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Original Article

Red, gold, and green: comparative genomics of polymorphic leopards from South Africa

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Abstract

An important goal of comparative and functional genomics is to connect genetic polymorphisms to phenotypic variation. Leopards (*Panthera pardus*) from northern South Africa are particularly diverse, as here a unique color morph occurs, as well as two deeply diverged southern (SA) and central African (CA) mitochondrial clades, stemming from Pleistocene refugia. Here, we present the first whole genomes of a red leopard and a black (captive) leopard, and wildtypes belonging to the CA and SA mitochondrial clades, to evaluate genome-wide diversity, divergence, and high-impact mutations that may relate to their phenotype. In the black leopard, we found long runs of homozygosity (ROHs), low nucleotide diversity across the genome, and a large number of homozygous structural variants, likely resulting from inbreeding to maintain this color morph in captivity. In red leopards, runs of homozygosity were slightly longer compared to wildtype leopards, with potential deleterious mutations relating to its phenotype, including impaired vision. When assessing population structure, we found no divergence between CA and SA leopards and the rest of Africa, whether comparing single nucleotide or structural variants. This illustrates the homogenizing effect of introgression, and highlights that although leopards in northern South Africa may be phenotypically unique, they are not genetically different.

Keywords: color morph, genomic divergence, *Panthera pardus*, structural variants, natural selection, whole genome sequencing

Introduction

An important goal of comparative and functional genomics is to connect genetic polymorphisms to phenotypic variation (Peters & Musunuru, 2012). Due to rapidly advancing genomic methods, the field has substantially increased our understanding of the basis of coat color-related traits and other forms of inherited diversity in recent years (Caro & Mallarino, 2020; Orteu & Jiggins, 2020). For instance, the assembly of reference genomes has enabled genotype–phenotype association studies (Andrade et al., 2019; Rodrigues et al., 2016), which have been used to unravel phenotypically plastic change in lizards (Sabolić et al., 2024), divergent selection of plumage pigmentation in birds (Campagna et al., 2017), and niche-specific adaptive radiation of cichlid fish (Sommer-Trembo et al., 2024). Whole genome analysis has also allowed a more accurate, in-depth understanding of species' evolutionary history and population genetic structure, due to large datasets of independent loci and the inclusion of protein-coding genes (Rokas et al., 2003). Because single nucleotide polymorphisms (SNPs) allow a more fine-scale resolution and reflect the role of selection, SNP data have revealed population-level variability that was overlooked when using traditional methods such as mitochondrial genes (mtDNA) and nuclear microsatellites (Downing et al., 2012; Evans & Cardon, 2004; Szatmári et al., 2021; Zimmerman et al., 2020). Because functional genomic analysis can be used to unravel how neutral and selective processes affect phenotypic and genotypic variation (Cano-Gamez & Trynka, 2020), it is particularly interesting for polymorphic populations that are maintained in the wild. Considering the scope of research possibilities, leopards (*Panthera pardus*) from South Africa offer an excellent case study, due to their high phenotypic diversity and unique demographic history (Tensen et al., 2022).

Leopards evolved roughly three million years ago (mya) in East Africa and spread to Eurasia some 0.5 to 0.6 mya (Paijmans et al., 2018), which led to population divergence that follows an isolation-by-distance pattern (Uphyrkina et al., 2001). Eight subspecies are currently recognized, based on

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an assessment that grouped the North-Chinese leopard *P. p. japonensis* with the Amur leopard *P. p. orientalis* (Kitchener et al., 2017). The African leopard belongs to a single subspecies (*P. p. pardus*) but consists of three mitochondrial clades in West Africa, Central Africa (CA), and Southern Africa (SA) (Anco et al., 2018). The population in northern South Africa (NSA) comprises a contact zone between the CA and SA clades (Figure 1A), which are thought to have originated during climatic shifts in the Mid-Pleistocene, some 0.8 mya, based on mitochondrial genomes (Tensen et al., 2024). The Pleistocene refuge theory suggests that historic dispersal barriers occurred when savanna habitat was replaced by deserts, which promoted differentiation between populations in East and SA (Sithaldeen et al., 2015; Tensen et al., 2024). As such, South African leopards may reflect the unique climatic history of Africa, which has resulted in eminent genetic diversity (Morris et al., 2024), although this has yet to be examined using a whole genome approach. Mitochondrial DNA is known to be unsuitable for unraveling spatial genetic structure or isolation-by-distance (see e.g., Dávalos & Russell, 2014; Teske et al., 2018), and it is likely that the clades have homogenized in the face of introgression of nuclear genes, due to long-distance dispersal that potentially occurs across SA (Fattebert et al., 2013). For instance, whole genomes did not reflect the mtDNA phylogeographic structure of leopards in Central Africa, due to male dispersal, gene flow, and introgressive hybridization (Paijmans et al., 2021; Pečnerová et al., 2021).

