










# Simulated genetic efficacy of metapopulation management and conservation value of captive reintroductions in a rapidly declining felid

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## Keywords

*Acinonyx jubatus*; endangered species; genetic diversity; rewilding; single nucleotide polymorphisms; population viability; reintroductions; translocations.

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## Abstract

In South Africa, cheetah (*Acinonyx jubatus*) occur as a relictual, unmanaged population of 'free-roamers', a managed metapopulation across fenced reserves, and in various captive facilities. To ensure that the Cheetah Metapopulation Project (CMP) is not at risk of losing overall genetic variation to drift or inbreeding, we propose various interventions, including exchanges between free-roamers and the metapopulation or supplementation with unrelated individuals from captivity. Simulated trajectories of genetic diversity under such intervention strategies over time could directly inform conservation action and policy towards securing the long-term genetic integrity of the CMP. Single Nucleotide Polymorphisms (SNPs) were genotyped for 172 adult cheetahs across the free-roamer population, the metapopulation, and three major captive facilities. Management intervention trajectory models were tested including, (1) no intervention, (2) genetic exchange between free-roamers and the metapopulation, (3) translocation from a single captive facility and (4) translocation from several captive facilities into the metapopulation. Discriminant Analysis of Principal Components (DAPC) showed that two captive populations are highly differentiated from the metapopulation and each other, whilst the third captive and free-roamer populations are genetically more similar to the metapopulation. Simulated genetic variation over 25 generations indicated that models 1 and 2 show significant losses of heterozygosity due to genetic drift and present a proportional increase in the frequencies of 1st- and 2nd-degree relatives, whilst this variation and pairwise relatedness remain relatively constant under models 3 and 4. We emphasise the potential importance of captive facilities as reservoirs of genetic diversity in metapopulation management and threatened species recovery.

## Introduction

Many species are threatened by human activity, such as land transformation, exploitation, pollution and climate change (Tilman *et al.*, 2017) and in an attempt to slow or reverse this loss of global biodiversity, conservation translocations and metapopulation management have been implemented to

restore many wild populations of dwindling or extirpated species (Bubac *et al.*, 2019). Human activity often causes population fragmentation, possibly resulting in a metapopulation system of geographically distinct subpopulations (Bull & Maron, 2016). Restoring gene flow within a metapopulation system is an important conservation strategy as it delays or even reverses increasing differentiation between these

connected populations (Kunz *et al.*, 2021). Metapopulations require information about how much migration would benefit the populations, in order to maintain them, and future projections may prove to be particularly informative in making these conservation decisions. Therefore, forward-time simulations of genetic data together with population models represent excellent tools to investigate genetic differentiation between populations and subpopulations, particularly within closed metapopulation systems (Kunz *et al.*, 2021).

With approximately 7,100 adult individuals remaining as five recognised subspecies across Africa and Asia, cheetahs (*Acinonyx jubatus*) are considered 'vulnerable' under the International Union for the Conservation of Nature (IUCN; Durant *et al.*, 2015, 2017). Now confined to only 9% of their historical distribution, 77% of which occurs outside of formally protected areas (PAs), cheetahs face a variety of threats, including habitat loss (Jeo *et al.*, 2017), competition with other large carnivores (Buk *et al.*, 2018), poaching (Tricorache *et al.*, 2018; Tricorache, Yashphe, & Marker, 2021), illegal trade (Naude *et al.*, 2020) and human-wildlife conflict (Durant *et al.*, 2017; Dickman *et al.*, 2018). Cheetahs in South Africa occur as unmanaged free-roamers, a managed metapopulation of fenced reserves and individuals in captive breeding facilities. Free-roamers occur predominantly along the northern border with Namibia, Botswana and Zimbabwe, as well as the eastern border with Mozambique, where suitable habitat occupied by cheetahs equates to 99,208 km<sup>2</sup> of which only 30% falls within formally PAs (Marnewick *et al.*, 2007). Whilst Waterberg and Kalahari free-roamer populations persist with suspected gene flow through Botswana (Kotze *et al.*, 2008), sighting records of free-roamers outside of PAs, especially in the Lowveld and Kalahari are in decline (Durant *et al.*, 2017). The Cheetah Metapopulation Project (CMP) was established in 2011 by the Endangered Wildlife Trust (EWT), to ensure the genetic and demographic integrity of the cheetah metapopulation by coordinating translocations between participating reserves and increasing resident range through reintroductions into their historical distribution. The current metapopulation comprises >460 cheetahs on 63 fenced reserves, distributed as five geographic clusters across South Africa and are considered wild as they are required to hunt, are exposed to diseases and co-exist with competing predators (Buk *et al.*, 2018). Whilst the National Cheetah Conservation Forum (NCCF) recorded 44 captive facilities holding >524 cheetahs across 8 'zoological parks' and 36 'breeding operations, rehabilitation centres or safari parks', of which 11 facilities recorded actively breeding cheetahs in 2004, the current captive population in South Africa is estimated at >600 cheetahs across 70 facilities (Marnewick *et al.*, 2007).

Before the CMP was established, cheetah reintroductions were largely uncoordinated and opportunistic. Between 1965 and 1998 for instance, 188 'problem' cheetahs from Namibia were relocated into nine South African reserves, within these, cheetah persisted in only two, with surplus animals from one of these subsequently being relocated to 17 additional metapopulation reserves (Rowe-Rowe, 1992; Hofmeyr & van Dyk, 1998). The low success rate of such relocations

was attributed to animals escaping from inadequately fenced reserves, a reduction in prey populations and high mortality rates due to high densities of competing predators in many reserves (Pettifer, 1980). In 1995, a managed metapopulation strategy for Southern Africa was proposed (Lindsey *et al.*, 2009), by 1998, Namibia implemented new regulations prohibiting further cheetah reintroductions into South Africa. Between 1999 and 2009, the NCCF aimed to reduce cheetah-farmer conflict by removing wild cheetahs from commercial farms. Over 10 years, 157 'problem' cheetahs were captured on farmland and relocated to 37 fenced reserves across South Africa, however, this practice was discontinued in 2009, as free-roaming cheetahs of high conservation value were excessively harvested following an incentive scheme whereby the NCCF paid commercial farmers for live-captured cheetah caught killing livestock on their properties (Lindsey *et al.*, 2009). Many reintroduced cheetahs thrived on these fenced reserves and produced offspring that form part of the current metapopulation (Buk *et al.*, 2018). Between 1965 and 2009, a total of 345 cheetahs were translocated to establish the metapopulation and decreased to 217 by 2012 after supplementation from free-roaming populations ceased. This decrease in population size was largely attributed to the trade of metapopulation cheetah to captivity, single-sex reintroductions and the use of contraception; practices which were effectively halted by participating reserves agreeing to a code of ethics later that year. The number of cheetahs in the metapopulation has since doubled. By 2017, the number of unrelated wild cheetahs being moved into the metapopulation fell below the threshold of four individuals per year and relocating some slightly related individuals (2nd cousins) into the same reserves became unavoidable. To ensure the long-term genetic integrity and health of the growing metapopulation, supplementation with unrelated captive individuals has been proposed. However, before any such large-scale conservation intervention can be considered, the genetic status of both the captive population and the metapopulation needs to be established and the benefits of genetic supplementation using captive cheetahs empirically demonstrated.

