 X) of DreamTaq Green PCR Master Mix; 1 μL (10 pmol) of forward and reverse primers (Integrated DNA technologies), 9.5 μL of double distilled water (ddH_2O) and 1 μL of template DNA (20 ng). The cycling conditions for blood and tissue samples were as follows: one cycle at 95°C for 5 min; then 30 cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min followed by one cycle at 72°C for 10 min. For museum and feather samples the annealing temperature was lowered to 45–50°C. PCR was performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., CA). All PCR products generated were purified using alkaline phosphatase (Thermo Scientific) and exonuclease I (Thermo Scientific) before cycle sequencing. This was achieved by adding 1 μL FastAP (1 U/μL) and 0.25 μL exonuclease I (20 U/μL) to 10 μL of PCR product. Incubation was conducted at 37°C for 15 and then 85°C for 15 min using a T100™ Thermal Cycler (Bio-Rad Laboratories, USA). Following PCR product clean-up, cycle sequencing was conducted using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA). The 10 μL total reaction volume contained: 0.7 μL of BigDye®, 2.55 μL of Sequencing buffer, 0.75 ddH_2O, 1 μL of reverse or forward primer (10 pmol) and 5 μL of purified PCR product. This reaction was carried out in a T100™ Thermal Cycler (Bio-Rad Laboratories, CA). The annealing temperature for all gene regions was 55°C. The Zymo Research (ZR) DNA Sequencing Clean-up Kit™ was used to purify products of cycle sequencing following the manufacturers protocol. Analysis of the sequences was carried out on the ABI 3500 genetic analyser (Applied Biosystems, Inc., Foster City, CA). Sequence-Analysis v. 4.0 software (Applied Biosystems, Inc., USA) was used to visualise the sequences obtained.

DNA sequence trace files were edited manually using GENEIOUS v8.1.6 (Biomatters, LTD, Auckland, NZ). Consensus sequences for forward and reverse sequences of each mtDNA gene were then aligned using MUSCLE (Edgar, 2004) in GENEIOUS. Molecular Evolutionary Genetic Analysis (MEGAX; Kumar et al., 2018) was used for model selection for each gene fragment (best-fit substitution model) using a Maximum Likelihood (ml) approach, with 1000 bootstrap replications and under Bayesian Information Criterion (BIC) using default parameters (See Table S2). The best fit model was selected for use in Bayesian inference phylogenetic analysis for each gene partition for the mtDNA dataset (12 S, COI, CYTB and ND2) using BEAST v1.8.4 (Drummond et al., 2012) as implemented with BEAGLE (Ayres et al., 2012). The XML input file was configured in BEAUti v1.8.0 (Drummond et al., 2012) for running with10 million Markov Chain (MC), generations with a sampling frequency of 10 000 generations and a final burn-in of 10%. A maximum clade of credibility tree (MCC) was constructed using TreeAnnotator v1.8.4 (Drummond et al., 2012) and visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). All pairwise distances, within and among groups, were calculated using the p-distance method in MEGAX; standard error estimates were obtained by a bootstrap procedure (1000 replicates). Haplotype diversity (h), nucleotide diversity (π) and levels of gene-flow were calculated using DNASP (Rozas et al., 2003). Haplotype networks were constructed using PopART v. 1.7 (Leigh and Bryant, 2015) that employs an agglomerative approach where clusters are progressively combined with one or more connecting edges.

2.4. Divergence time estimation

Phylogenetic and molecular dating analyses of the evolution of the SGHs and the genus *Bucorvus* were done using all six major clades of hornbills (Families: Bucerotidae and Bucorvidae) as described by Gonzalez et al. (2013). Molecular clock calibration was done using Bayesian evolutionary analysis in BEAST v1.8.4 only using the mtDNA CYTB dataset with downloads from GenBank. This was due to the unavailability of published hornbill reference sequences for the other gene regions analysed in this study. The divergence times were calibrated using published date estimates of hornbill fossils (Boev and Kovachev, 2007; Gonzalez et al., 2013; Prum et al., 2015) where there is a general agreement of an early to mid-Miocene (15–19 Ma) origin although monophyly or phylogenetic interrelationships have been controversial. Therefore, an average node age of 17 million years ago (Ma) (95% CI 15–19) was used for the root to represent the Miocene origin of hornbills. Five species used by Gonzalez et al. (2013) from closely related coraciiform families (*Phoeniculus purpureus* - Phoeniculidae; *Coracias caudata* - Coraciidae, and *Todiramphus sanctus* - Alcedinidae) and two other distantly related bird taxa (*Rallus longirostris* – Rallidae; and *Morphnus guianensis* - Accipitridae) were downloaded from GenBank and included as outgroups. Published CYTB sequences of 13 hornbill species from 13 genera of the Bucerotidae (*Aceros, Anorrhinus, Anthracoceros, Berenicornis, Buceros, Bycanistes, Ceratogymna, Ocyceros, Penelopides, Rhinoplax, Rhyticeros, Tockus* and *Tropicranus*) were also included as a closely related outgroup. The two species of Bucorvidae, *Bucorvus abyssinicus* and *B. leadbeateri* (haplotypes of *B. leadbeateri* generated by this study) were included as the ingroup [Information of all additional GenBank sequences used is given in Table S3]. The data was run using a random starting tree under the following parameters; 10 million generations, with 10,000 tree sampling frequency with the first 10% discarded as burn-in, a strict clock model, a speciation (Yule) process and TN93+G as the best fitting nucleotide substitution model for the CYTB data under the BIC.