In NSA, where the SA and CA clades are admixed, a unique coat color variant also occurs (Figure 1B), referred to as the red or strawberry leopard (Pirie et al., 2016). Interestingly, the red leopard mtDNA genome was found to be quite distinct from other leopards, and sister to the CA clade (Tensen & Camacho, 2024). Although mitochondrial lineages tend to be incongruent with nuclear genes that relate to the occurrence of phenotypic variation (Lin et al., 2009), polymorphic species are commonly ancestral in phylogenetic trees and may give rise to monomorphic daughter species (Corl et al., 2010; Hugall & Stuart-Fox, 2012; Van Gossum & Mattern, 2008; Williams et al., 2015). This is because polymorphic species often speciate more rapidly as a result of niche segregation and assortative mating (Heuer et al., 2024; Hugall & Stuart-Fox, 2012). The most famous example of color polymorphism in leopards is the melanistic "black" form, which has reached

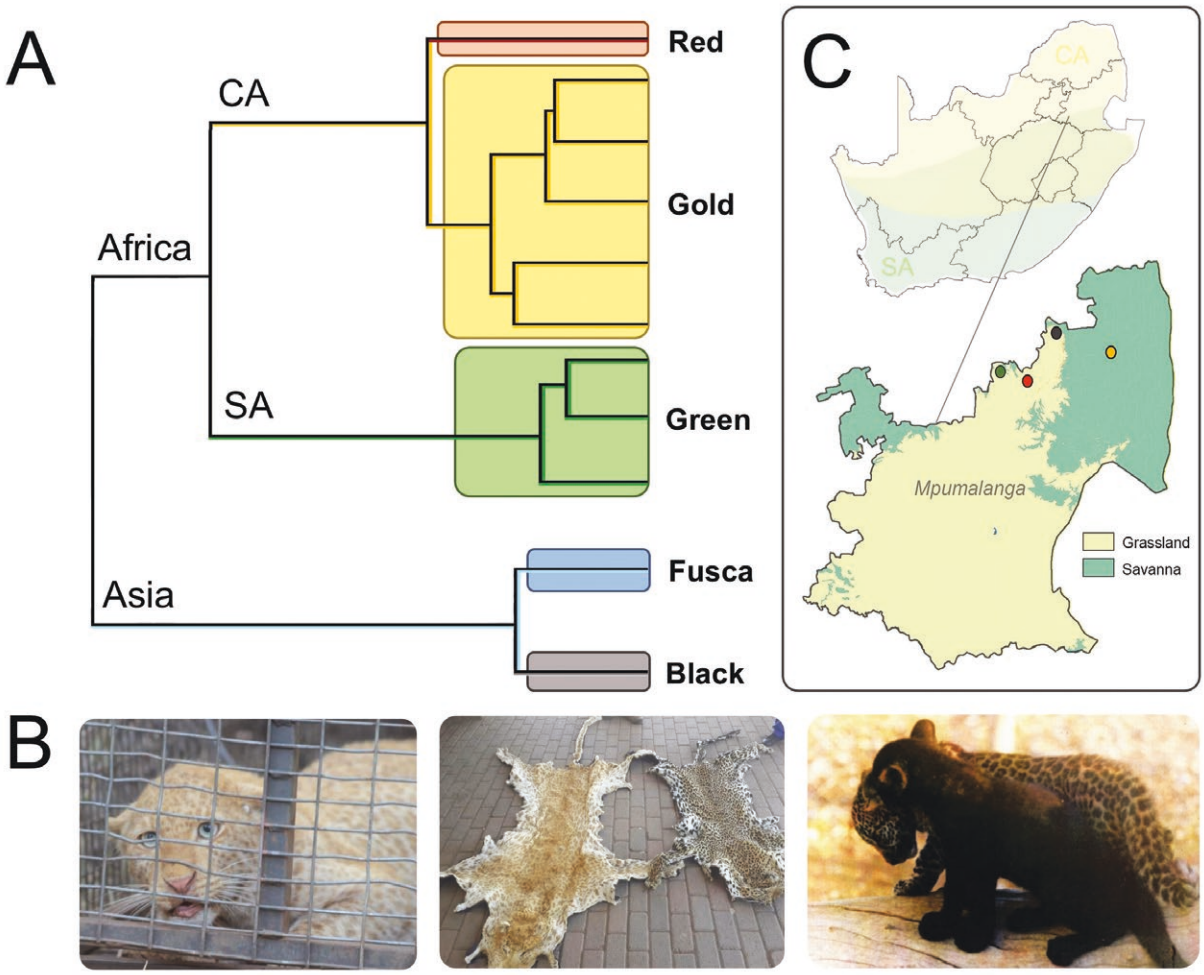

Figure 1. Diversity of South African leopards. (A) An abbreviated Bayesian tree generated with mitochondrial genomes (Tensen & Camacho, 2024; Tensen et al., 2024), illustrating two deeply diverged SA and CA clades. (B) The red or strawberry leopard color morph is characterized by a pink skin, pale fur, and light eyes caused by a TYRP1 mutation, which is prevalent in northern provinces (photos by Vasti Botha and Gerrie Camacho). The black leopard on the right was taken of cubs that were thought to be distanced relatives of a wild black leopard from South Africa. (C) A map with sample locations. The colors refer to a red leopard, gold (CA clade), green (SA clade), and a black leopard at the Sebaka Game Reserve.