A common objective of population genetics is to infer the evolutionary forces that have shaped genetic variation in a population (Tataru, Bataillon, & Hobolth, 2015). Amongst the most widely used theoretical frameworks for this purpose is the Wright-Fisher model (Nielsen & Slatkin, 2013), which characterises evolution in populations of the finite size that mate randomly with non-overlapping generations, and describes the behaviour of allele frequencies over time (Tataru *et al.*, 2015). To infer the history of a population from its allelic frequencies, it is necessary to consider the effects of mutation and migration rates, selection, random genetic drift, and changes in population size. Allele frequencies in any finite population change from one generation to the next due to random sampling, whilst migration, mutation and selection determine the probability of sampling certain alleles (Tataru *et al.*, 2015). Migration would be a particularly important consideration in large wild cheetah populations, as young males are known to disperse up to 200 km

from their natal range (Marker, Fabiano, & Nghikembua, 2008). However, this does not apply to populations separated by fences where the only migration is human-mediated, as in the South African metapopulation. Effective population size ( $N_e$ ) aids in the interpretation of such dynamic interactions (i.e. populations with variable and fluctuating size, non-discrete generations or complex demographic structures) and is defined as the number of individuals in a Wright-Fisher model that would experience the same amount of genetic drift as in the real population (Nielsen & Slatkin, 2013; Tataru *et al.*, 2015). The burgeoning availability and resolution of population-wide ecological and genetic data over the past two decades, has transformed our ability to model a variety of genetic mechanisms from theoretical simulations of evolutionary biology to real-world modelling applications and is increasingly used for conservation management and policy development (Cullingham *et al.*, 2008). Forward-time simulations have thus become a globally important tool in determining population viability and species extinction risk (Haller & Messer, 2019).

In this study, we determine the genetic integrity and future viability of the South African cheetah metapopulation by genotyping 240 Single Nucleotide Polymorphisms (SNPs) in 172 cheetahs from the free-roaming population, the metapopulation and three captive facilities, and extrapolate using forward-time simulations to predict the future effects of proposed translocations on the metapopulation. Four models are tested including (1) no intervention, (2) genetic exchange between free-roamers and the metapopulation, (3) supplementation from a single captive facility into the metapopulation and (4) supplementation from three captive facilities into the metapopulation. Under current rates of global biodiversity decline, such metapopulation management and range-reintroduction with genetic supplementation from captivity are considerations for the effective conservation of many threatened species, exemplified by cheetahs in this case, and require ongoing empirical evidence to support such drastic interventions.

## Methods

### Sample collection and DNA extraction

Cheetah blood samples collected throughout South Africa have deposited in the South African National Biodiversity Institute (SANBI) Biobank (at  $-80^{\circ}\text{C}$ ). A sample subset was selected for this study ( $n = 172$ ) based on availability and maximum spatial coverage (Fig. 1), to represent the free-roaming population (FRM;  $n = 12$ ), the metapopulation (MET;  $n = 40$ ) and three captive facilities (CPT), namely Ashia Cheetah Center (ACC;  $n = 40$ ), Ann van Dyk Cheetah Centre (AVD;  $n = 40$ ) and Hoedspruit Endangered Species Centre (HSC;  $n = 40$ ), in South Africa (Table S1). Due to the low number of free-roaming individuals in South Africa (despite their large distribution), only 12 free-roaming samples were available for analysis and exact locations were not available, therefore, information on where the sample was taken (usually a wildlife clinic) was used.

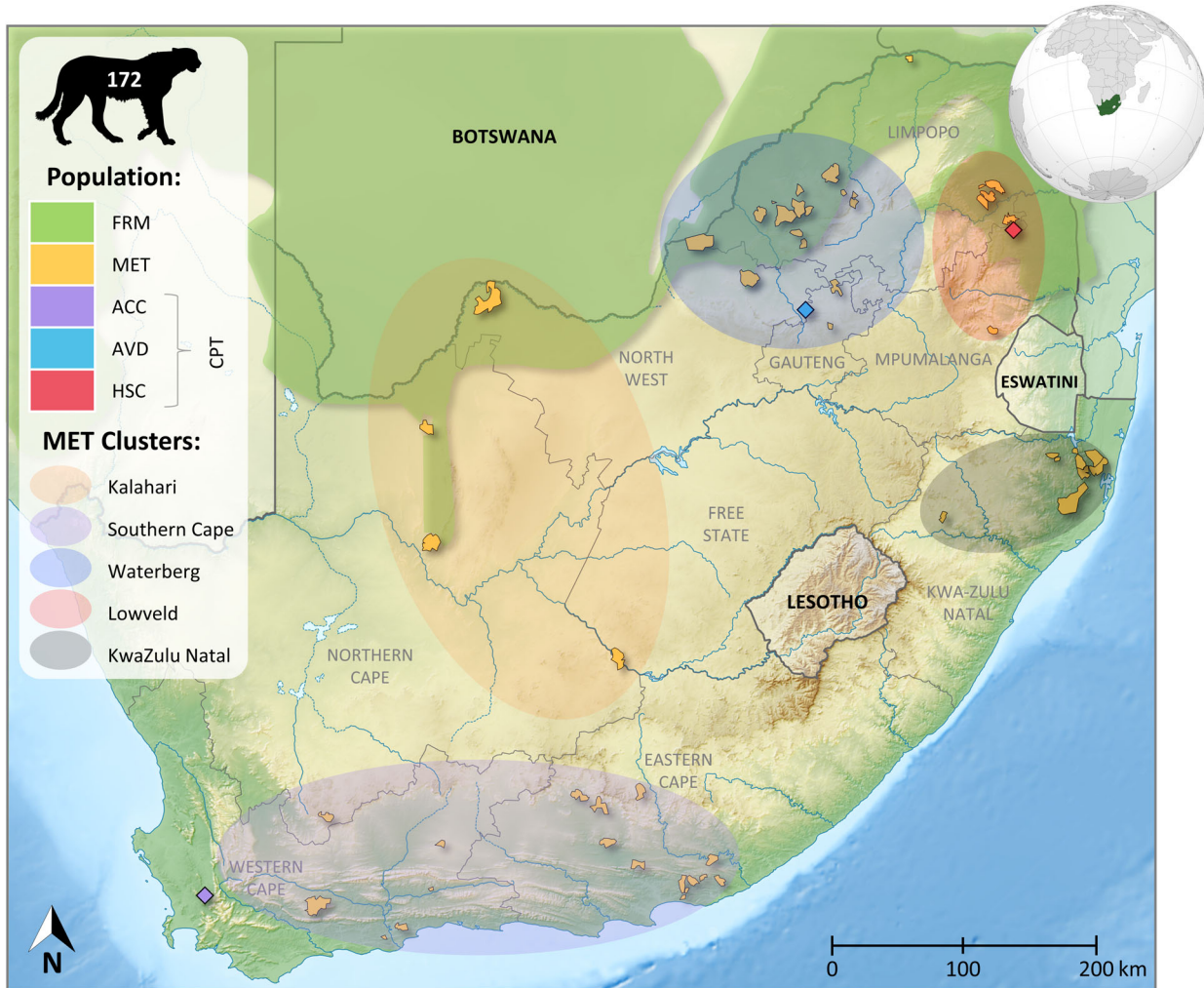
Genomic DNA was isolated from blood samples using the Zymo Research Quick-DNA™ Miniprep Plus Kit, following the manufacturer's instructions, whilst the quality and quantity of DNA were determined using a NanoDrop Spectrophotometer ND-1000. Ethical approval was obtained from the University of the Free State Animal Research Ethics Committee (#UFS-AED2018/0040) and the SANBI Research Ethics and Scientific Committee (#SANBI/RES/P2018/20), and actions permitted under Section 20 of the Animal Diseases Act, 1984 (Act 35 of 1984) from the Department of Agriculture, Forestry and Fisheries (#12/11/1/18), South Africa.