3. Results

3.1. Nuclear analysis

For the 16 microsatellite markers examined in this study, a total of 110 alleles were scored. All loci were polymorphic, with the number of alleles varying from two (Buco2) to 13 (Buco24). Five markers (Buco9, Buco16, Buco25, GHB15, and GHB19) showed evidence of null alleles. One marker, Buco4, showed significant ($P>0.05$) evidence for pairwise gametic disequilibrium. Significant deviations from HWE was detected in five markers (Buco16, Buco24, Buco25, GHB15, and GHB19). As the GHB population was analysed as a single unit, heterozygote deficiencies may be attributed to a variety of factors such as inbreeding and population substructuring (Lade et al., 1996). On the other hand, null alleles could be due to nucleotide sequence variation in primer annealing sites (Callen et al., 1993). Thus, due to the presence of null alleles and deviations from HWE all subsequent analysis was conducted with and without these markers. Results provided here are those for which analysis included only markers that did not deviate from HWE. Genetic diversity estimates were determined as a single population with the following results (Table 1): observed heterozygosity was 0.546±0.068, expected heterozygosity was 0.559±0.072 and unbiased heterozygosity was 0.563±0.072. The average number of alleles was 5.889±1.020 and the number of effective alleles was 2.801±0.437.

Assignment tests of individuals (n=83) supported two clusters (Fig. 3, Figs. S1 and S2) as at higher values of K, there was non-informative symmetry of assignment across all individuals, justifying the selection of K=2 as the correct number of populations. STRUCTURE analyses with and without USEPOPINFO and POPFLAG options that assumed correlated and independent allele frequencies provided similar results (Figs. S1 and S2). Pie charts representing assignment probability of belonging to each of K=2 clusters identified by STRUCTURE are presented in Fig. 3. In general, individuals from Kenya, the Democratic Republic of Congo, Tanzania, Zambia, Namibia, and Botswana assigned to the northern cluster while individuals mostly from South Africa (Eastern Cape Province, Kruger National Park, and Kwazulu-Natal Province) clustered with the southern cluster. A geographically intermediate group, mostly representing individuals sampled from Mozambique, Zimbabwe and Limpopo Province, South Africa) were observed to be admixed. The two clusters observed in STRUCTURE were less distinct in the PCA (Fig. S3). Here, the resulting principal component 1 (PC1) accounted for 13.99% of the variance, and principal component 2 (PC2) accounted for 11.32% of the variance, with other values representing less variation. Populations were found to be generally overlapping with the largest variation being between individuals from Tanzania and Kwazulu-Natal Province (South Africa).

Separate PCAs and STRUCTURE analyses was conducted for each cluster to identify potential population substructure within the northern and southern clusters (results not shown). However, no further population substructure was found. Within the southern and northern clusters, STRUCTURE and PCA analyses supported a single population.

The AMOVA results based on analysis per population, where markers that deviated from HWE and/or displayed null alleles were removed, revealed that most variability was found within individuals (98%, Table 2) and the among populations component was relatively small (5%). Global F_{ST} (0.05, Table 2) was statistically insignificant, however, F_{ST} values between sampling locations were higher and ranged from 0 to 0.22 (Table 3A). In general, values between populations from South Africa and populations from Kenya and Tanzania were higher (0.04–0.19) and significantly different from zero ($P<0.05$). Differentiation between South African populations (excluding KwaZulu-Natal) and populations from Angola and Democratic Republic of Congo were generally low (0–0.02) and were non-significant which may be due to low sample size in the latter populations. Overall, South African populations from KwaZulu-Natal showed high and significant differentiation from populations in Angola, Botswana, Kenya, Namibia, Tanzania and Zambia (0.18–0.22, $P < 0.05$). Further, populations from Zimbabwe were differentiated from populations in Angola, Botswana, Kenya, Namibia, Tanzania (0.09–0.11), however this was not significant. Meanwhile, the Nm values varied from 1.74 to infinite (Table 3B)

Table 1
Genetic diversity estimates of each SGH sampling location indicating the sample location, number of alleles (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He) and unbiased expected heterozygosity (uHe).