### SNP genotyping

A validated 240 SNP array for cheetah was used to genotype all samples (Magliolo *et al.*, 2021). DNA extracts and TaqMan OpenArray MasterMix were added in equal volumes to 96-well plates and transferred to 384-well plates, where both steps were followed by centrifugation at 4,100 rpm for 1 minute. Samples were located by OpenArray® Sample Tracker and the QuantStudio™ TaqMan® OpenArray® Accu-Fill™ was used to load the SNP array. Once loaded, the SNP array was sealed with the OpenArray® Case Lid, using the QuantStudio™ 12 K Flex OpenArray® Plate Press 2.0. After even coverage with immersion fluid, the SNP array was immediately loaded into the Applied Biosystems™ QuantStudio™ 12 K Flex Real-Time PCR System and run according to manufacturer-recommended operating conditions at 240 SNPs per sample in sets of 12 samples. Genotype data were analysed using TaqMan® Genotyper v1.0.5. (Applied Biosystems, CA, USA).

### Genetic diversity and population structure

SNP data that did not meet specific thresholds determined in PLINK (i.e., MAF  $>0.05$ , SNP call rate  $>0.95$  and individual genotype call rate  $>0.95$ ), were removed from all subsequent analyses (Purcell *et al.*, 2007). Observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity were calculated using GenALEX (Peakall & Smouse, 2006; Smouse & Peakall, 2012). Deviations from Hardy-Weinberg Equilibrium (HWE), instances of Linkage Disequilibrium (LD) and population-specific inbreeding coefficients ( $F_{IS}$ ) were determined using GENEPOP (Raymond & Rousset, 1995; Rousset, 2008). Polymorphic Information Content (PIC) was calculated using CERVUS v3.0.7 (Kalinowski, Taper, & Marshall, 2007). Arlequin 3.5 (Excoffier & Lischer, 2010) was used to compute pairwise  $F_{ST}$  and their  $P$ -values (20,000 permutations). Analysis of Molecular Variance (AMOVA) and Discriminant Analysis of Principal Components (DAPC) were conducted using the R adegenet package (Jombart, 2008; Jombart, Devillard, & Balloux, 2010; Jombart & Ahmed, 2011). Clusters and assignment probability were determined by DAPC scatterplot, where the number of retained PCs was selected by predicting the maximum  $\alpha$ -score with the `optim.a.score` function (20 replicate  $\alpha$ -scores were calculated) to reduce over or under discrimination (Jombart *et al.*, 2010).



**Figure 1** Map of the study area indicating the five cheetah populations in South Africa (FRM: free-roaming, green; MET: metapopulation, yellow; ACC: Ashia Cheetah Center, purple; AVD: Ann van Dyk Cheetah Centre, blue; HSC: Hoedspruit Endangered Species Centre, red). Indicated also are the five major biogeographical regions in which the cheetah metapopulation occurs (Kalahari, transparent yellow; Southern Cape, transparent purple; Waterberg, transparent blue; Lowveld, transparent red; KwaZulu Natal, transparent grey).

### Forward-time simulations

Simulations were conducted using simuPOP (Peng & Kimmel, 2005), where individual identification and sex were manually defined, and simulation starting population sizes were set to the genetic sample size. All individuals were assumed to be from a single generation, as estimated age data were limited, and were set as 'generation zero' for simulation, with each new generation resulting in the previous generation being discarded, assuming that no mutation occurred and that mating was random. Discrete generations were chosen for modelling efficiency, although it is likely that in natural conditions generations do overlap. Simulations deviated from Wright-Fisher in that migration was permitted in models 2–4 and population size was allowed to change by generation across all models allowing for the populations to grow or shrink over time. The mating scheme permitted a

random number of mating events scaled proportionally by population size per generation and mating pairs were randomly chosen with replacement (i.e. a single parent could contribute to multiple sets of cubs). Each mating event resulted in random litter sizes of 0–4 wild or 1–4 captive (assuming constant, exponential growth) offspring per mating. Litter sizes were set to match the recorded survival rates of cubs in captivity versus those in the wild (Buk *et al.*, 2018). If overlapping generations had been used, both average lifespan and death rate would need to have been included in these models, but as litter sizes were set to be equal in both the captive and wild populations, this variation is accounted for. As discrete generations were used, these simulations also assume that all cubs that are born, survive to reproductive age and can produce offspring. For this reason, the mean cub number for the wild population matched the mean number of cubs a female cheetah raises to

adulthood in her lifetime (i.e.  $\pm 1.7$  cubs in the Serengeti; Kelly *et al.*, 1998), even though litter sizes for cheetah are generally 2 to 6 cubs (Wachter *et al.*, 2018). More cubs survive to maturity in captive populations, and a longer average lifespan means additional mating opportunities, thus the mean number of cubs was set to 2.5 (i.e., between 1 and 4). The generational period ran in parallel for all populations and migration was set to occur before breeding, such that breeding pairs were of the same generation and allowed for migrational contribution to the next generation in the recipient population whilst simultaneously removing it from the donor population. All simulations were run for 25 continuous generations and averaged over 10 replicates each.