	Na	Ne	Ho	He	uHe
ANG	1.444	1.444	0.444	0.222	0.444
BOT	2.778	2.219	0.519	0.426	0.511
KEN	2.889	2.093	0.426	0.420	0.458
MOZ	1.889	1.889	0.889	0.444	0.889
NAM	3.222	2.571	0.606	0.517	0.578
TAN	4.222	2.462	0.567	0.525	0.549
ZAM	2.889	2.444	0.741	0.546	0.719
ZIM	2.556	1.871	0.426	0.428	0.500
DRC	1.889	1.807	0.500	0.333	0.481
SAE	2.889	2.155	0.559	0.462	0.514
SAG	4.333	2.609	0.587	0.549	0.562
SAK	3.111	2.288	0.488	0.453	0.482
SAL	3.444	2.251	0.509	0.478	0.520
Total	5.889	2.801	0.546	0.559	0.563

ANG = Angola, BOT = Botswana, KEN = Kenya, MOZ = Mozambique, NAM = Namibia, TAN = Tanzania, ZAM = Zambia, ZIM = Zimbabwe, DRC = Democratic Republic of Congo, SAE = South Africa Eastern Cape, SAG = South Africa Kruger National Park, SAK = South Africa KwaZulu-Natal, SAL = South Africa Limpopo.

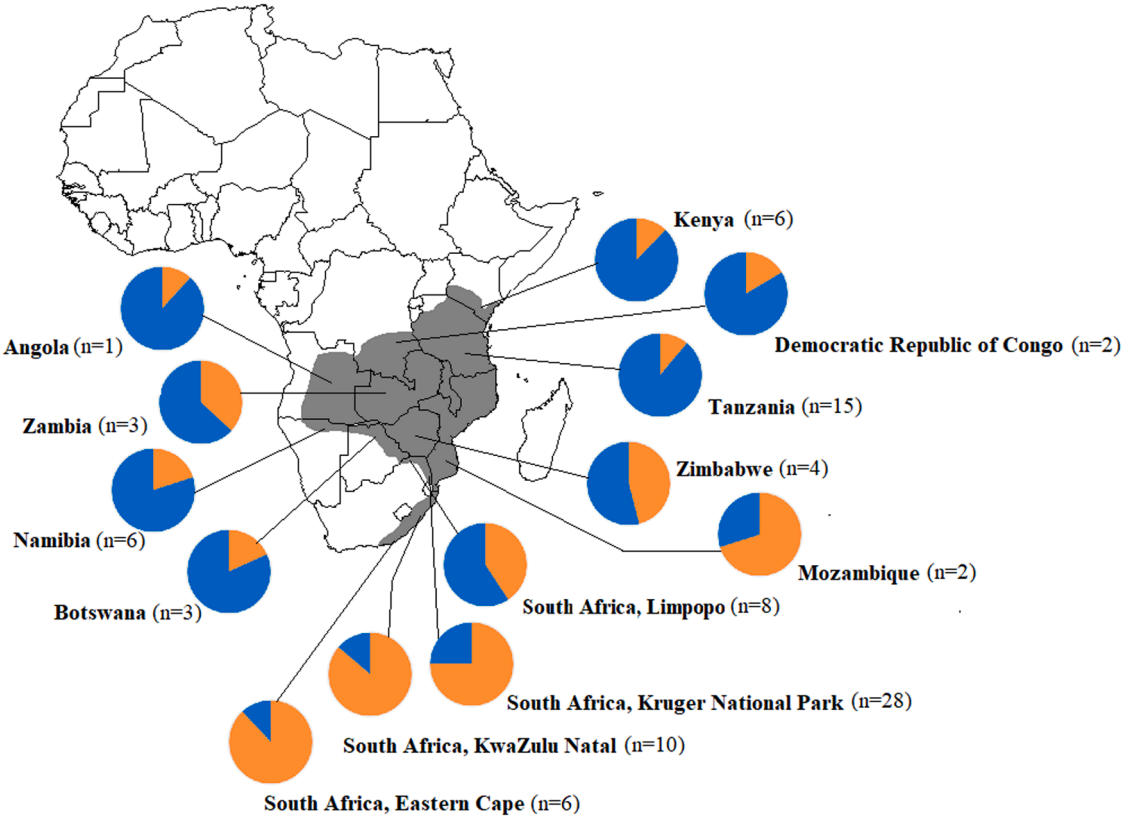

Fig. 3. Assignment of Southern Ground-Hornbill population by sampling site for K = 2 as identified by STRUCTURE. Pie charts represent probability of each sampling site belonging to each of the two clusters.