### Conservation intervention strategies

Four metapopulation management models were investigated to simulate the likely genetic consequence of each approach through 25 generations; namely (1) no intervention, (2) genetic exchange between free-roamers and the metapopulation, (3) translocation from a single captive facility and (4) translocation from several captive facilities into the metapopulation. In model 1 (no intervention), the metapopulation was simulated with no migration or additions from captive facilities. In model 2 (genetic exchange with free-roamers), the simulation allowed  $\leq 5\%$  of individuals to randomly migrate between the free-roaming population and the metapopulation per generation. Model 3 (translocation from one captive facility) allowed 10 random individuals to be donated from ACC to the metapopulation per generation. Model 4 (translocation from multiple captive facilities) allowed 10 random individuals to be donated from captivity ( $n_{ACC} = 4$ ;  $n_{AVD} = 3$ ;  $n_{HSC} = 3$ ) to the metapopulation per generation. The reason for the focus on ACC in model 3, was that the individuals sampled from this population are all currently considered ecologically competent candidates for reintroduction, whilst the specific viability of individuals from the other captive populations is undecided.

### Simulation data analysis

Metapopulation genetic summary statistics were calculated and averaged for each replicate simulation using GenAlEx (Peakall & Smouse, 2006; Smouse & Peakall, 2012). Observed unbiased heterozygosity ( $uH_e$ ) was then compared to that expected for the metapopulation given the change in population size ( $N$ ) over 25 generations, where the harmonic mean size of each population was calculated (1), to account for generational changes in effective population size ( $N_e$ ; Kliman, Sheehy, & Schultz, 2008):

$$\frac{1}{N_e} = \frac{1}{t} \sum_{i=1}^t \frac{1}{N_i} \quad (1)$$

where  $t$  is the number of generations and  $N_i$  represents the number of individuals in the population at generation  $i$ . The expected change in  $uH_e$  was thus calculated for each change in  $N_e$  over 25 generations (2) and compared between model simulations:

$$H_t = \left(1 - \frac{1}{2N_e}\right)^{(t-1)} \times H_0 \quad (2)$$

where  $H_t$  is the  $uH_e$  of the generation being calculated,  $H_0$  is the  $uH_e$  of the initial generation,  $t$  is the number of generations and  $N_e$  is the effective population size under consideration. The program *Laden* estimates  $N_e$  from linkage disequilibrium using the Pearson correlation estimate and was calculated for each simulated population (Waples, 2006; Waples, Larson, & Waples, 2016). DAPCs (Jombart *et al.*, 2010) were then conducted for all repeat simulations where the final generation of each model was compared to that of the first generation of the sampled metapopulation, using the R-based ‘*adegenet*’ package (Jombart, 2008; Jombart & Ahmed, 2011).

### Relatedness analysis

Pairwise relatedness between all individuals in each generation was calculated for each model, using the Wang relatedness estimate ( $r_w$ ) in SPAGeDI (Hardy & Vekemans, 2002; Wang, 2002). Amongst relatedness indices, this estimate shows low sensitivity to sampling error (introduced by estimating population allele frequencies) and shows a low sampling variance (Blouin, 2003). This relatedness coefficient ( $r_w$ ) ranges from 0 to 1, where 0 indicates that candidate pairs are unrelated, whilst 0.5 indicates highly related pairs (e.g., parent-offspring or full-sibling), however, such estimates can range from 0.37 to 0.61 (Visscher *et al.*, 2006), thus cheetah of known relation we used to ground-truth  $r_w$  variability (Magliolo *et al.*, 2021).

Pairwise relatedness and upper-lower estimate bounds were averaged across model replicates within each population per generation. Mean  $r_w$  was then categorised per pair as 1st- and ( $r_w \geq 0.25$ ; parent-offspring or full-sibling pairs), 2nd-degree relatives ( $0.25 < r_w > 0.125$ ; half-sibling, grandparent-grandchild or sibling-nibling pairs) and unrelated individuals ( $r_w \leq 0.125$ ). The average proportion (%) of the population in each of these categories was then compared between models over 25 generations.

### Rarefaction analysis

A lack of sufficient biological replication to characterise the observed biodiversity in a population can be detected by rarefaction analysis (Gotelli & Colwell, 2001). To assuage concerns over small or biased sampling effort, as limited by availability or amplification success, a Python-based script (Fig. S1) was developed to randomly select, with replacement, a subset of individuals for each model dataset ( $n = 5, 10, 15, 20, 25, 30$  or 35) and replicate the simulation 1,000 times for each sample size. To assess the impact of decreasing sample size on genetic diversity estimates, mean heterozygosity values ( $H_o$  and  $uH_e$ ) for the bootstrap replicates were compared by sample size. To assess the effect of sample size on estimates of the degree of genetic differentiation amongst populations, average  $F_{ST}$  values were

calculated by comparing bootstrap replicate populations to the original populations by averaging across replicates.

## Results

### Genetic diversity and population structure

SNP profiles (209 SNP loci) were generated for 172 cheetahs (Table S2). After excluding eight individuals for having >5% missing data and four loci for missing data in >5% of all individuals, the final dataset included 205 SNP profiles for 164 cheetahs ( $n_{\text{FRM}} = 11$ ;  $n_{\text{MET}} = 38$ ;  $n_{\text{ACC}} = 37$ ;  $n_{\text{AVD}} = 39$ ;  $n_{\text{HSC}} = 39$ ). All populations had loci which deviated significantly from expected under Hardy–Weinberg Equilibrium (HWE;  $\text{FRM} = 30$ ;  $\text{MET} = 58$ ;  $\text{ACC} = 51$ ;  $\text{AVD} = 45$ ;  $\text{HSC} = 47$ ). Observed deviations from HWE were not locus-specific and varied per population, suggesting biological rather than technical determinants. Mean PIC was 0.365 (0.172–0.375), whilst mean  $uH_e$  and  $H_o$  (Table 1) were similar between populations (FRM:  $H_e = 0.478$ ,  $H_o = 0.535$ ; MET:  $H_e = 0.469$ ,  $H_o = 0.573$ ; ACC:  $H_e = 0.467$ ,  $H_o = 0.538$ ; AVD:  $H_e = 0.473$ ,  $H_o = 0.552$ ; HSC:  $H_e = 0.449$ ,  $H_o = 0.531$ ).

AMOVA showed that individual- rather than population-level differences explained the most variance (Table 2), however, there was a distinct genetic structure within and between populations (Fig. 2), with pairwise  $F_{\text{ST}}$  estimates revealing significant ( $P \leq 0.05$ ) differentiation amongst all five populations (Table 3). HSC was the most genetically distinct population (HSC-FRM $_{F_{\text{ST}}} = 0.059$ ; HSC-MET $_{F_{\text{ST}}} = 0.063$ ; HSC-ACC $_{F_{\text{ST}}} = 0.083$ ; HSC-AVD $_{F_{\text{ST}}} = 0.047$ ), whilst the FRM and MET populations were most similar (FRM-MET $_{F_{\text{ST}}} = 0.005$ ;  $P = 0.024$ ) and AVD was the most similar to all other populations ( $F_{\text{ST}} = 0.015$ – $0.043$ ).

### Forward-time simulations

Changes in simulated  $uH_e$  illustrate the effect of genetic drift on each model population over 25 generations (Fig. 3a). Model 1 followed the expected negative trend of  $uH_e$  loss to genetic drift alone ( $N_e = 15.4$ ). In model 2, migration was slow (i.e., only a few free-roamers per generation, if any), resulting in some contribution to  $uH_e$ , which reduced the effects of genetic drift, but  $N_e$  declined over time as

**Table 2** Analysis of Molecular Variance (AMOVA) across five cheetah populations in South Africa

Source of variance	Df	Sum of squares	Mean of squares	Sigma	Percentage of variation
Between populations	4	1,562.64	390.66	4.90	4.90
Between individuals within population	159	12,412.08	78.06	-17.18	-17.16
Within Individuals	164	18,437.97	112.43	112.43	112.26
Total	327	32,412.68	99.12	100.15	100.00

expected ( $N_e = 23$ ). Substantial supplementation ( $n = 10$  individuals per generation) in models 3 and 4 increased both the overall diversity ( $uH_e$ ) and the number of potential breeding events within each generation. The resulting increase in  $N_e$  was evidenced by estimates in models 3 ( $N_e = 150$ ) and 4 ( $N_e = 1,500$ ) being larger than expected ( $N_e = 105$ ) and far greater than no intervention at all ( $N_e = 15.4$ ). Mean population sizes at the end of simulations are available in the supplementary material (Table S3).

DAPC of the final generation of each model compared to that of the first generation of the sampled metapopulation (Fig. 4) shows that individuals of a randomly selected 25th generation in models 1 and 2 are closely clustered. These populations are therefore genetically more similar to each other than the individuals in a randomly selected 25th generation of models 3 and 4, which are grouped more closely to the sampled metapopulation than models 1 and 2. Effective population size ( $N_e$ ) grew under all model simulations, being highest in models 3 (+81 individuals) and 4 (+262 individuals) and lower in models 1 (+60 individuals) and 2 (+23 individuals) relative to the sampled metapopulation (Fig. 4).

### Relatedness analysis

Pairwise relatedness ( $r_w$ ) between all individuals within each generation was calculated for each model (Fig. 3b) to determine the relative cost of reduced diversity ( $uH_e$ ) over 25

**Table 1** Summary statistics indicating the mean  $\pm$  SE sample size ( $N$ ), number of alleles ( $N_a$ ), number of effective alleles ( $A_e$ ), information index ( $I$ ), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ), unbiased expected heterozygosity ( $uH_e$ ) and fixation index ( $F$ ) across all SNP loci for each of the five South African cheetah populations (FRM: free-roaming; MET: metapopulation; ACC: Ashia Cheetah Center; AVD: Ann van Dyk Cheetah Centre; HSC: Hoedspruit Endangered Species Centre)

	$N$	$N_a$	$A_e$	$I$	$H_o$	$H_e$	$uH_e$	$F$
FRM	10.99 $\pm$ 0.01	2.00 $\pm$ 0.00	1.86 $\pm$ 0.01	0.65 $\pm$ 0.01	0.54 $\pm$ 0.02	0.46 $\pm$ 0.01	0.48 $\pm$ 0.01	-0.16 $\pm$ 0.03
MET	37.65 $\pm$ 0.06	2.00 $\pm$ 0.00	1.88 $\pm$ 0.01	0.65 $\pm$ 0.00	0.57 $\pm$ 0.01	0.46 $\pm$ 0.00	0.47 $\pm$ 0.00	-0.23 $\pm$ 0.03
ACC	36.86 $\pm$ 0.03	2.00 $\pm$ 0.00	1.87 $\pm$ 0.01	0.65 $\pm$ 0.01	0.54 $\pm$ 0.02	0.46 $\pm$ 0.00	0.47 $\pm$ 0.00	-0.15 $\pm$ 0.03
AVD	38.85 $\pm$ 0.03	2.00 $\pm$ 0.00	1.89 $\pm$ 0.01	0.66 $\pm$ 0.00	0.55 $\pm$ 0.01	0.47 $\pm$ 0.00	0.47 $\pm$ 0.00	-0.17 $\pm$ 0.03
HSC	38.91 $\pm$ 0.02	2.00 $\pm$ 0.00	1.82 $\pm$ 0.01	0.63 $\pm$ 0.01	0.53 $\pm$ 0.02	0.44 $\pm$ 0.01	0.45 $\pm$ 0.01	-0.18 $\pm$ 0.03



**Figure 2** Discriminant Analysis of Principal Components (DAPC) and individual assignment probabilities (inset) of each sampled cheetah in the free-roaming (FRM; green), metapopulation (MET; yellow), Ashia Cheetah Center (ACC; purple), Ann van Dyk Cheetah Centre (AVD; blue) and Hoedspruit Endangered Species Centre (HSC; red) populations of South Africa.

**Table 3** Pairwise  $F_{ST}$  distance matrix of five cheetah populations in South Africa (FRM: free-roaming; MET: metapopulation; ACC: Ashia Cheetah Center; AVD: Ann van Dyk Cheetah Centre; HSC: Hoedspruit Endangered Species Centre). The matrix below the diagonal indicates the pairwise  $F_{ST}$  values between groups, whilst those above indicate the pairwise  $P$ -values

	FRM	MET	ACC	AVD	HSC
FRM	-	0.024	<0.001	<0.001	<0.001
MET	0.005	-	<0.001	<0.001	<0.001
ACC	0.028	0.032	-	<0.001	<0.001
AVD	0.015	0.030	0.043	-	<0.001
HSC	0.060	0.063	0.083	0.048	-

generations. In model 1, all simulated first-generation cheetahs were unrelated, however by the 25th generation, only 58% of the population comprised of unrelated individuals, whilst the remainder were either 1st-degree (37%) or 2nd-degree (5%) relatives. A similar pattern was observed for model 2, with 74% unrelated cheetahs and high proportions of 1st-degree (16%) and 2nd-degree (10%) relatives by the 25th generation. In contrast, models 3 and 4 showed the majority of the population (99%) remaining unrelated by the 25th generation, with the number of 2nd-degree relatives