Table 2
Analyses of molecular variance (AMOVA) among populations.

AMOVA	Sum of Squares	Explained variance (%)	F_{ST} (P value)
Among populations	21.98	5.09	0.051 (0.23)
Within individuals within populations	77.21	-3.23	
Within individuals	98.00	98.14	

and were generally lower in the South African populations from KwaZulu-Natal (1.74–5.68), except between KwaZulu-Natal and Eastern Cape where geneflow was infinite. Geneflow estimates were also lower between South Africa and Kenya (2.07–8.57) as well as between South Africa and Tanzania (2.25–13.93). The distance at which genetic similarity between individuals became statistically independent was assessed by application of a spatial autocorrelation analysis. In the correlogram with smaller distance classes (Fig. S4), the correlation between genetic and geographic distance was positive and significant at 211 km, however at larger distances (from 772 km), r values increasingly become negative and in general was not significant. The overall Mantel test however showed no significant correlation however between genetic and geographic distance, rejecting the hypothesis of isolation-by-distance (Rxy = 0.079; P = 0.14). Similarly, a non-significant correlation was observed in female (Rxy = 0.055; P = 0.45) and male (Rxy = 0.024; P = 0.06) birds (Fig. S5).

3.2. *Mitochondrial analysis*

Single gene region mitochondrial DNA trees are provided in Fig. S6. The gene regions 12 S, COI and CYTB show a single well-supported clade whereas the gene region ND2 includes two clades, one which includes individuals from the Eastern Cape, South Africa and a second clade that includes all other areas. The concatenated mitochondrial DNA sequence was approximately 2016 bp and the resultant tree was monophyletic (100% PP) for *S. leadbeateri* with zero support for internal branches. The total haplotype and nucleotide diversity for the concatenated mitochondrial genes was Hd = 0.3313 and π = 0.0015. The Bayesian inference tree of the concatenated mtDNA sequences shows a single well-supported (Posterior probability = 100) clade for SGH from different areas (Fig. 4 A). The haplotype median-joining network (Fig. 4B) had seven unique *B. leadbeateri* haplotypes (H02-H08) with one common

Table 3
A) Genetic differentiation (F_{ST} values) among sampling areas. Significant values ($p<0.05$) are given in bold type. B) Geneflow (Nm) among sampling sites.

A.

	ANG	BOT	KEN	MOZ	NAM	TAN	ZAM	ZIM	DRC	SAE	SAG	SAK
ANG												
BOT	-0.044											
KEN	-0.195	0.076										
MOZ	-0.333	-0.091	-0.117									
NAM	-0.139	0.016	-0.007	-0.062								
TAN	-0.146	-0.013	0.019	-0.031	0.015							
ZAM	0.084	0.064	0.070	-0.223	-0.015	0.068						
ZIM	0.101	0.086	0.106	-0.171	0.097	0.095	0.030					
DRC	-0.359	-0.077	-0.081	-0.173	-0.090	-0.082	0.043	0.034				
SAE	0.015	0.049	**0.087**	-0.049	**0.072**	**0.078**	0.087	0.017	-0.020			
SAG	-0.090	0.030	**0.073**	-0.027	0.006	**0.040**	0.059	0.032	-0.035	0.007		
SAK	0.223	**0.193**	**0.194**	0.121	**0.184**	**0.182**	**0.220**	0.081	0.115	-0.023	0.094	
SAL	-0.03	-0.003	**0.055**	-0.117	-0.030	**0.035**	0.034	0.037	-0.077	0.027	0.001	0.105

B.

	ANG	BOT	KEN	MOZ	NAM	TAN	ZAM	ZIM	DRC	SAE	SAG	SAK
BOT	inf											
KEN	inf	6.042										
MOZ	inf	inf	inf									
NAM	inf	31.6	inf	inf								
TAN	inf	inf	26.66	inf	32.48							
ZAM	5.423	7.345	6.692	inf	inf	6.877						
ZIM	4.43	5.315	4.224	inf	4.684	4.769	16.21					
DRC	inf	inf	inf	inf	inf	inf	11.27	14.2				
SAE	32.83	9.772	5.268	inf	6.406	5.952	5.255	29.62	inf			
SAG	inf	16.15	6.389	inf	90.87	12.09	8.055	15.03	inf	67.69		
SAK	1.742	2.098	2.074	3.615	2.224	2.253	1.774	5.675	3.834	inf	4.812	
SAL	inf	inf	8.57	inf	inf	13.93	14.34	12.9	inf	18.4	983.3	4.26

Inf = high-level gene flow (infinite). ANG = Angola, BOT = Botswana, KEN = Kenya, MOZ = Mozambique, NAM = Namibia, TAN = Tanzania, ZAM = Zambia, ZIM = Zimbabwe, DRC = Democratic Republic of Congo, SAE = South Africa Eastern Cape, SAG = South Africa Kruger National Park, SAK = South Africa KwaZulu-Natal, SAL = South Africa Limpopo.