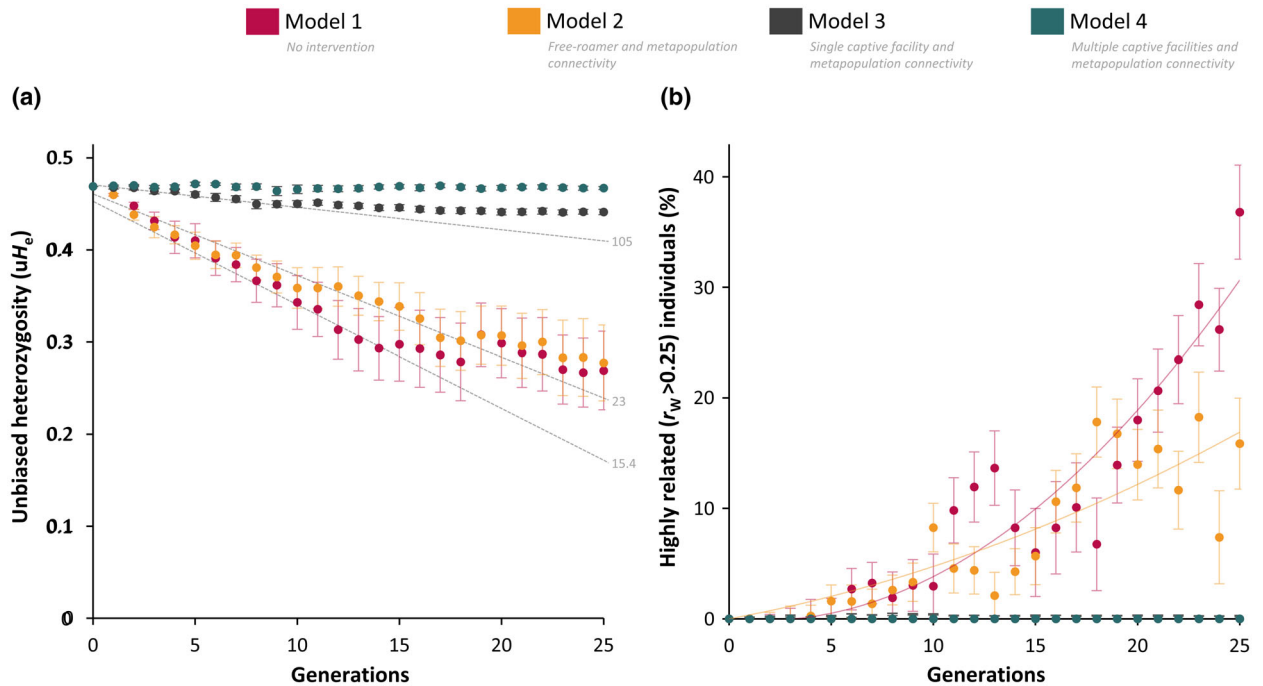
in model 3 (2%) being slightly higher than that of model 4 (<1%).

### Rarefaction analysis

The potential impact of limited biological replication on forward-simulated genetic diversity estimates was determined through resampling with replacement using subsets of 5, 10, 15, 20, 25, 30 and 35 individuals in each population. The difference between and variability within mean heterozygosity ( $H_o$  and  $uH_e$ ) and  $F_{ST}$  estimates for all bootstrapped populations decreased as the number of randomly resampled individuals increased and stabilised between 20–25 randomly resampled individuals (Table S4).

### Discussion

This study demonstrates how recent developments in forward-time simulation can directly inform metapopulation management policy towards securing genetic diversity and ultimately, the success of proposed conservation intervention strategies. Heterozygosity ( $H_o$  and  $uH_e$ ) is similar between all populations (Table 1), suggesting that the genetic

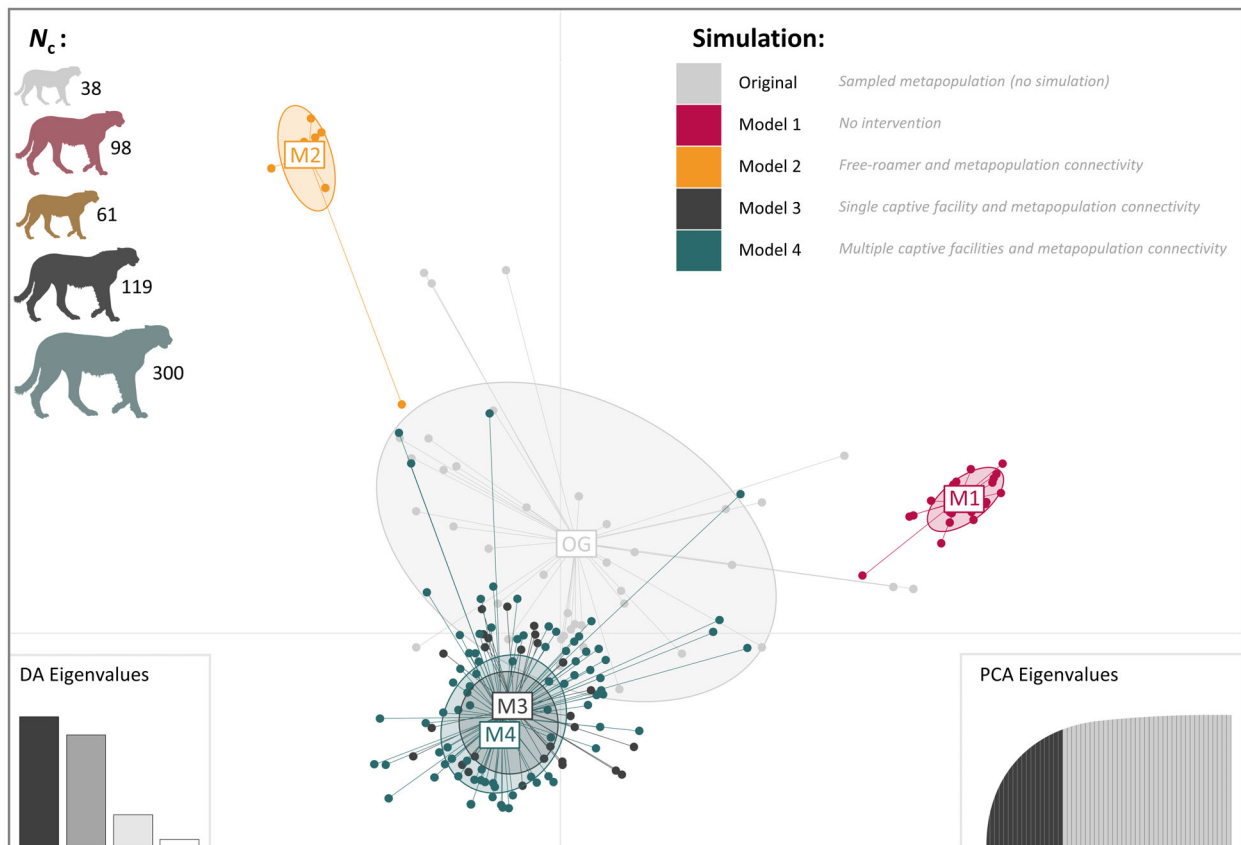


**Figure 3** Change in average unbiased heterozygosity ( $uH_e$ ; coloured dots with ball and whisker), relative to the expected  $uH_e$  (grey dashed lines with values given on the right) given specific effective population sizes ( $N_e$ ) for each metapopulation model (a) and the proportion (%) of highly related ( $r_w > 0.25$ ) individuals simulated over 25 generations (b). Model 1 (red) indicates no intervention, model 2 (yellow) allows for free-roamer and metapopulation connectivity, whilst models 3 (grey) and 4 (teal) allow for connectivity between single and multiple captive facilities, respectively.

diversity of the metapopulation has thus far been maintained. This population comprises multiple sources, including Namibian cheetah translocated to South Africa (1965–1998) as human-wildlife conflict resolution (Rowe-Rowe, 1992; Hofmeyr & van Dyk, 1998) and remnants of free-roaming populations in South Africa (Buk *et al.*, 2018). The three South African free-roaming populations (i.e. Kalahari, Waterberg and Lowveld) are likely connected to those in Namibia through the large, contiguous free-roaming population of Botswana (Kotze *et al.*, 2008). However, the Lowveld population has likely become increasingly disconnected from these northern populations by recent anthropogenic landscape transformation (Durant *et al.*, 2017). Historically (1999–2009), genetic exchange between the metapopulation and free-roamers was possible (and actively pursued by NCCF), thus outbreeding may have maintained the comparably high levels of genetic diversity ( $uH_e$ ) observed within the metapopulation. This exchange is corroborated by the free-roaming population being the most similar ( $F_{ST}$ ) to the metapopulation (Table 3) and evident in the assignment probability overlap between them, with several AVD cheetah also descending from South African free-roamers (Fig. 2). It should, however, be noted that the excess in heterozygosity relative to HWE proportions, leading to the highly negative  $F$  values observed likely results from an SNP array design consisting of only high heterozygosity markers selected to maximise information content for individual identification (Magliolo *et al.*, 2021).

Current levels of genetic diversity are similar between all populations (Table 1), however, forward-time simulations demonstrate that metapopulation will suffer if no future interventions are implemented (model 1). The effective population size is predicted to fall below half of the original sample size ( $n = 38$ ;  $N_e = 15.4$ ) and genetic diversity is expected to drop by 54% ( $uH_e = 0.469$  to  $0.215$ ) over 25 generations. Simulations also suggest that even if viable corridors between the South African free-roamers and the metapopulation were possible (model 2), such an intervention would not be sufficient to secure the current genetic diversity of the metapopulation against genetic drift (Fig. 3a). The free-roaming population in South Africa is small and capture of these animals for release into the metapopulation would be rare and inconsistent, therefore, low migration rates were chosen for this model. However additional modelling was conducted where the free-roaming population was simulated for up to 40 individuals (i.e. to match that of the other populations) and directly compared with equal migration rates. It was found that the free-roaming population still contributed less to the genetic diversity of the metapopulation overall (Fig. S2). This cost is especially evident in the proportional increase of 1st- and 2nd-degree relatives after 25 simulated generations (Fig. 3b), being the highest in model 1 (37%) and substantial in model 2 (16%). However, a metapopulation study of pink salmon (*Oncorhynchus gorbuscha*) found that such low levels of ‘straying’ or migration could be beneficial to the robustness of the metapopulation, provided that





**Figure 4** Discriminant Analysis of Principal Components (DAPC) presenting a randomly selected 25th generation simulated outcome of metapopulation models 1 (red; no intervention), 2 (yellow; genetic exchange between free-roamers and the metapopulation), 3 (grey; translocation from a single captive facility) and 4 (teal; translocation from several captive facilities into the metapopulation) relative to the sampled metapopulation (grey). Indicated also is the proportionally simulated census population size ( $N_c$ ) of each simulated metapopulation model outcome (top left by colour).

it was consistent (Yeakel *et al.*, 2018). In contrast, forward-time simulations suggest that genetic variability in the metapopulation will remain relatively stable if the population is supplemented with at least 10 unrelated cheetahs from captive facilities per generation, with a predicted near 100-fold increase in the effective population size ( $N_e = 15.4$  to 1,500) relative to no intervention over 25 generations (Fig. 3a). After controlling for population growth in models 3 and 4 (i.e., simply taking the change in population size into account when calculating expected effective population sizes), the addition of these captive individuals contributes to an increase in the overall genetic diversity of the metapopulation, which presents greater intra-individual diversity and retains more original diversity than models 1 and 2 (Fig. 4), without a proportional increase in 1st- and 2nd-degree relatives after 25 simulated generations (Fig. 3b). We have shown that genetic supplementation of the metapopulation with cheetah from these three captive populations will maintain current genetic diversity and ensure the long-term genetic integrity of the metapopulation, thus fulfilling a primary objective of the CMP. Our simulations show similar results to a study of Western capercaillie (*Tetrao urogallus*),

suggesting that increased migration rates between these populations can counteract the loss of genetic diversity and differentiation (Kunz *et al.*, 2021).

These simulations are estimated projections of sampled genetic diversity under theoretical model parameters and cannot account for demographic and stochastic effects within these populations. Biological sampling was limited and only partially representative of these populations (FRM = 9%; MET = 9%; CPT = 20%). However, based on coalescent theory, a sample of 20 unrelated, diploid individuals should have a 95% probability of including the most recent common ancestor in any population and thus be representative of its genetic variation and genealogical structure (Hein, Schierup, & Wiuf, 2004). Here, rarefaction analyses demonstrated that relatively small sample sizes of 20–25 individuals are sufficient to obtain accurate estimates of genetic diversity and differentiation following theoretical expectations (Table S4). Not all captive facilities were included in these analyses and data regarding trade between breeding facilities was limited. Simulations thus assumed no migration between facilities, potentially underestimating their diversity. As discrete, rather than overlapping generations were used, these

could represent anything between reproductive age (2–3 years) and total lifespan (>12 years), with no backcrossing and potentially underrepresented reproduction as cheetahs often produce several litters (Buk *et al.*, 2018). Captive cheetahs are not exposed to the same selection pressures as those in wild conditions and as such, artificial selection acting on captive cheetahs may select for traits that are undesirable in wild populations (Willoughby & Christie, 2019; Wemer *et al.*, 2021). Regardless, any captive cheetah released into the metapopulation will be exposed to natural evolutionary pressures (e.g. disease and competition with competing predators) which should eliminate captive animals not suited for wilding (Williams & Hoffman, 2009). Determination of post-release performance of captive-raised would therefore determine the relative value of adding diversity from captive populations into the metapopulation.