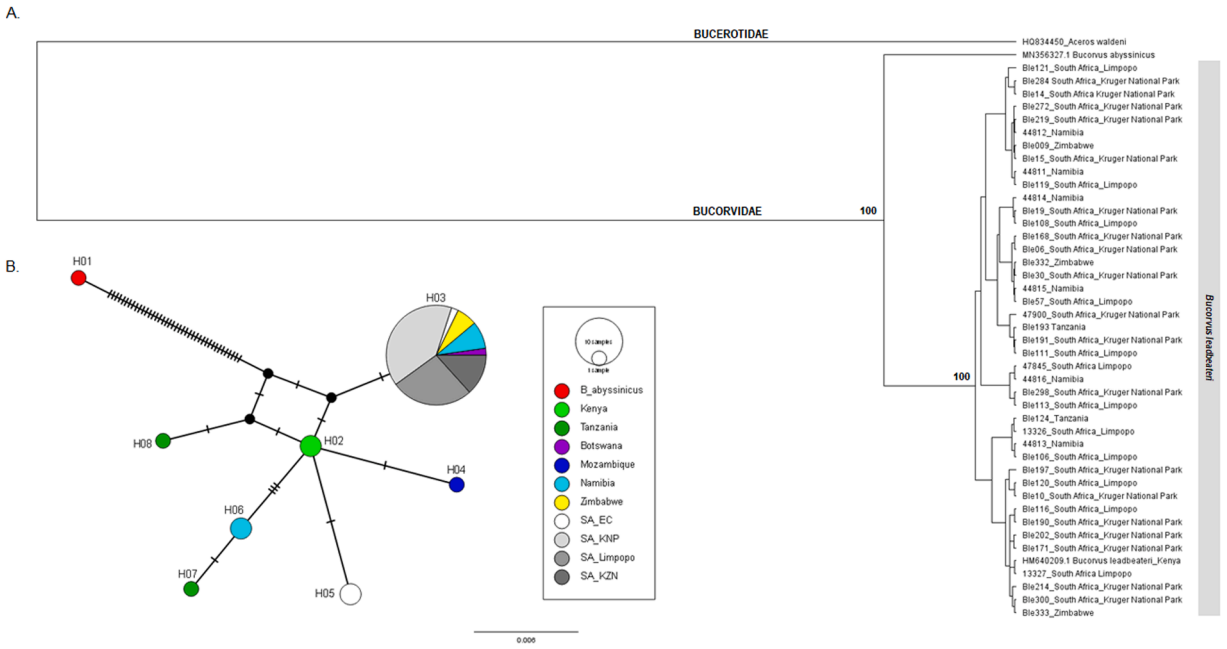

Fig. 4. (A) Maximum likelihood tree of concatenated mitochondrial DNA sequences conducted in MEGAX. Numbers indicate bootstrap values. (B) Median-joining haplotype network for concatenated mtDNA. Areas of circles represent different sampled haplotypes in proportion to their frequencies. Distances between haplotypes are proportional to the number of base differences.

widespread haplotype that was shared by 45 individuals from seven areas. Rare haplotypes were detected for both the northern (Kenya and Tanzania) and southern edge populations across the species distribution range (South Africa specifically the Eastern Cape, Mozambique and Namibia). These haplotypes had well supported clades in the BI tree. The number of mutations separating the different haplotypes was low. The genetic distances (Table 4) recorded between Botswana, Kenya, Mozambique, Namibia, South Africa, Tanzania and Zimbabwe were all low (range 0.000–0.004). The time calibrated tree (Fig. 5) revealed support for the hornbill phylogenetic relationships proposed in previous studies that will not be discussed in this paper as non-target taxa. There was strong support (Posterior probability 100) for the Miocene evolution of the two hornbill families Bucorvidae and Bucerotidae from a shared common ancestor (monophyletic) approximately 14.45 Ma (95% CI: 11.5–18.1). Bucorvidae was a well-supported monophyletic clade with the two sister taxa evolving around 2.52 Ma (95% CI: 1.5–3.8) in the late Pliocene to early Pleistocene. The diversification of *B. leadbeateri* is more recent and estimated around 0.67 Ma (95% CI: 0.3–1.1) in the Pleistocene.