The current approach can certainly be further adjusted to better serve conservation action in the field. For one, despite the number of samples and complexity of modelling, this is a preliminary investigation into the simulated effects of current management intervention options and modelling approaches could be improved upon once more ecological information becomes readily available. A good start would be to incorporate known cheetah ages and use a larger dataset to allow for overlapping generations in simulations, which would provide an improved overview of breeding dynamics. As these populations are actively managed, one could get a better idea of which individuals are most likely to breed (and with which other individuals) and incorporate those chances into the considerations for parent pair selection (though detailed information may be difficult to attain in some reserve settings where animals are not consistently monitored). Having more information about the areas of FRMs sampled, as well as including a few more sampled individuals could improve the comparisons between FRM and CPT populations in terms of their relative genetic contributions. Including a wider variety of genes in such simulations (e.g. not just highly diverse regions of the genome) may improve our understanding of true diversity and therefore which populations would contribute most to the integrity of the MET—as not all diversity is good, some stability is necessary for the maintenance of health, or environmental adaptation of a species. These simulations also did not take into consideration translocation success rates, meaning the contribution to diversity that the captive individuals provide may be overestimated, as some animals may not survive to breed in the metapopulation. Translocation success rates were not included as these can be difficult to evaluate and highly case-dependent (Boast *et al.*, 2018). A recent study was done in Namibia on the release of wild cheetah into new free-ranging areas with a 40% success rate (Weise *et al.*, 2015). The majority of these deaths were caused by human-wildlife conflict (Weise *et al.*, 2015), however, wild cheetahs that are released into areas with predator-proof fencing have been found to have greater reproductive success than those released into free-ranging environments (Chelysheva, 2011) and present higher survival rates (Boast *et al.*, 2018). In this study, we consider the translocation of captive-raised cheetah

into a wild metapopulation of fenced reserves, so whilst such animals have shown a higher survival rate when released into fenced reserves, estimates from those studies would not apply directly to this scenario. However, captive individuals have been successfully reintroduced into wild populations before (Wemer *et al.*, 2021). Important considerations for release into reserves would also need to include information about the existing cheetah populations in these reserves, prey densities, habitat suitability and other existing predator densities (Boast *et al.*, 2018). Such assessments would need to be made for each individual translocated before a reserve is chosen. This study provides an overview to determine if translocating captive cheetahs would benefit the metapopulation genetically, and there is still a need to investigate the relocation of each individual cheetah. As more information on these and other similar metapopulation translocations becomes available, it will be increasingly possible to incorporate more detail in future simulations to improve model accuracy and relative intervention value. By establishing and projecting the genetic status of free-roaming, metapopulation and captive cheetah populations in South Africa, we show that the long-term genetic integrity and health of a growing metapopulation cannot be secured by intrinsic diversity or conventional migration alone. Instead, this requires supplementation with unrelated individuals, such as those currently held in captivity. Whilst initially, cheetah population and habitat viability were informally assessed (Lindsey *et al.*, 2009), with follow-up studies exploring relocation success (Johnson *et al.*, 2010), minimum prey and area requirements (Lindsey *et al.*, 2011), demography (Bisset & Bernard, 2011), self-sustained growth (Buk *et al.*, 2018), supplementary feeding (Warmenhove *et al.*, 2020), predatory naïveté (Wemer *et al.*, 2021) and release of captive-raised cheetah (Walker *et al.*, 2022), to our knowledge, this is the first study using genetic data representing all three South African subpopulations and which simulates the efficacy of metapopulation management and conservation value of captive reintroductions. Such large-scale conservation intervention should however be supported by intensive rewilding processes, as well as rigorous health and genetic screening to maximize individual survival and therefore genetic contribution. Forward-time simulations are integral to the effective monitoring and adaptive genetic management of metapopulations (Laikre, 2010). The methods developed herein can be applied to a multitude of threatened species such as African wild dog (*Lycaon pictus*; Nicholson *et al.*, 2020), African lion (*Panthera leo*; Becker *et al.*, 2022; Bertola *et al.*, 2021; Dolrenry *et al.*, 2014), Tasmanian devils (*Sarcophilus harrisii*; Hogg *et al.*, 2017) and piping plovers (*Charadrius melodus*; Catlin *et al.*, 2016), that are currently under metapopulation management, where such projections will be crucially informative to supporting applied conservation decisions to secure the future of these species.

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**Michelle Magliolo:** conceptualization, methodology, Software, validation, formal analysis, investigation, data curation, writing—original draft, writing—review and editing and visualisation. **Vincent N. Naude:** conceptualization, methodology, Software, validation, formal analysis, investigation, data curation, writing—review and editing, visualisation and supervision. **Vincent C. van der Merwe:** conceptualization, resources, investigation, data curation, writing—review and editing. **Stefan Probst:** methodology, Software, validation, resources and writing—review and editing. **Pablo Orozco-terWengel:** conceptualization, methodology, Software, validation, formal analysis, investigation, writing—review and editing, visualisation and supervision. **Pamela A. Burger:** conceptualization, methodology, resources, writing—review and editing and supervision. **Antoinette Kotze:** conceptualization, methodology, resources, writing—review and editing, supervision and project administration. **J. Paul Grobler:** conceptualization, methodology, resources, writing—review and editing and supervision. **Desiree Lee Dalton:** conceptualization, methodology, resources, writing—review and editing and supervision.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Python script developed to randomly select, with replacement, a subset of individuals for each model dataset ( $n = 5, 10, 15, 20, 25, 30$  or  $35$ ) and replicate the simulation 1,000 times for each sample size.

**Figure S2.** Change in average unbiased heterozygosity ( $uH_e$ ), relative to the expected  $uH_e$  given specific effective population sizes ( $N_e$ ) for each metapopulation model when the free-roaming population was simulated up to 40 individuals and an equal migration rate was used (same as the

migration rate from the captive populations). Colours represent the scenarios as follows: light blue (No change—no migration of animals into the metapopulation), orange (Migration of individuals from a single population—specifically FRM the free-roaming population), grey (Migration of individuals from a single population—specifically ACC the Ashia Cheetah Center), yellow (Migration of individuals from a single population—specifically AVD the Ann van Dyk Cheetah Centre), dark blue (Migration of individuals from a single population—specifically HSC the Hoedspruit Endangered Species Centre) and green (Migration of individuals from all captive facilities—ACC, AVD and HSC).

**Table S1.** Individual-based sample information ( $n = 172$ ), including South African Biodiversity Institute (SANBI) Bio-Bank catalogue sample numbers, sex, population classification, submission author and geographic origin.

**Table S2.** Individual-based Single Nucleotide Polymorphism (SNP) profiles ( $n = 172$ ), including South African Biodiversity Institute (SANBI) BioBank catalogue sample numbers for a 240 SNP array.

**Table S3.** Mean population sizes per model in the 25th generation of simulations

**Table S4.** Rarefaction analysis showing average  $H_o$ ,  $uH_e$  and  $F_{ST}$  (SE) values determined for a random combination of individuals by specified sample size class over 1000 bootstrap replicates compared to samples used in this study amongst five original cheetah populations in South Africa (FRM: free-roaming; MET: metapopulation; ACC: Ashia Cheetah Center; AVD: Ann van Dyk Cheetah Centre; HSC: Hoedspruit Endangered Species Centre).