4. Discussion

This is the first account of the genetic diversity of a large, flighted African savanna bird. The only other savanna bird investigated at this scale, the Common Ostrich (*Struthio camelus*), is flightless, and as a consequence exhibits a higher variance (64%) among populations of sub-species groups from a similarly sized range (Miller et al., 2011). Allelic diversity for the Ostrich ranged from 2 to 27, while Southern Ground-Hornbills allelic diversity was much lower (2−13). The moderate genetic diversity found for the Southern Ground-Hornbill ($H_o = 0.546$) was comparable with those for three ostrich sub-species found in southern ($H_o = 0.556$), east ($H_o = 0.576$) and North Africa ($H_o = 0.664$), but greater than that of the sub-species found in Ethiopia ($H_o = 0.302$). The range of southern African Ostrich sub-species is reasonably comparable to that of the Southern Ground-Hornbill in both extent and location, except for the Ostrich with an expected heterozygosity much greater than observed, which suggests inbreeding (Miller et al., 2011). The SGH indicated an absence of significant differences between expected and observed values suggesting low inbreeding. Hailer et al. (2006) suggested that maintenance of high levels of genetic diversity commonly occurs in populations of long-lived avian species as their longevity acts as a buffer to the normal genetic impacts often found in population declines of a species. The links between these molecular metrics and fitness should be investigated as they were not found to be robust predictors in models of fitness for other cooperative breeders based on breeding group formation, dominance, reproductive success or lifespan of individuals (Spiering et al., 2010). The mtDNA diversity in SGH was low ($Hd = 0.33$, $\pi = 0.0015$) comparable to several other threatened avian species such as the red kite (*Milvus milvus*, $Hd = 0.61$, $\pi = 0.0032$ Roques and Negro 2005) and crested ibis (*Nipponia nippon*, $Hd = 0.39$, $\pi = 0.0007$, Zhang et al., 2004).

Assessments of genetic structure of local populations via molecular approaches provide important information on genetic diversity and adaptability to the environment (Reed and Frankham, 2003). In general, high rates of dispersal is required to reduce the risks of inbreeding and maintain a diversified population genetic structure (Janecka et al., 2014). In this study, we identified weak to moderate genetic differentiation among populations of SGHs based on F_{ST} and STRUCTURE with populations at the edge of the range being genetically differentiated. It should be noted that sample sizes of our populations were unbalanced (ie only one sample for Angola) due to difficulty in accessing sampling sites and restrictions in budget which can lead to incorrect assignment of individuals to clusters in STRUCTURE (Meirmans, 2019). Although additional sampling is advocated, it is unlikely that unbalanced sample size affected our study, as results were not unexpected or biologically difficult-to-explain and were further supported by all analysis conducted. The values of Nm indicate a normal gene flow among the populations, although gene flow was generally lower between South Africa and Tanzania as well as Kenya. The subdivision was considered weak based on PCA and AMOVA analysis with an overwhelming majority of variation in SGH being observed within individuals (98%). This finding is corroborated by the lack of any morphological differences being described (L. Kemp pers. obs.). In addition, birds from the far south of the species range (Eastern Cape, South Africa) and the Masai Mara (Kenya) respond to recorded vocalisations with equal vigour, suggesting no regional dialects (Theron et al., 2013, L. Kemp pers. obs.). For forest dwelling species in Africa where more structuring is expected, such as the Cape Parrot (*Poicephalus robustus*), 25% variation was found among populations of species and sub-species (Coetzer et al., 2015), and just 14% within individuals. Lack of

Table 4
Tamura Nei genetic distance assessed by concatenated mtDNA. Below the diagonal the genetic distance values and above the diagonal standard errors.

	BOT	KEN	MOZ	NAM	SAE	SAG	SAK	SAL	TAN	ZIM	*Bucorvus abssinicus*
BOT		0.001	0.001	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.005
KEN	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.005
MOZ	0.001	0.002		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.005
NAM	0.000	0.002	0.001		0.001	0.000	0.000	0.000	0.001	0.000	0.005
SAE	0.002	0.003	0.002	0.002		0.001	0.001	0.001	0.001	0.001	0.005
SAG	0.000	0.001	0.001	0.000	0.002		0.000	0.000	0.001	0.000	0.005
SAK	0.000	0.001	0.001	0.000	0.002	0.000		0.000	0.001	0.000	0.005
SAL	0.000	0.001	0.001	0.000	0.002	0.000	0.000		0.001	0.000	0.005
TAN	0.002	0.004	0.003	0.003	0.003	0.002	0.002	0.002		0.001	0.005
ZIM	0.000	0.001	0.001	0.000	0.002	0.000	0.000	0.000	0.002		0.005
Bucorvus abssinicus	0.030	0.030	0.031	0.030	0.029	0.030	0.030	0.030	0.030	0.030	

BOT = Botswana, KEN = Kenya, MOZ = Mozambique, NAM = Namibia, SAE = South Africa Eastern Cape, SAG = South Africa Kruger National Park, SAK = South Africa KwaZulu-Natal, SAL = South Africa Limpopo, TAN = Tanzania and ZIM = Zimbabwe.

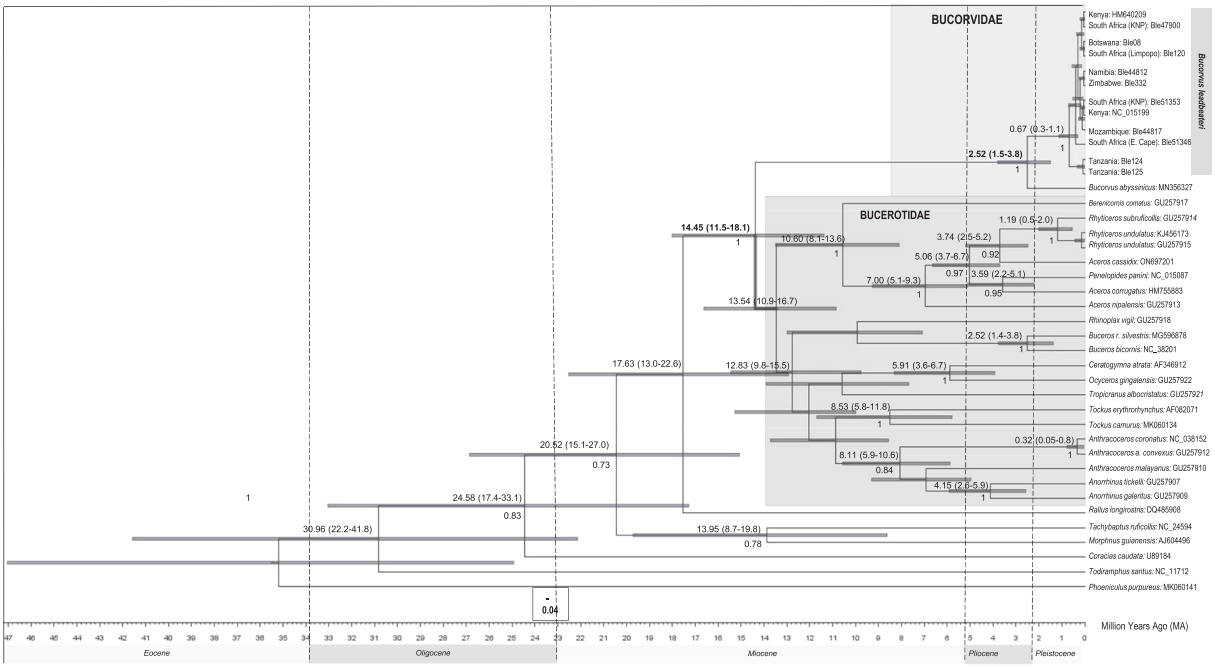

Fig. 5. Dated tree (CYTB) of hornbills. The divergence times correspond to the mean posterior estimate of their age in millions of years before present (Mya) with the grey bars representing the 95% HPD interval for the time estimates. Each node with a posterior probability greater than 0.9 is labelled on the branch with mean node age and its standard deviation in brackets.

population differentiation and weak structure within the population is as expected in species with generalist habitat requirements and high dispersal capability (Hailer et al., 2007). Genetic structure has been detected previously in wattled cranes (*Grus carunculatus*; Jones et al., 2006), with two genetic clusters, one in South Africa and the second in Zimbabwe/Botswana. Although this bird species is capable of long-distance flight, the Kalahari Desert may be a geographic barrier to gene flow as wattled cranes are niche dependant on wetland habitats for foraging (e.g., tubers and rhizomes of Cyperus and Eleocharis sedges and Nymphaea waterlilies) and breeding (Jones et al., 2006). Southern Ground-hornbills are a generalist species found in a variety of habitats such as woodlands, savannah grasslands, and agricultural landscapes (Leonard, 2005; Engelbrecht et al., 2007). In addition, SGH are faunivorous with diets consisting of reptiles, invertebrates, amphibians and land snails that are generally found on the surface. During dry periods, they source food by scratching or digging as a foraging method (Kemp, 1978). As such, the availability of food would not necessarily be a significant barrier to dispersal and subsequent gene flow for this species. The weak to moderate population structure, was supported by the mitochondrial DNA analysis, where we identified unique mitochondrial haplotypes in populations at the edge of the species range (Kenya, Tanzania, Mozambique, Namibia and South Africa (eastern Cape)). Hübner et al. (2003) previously suggested two populations from a smaller sample set of captive SGH. The Hübner et al. (2003) study was limited using only mitochondrial (CYTB) data and captive individuals with no origin locality information or details on whether wild caught or captive-bred. In this study, a higher and significant genetic correlation among individuals was detected at smaller geographic scales (approximately 211 km), providing support that SGHs demonstrate kin clustering. SGHs are reported to be highly territorial, with low dispersal rates between groups (one group per 50–250 km^2) (Kemp, 1995). Our results further support long distance dispersal due to the observed weak patterns of IBD and lower genetic correlation among individuals at larger geographic scales. However, long distance dispersal may be restricted to only a few individuals as supported by lower gene flow and higher differentiation between distant populations. Restricted dispersal leading to population genetic structuring is most likely driven by other factors and is not purely based on IBD. Here, population diversification was estimated to have occurred in the Pleistocene (0.67 Ma), a period with changing climatic conditions (inter-glacial oscillations). Because Africa has a very variable topography and a wide range of climates, these climatic fluctuations promoted isolating mechanisms among habitats for widespread species populations as recorded by other studies on birds. Shallow phylogeny and moderate population structure has been identified in other avian species such as the highly dispersive New Zealand's alpine parrot, the kea (*Nestor notabilis*; Dussex et al., 2014). The authors attributed this finding to historical postglacial expansion out of a single refugium followed by extensive gene flow between populations. In addition, Grosser et al. (2017) identified genetic differentiation in blue duck (*Hymenolaimus malacorhynchos*) populations from North and South islands in New Zealand due to divergence in the late Pleistocene followed by high levels of gene flow and male–juvenile dispersal. Carrera et al. (2022) investigated the effect of past climate oscillations, on six bird species currently distributed in Western Palearctic and Africa. The authors indicated that various bird species adopted different strategies to cope with changing Pleistocene climatic conditions based on different ecological requirements. For example, range expansion and contraction of the trumpeter finch (*Bucanetes githagineus*) during Pleistocene climatic changes lead to genetic structure between geographically close populations (Barrientos et al., 2014). Understanding adaptive responses and distributional shifts of

different bird species in the past is of critical importance in order to understand the future effects of climate change. However, the limited availability of data on sub-Saharan African fossil records, hampers research on past geographic distribution reconstruction (Carrera et al., 2022).

5. Population management

Our study contributes important genetic information with implications for future conservation planning. Mitochondrial DNA results showed shared and unique haplotypes across all sampling sites. In contrast, the STRUCTURE results at K=2 showed weak differentiation between populations that are not driven by isolation-by-distance. The inclusion of molecular genetic results into conservation planning is not always possible due to the need to make rapid decisions before all planned studies are completed (Wyner et al., 2002). South Africa, however, followed a rather stringent Precautionary Principle (as there is no current need to mix founders from the extremes of the range due to the ease of localised harvest of redundant second-hatched chicks from wild nests we can maintain current practises without risking any potential adverse genetic impacts) for captive breeding and reintroduction programmes by managing individuals from the two extremes of the distribution range separately (Meyer, 2016). Although weak genetic structure was identified, population management should continue to follow the Precautionary Principle until further genetic analysis can be conducted to fill the gaps in our knowledge. A full genomic assessment using Single Nucleotide Polymorphisms (SNPs) is suggested and will allow managers to make a final decision on whether the population should be monitored and managed separately. Further, additional sampling is recommended especially for some localities such as the Democratic Republic of Congo, Angola and Mozambique. Identification of SGHs as a panmictic population implies that pair selection requirements in captive breeding can then be relaxed to maintain genetic diversity. This creates an opportunity for inclusion of offspring from founders in international captive facilities for *in-situ* conservation restoration projects as they no longer need to be related back to their population of origin.

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CRediT authorship contribution statement

Prudent S. Mokgokong: Formal analysis, Methodology, Software, Writing – review & editing. **Raymond Jansen:** Investigation, Supervision, Writing – review & editing. **Antoinette Kotze:** Supervision, Writing – review & editing. **Desire Dalton:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – review & editing. **Lucy Kemp:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – original draft. **Monica Mwale:** Methodology, Software, Writing – review & editing. **J. Paul Grobler:** Supervision, Writing – review & editing. **M. Thabang Madisha:** Formal analysis, Methodology, Writing – review & editing. **Anna M. van Wyk:** Formal analysis, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Bucorvus leadbeateri sequences are available on GenBank, accession numbers: OP046521-OP046574, and OP122231 - OP122407

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2024.e02963.

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