near-fixation in South East Asia (Da Silva et al., 2017). The persistence of the black phenotype in leopard populations implies a selective benefit, and a balance between advantages and disadvantages compared to the "golden" wildtype (Fisher, 1930). For instance, melanistic morphs are better camouflaged in dense, tropical forests, causing disruptive selection (Allen et al., 2011; Da Silva et al., 2017; Schneider et al., 2015). To the contrary, the red leopard has only been observed since 1974 and its increasing occurrence in South Africa was suspected to be linked to a heterozygote deficiency instead (Tensen et al., 2022). The occurrence of rare color variants has been found in many small and fragmented carnivore populations through the expression of rare recessive alleles (Tensen & Fischer, 2024). Therefore, whole genome analyses of red leopards would be beneficial to determine whether there are deleterious mutations that are in close proximity (genetically linked) to the causative mutations related to this naturally occurring color morph.

The red leopard phenotype is caused by a deletion in the tyrosinase-related protein 1 (TYRP1), which down-regulates the albino-associated tyrosinase (TYR) protein-coding gene, which is crucial in the production of melanin, responsible for coloration in skin, fur, and eyes (Kobayashi et al., 1998). Because the TYRP1 mutation is recessively inherited, any form of inbreeding can increase the expression of this color variant (Tensen et al., 2022). More specifically, parental relatedness can cause inheritance of alleles that are Identical By Descent (IBD), which can result in homozygous stretches across the genome, referred to as Runs Of Homozygosity (ROHs), which increase the risk of recessive deleterious alleles to be co-expressed in the offspring (Bosse et al., 2015). This may reduce the viability of inbred offspring, and coat color variations such as albinism are generally considered a major disadvantage to animals in the wild (Acevedo et al., 2009; Ueda, 2000). A lack of pigment in the eyes, as the case for red leopards and albino individuals, can also cause visual impairment, and thus make it harder for animals to find prey (Rymer et al., 2007). Likewise, although melanistic morphs have been described in over 14 Felidae species, they are commonly lost from natural populations due to a selective disadvantage (Gray & McKinnon, 2007). Melanism is polymorphic in wild cats and can be caused by several mutations in the Agouti Signaling Protein (ASIP) or Melanocortin-1 receptor (MC1R), depending on the species (Eizirik et al., 2010). For instance, melanism in Indo-Chinese leopards (*P. p. delacouri*) is caused by a nonsense mutation in the ASIP exon 4 (c.333C>A) (Schneider et al., 2012), different from the ASIP mutation (c.353C>A) in Sri Lankan leopards (*P. p. kotiya*) (Sooriyabandara et al., 2023). There has been a long-standing myth that black leopards occurred in South Africa as well, remnants of which still remained in captivity, although mitogenomic analysis revealed that these were in fact of Indian descent (*P. p. fusca*) (Tensen & Camacho, 2024).

Here, we present the first whole genomes of leopard wildtypes belonging to the CA ("gold") and SA ("green") mitochondrial clades, as well as a red leopard and a black (Indian) leopard, to evaluate their genome-wide diversity, divergence, and high impact mutations (HIMs) that may relate to their phenotype. We test the following hypothesis: (1) introgression and recombination have led to homogenization of the CA and SA clades at the nuclear level; (2) genomic variation is expected to be lower in color variants; and (3) potential causative mutations differ between wildtype, red and black leopards. We compare single nucleotide variants (SNVs), including SNPs and single base-pair indels, and structural variants (SVs), including insertions, deletions, and duplications. We included SVs because they are most frequently associated with adaptive phenotypes and the maintenance of population differentiation in the face of gene flow (Mérot et al., 2020). The genetically and phenotypically diverse leopards of South Africa offer a valuable experimental platform for phenotype marker-assisted research in the future and theoretical studies on mechanisms that maintain biological diversity throughout evolutionary history.

Methods

Sample collection

Four leopard samples were collected in the Mpumalanga province, NSA, including a red leopard, wildtype leopard from the CA clade (gold), wildtype leopard from the SA clade (green), and a black leopard from the Sebaka Game Reserve (Figure 1; Supplementary Table S1). We added a genome of a wildtype Indian leopard (*P. p. fusca*) for comparison (PREJB43565; Paijmans et al., 2021). A blood sample was collected from the captive black leopard, whereas tissue samples were collected of the other leopards that were opportunistically collected from roadkills. No animal was killed for this study. The samples were stored at the Wildlife Genomics laboratory at −20 °C before DNA extraction. DNA was isolated using the QIAgen Blood and Tissue kit (Qiagen, Valencia, CA, United States), applying the support protocol for blood and tissue samples. Double-stranded DNA was quantified using QuBit, and samples were run on an agarose gel to assess possible DNA fragmentation visually.

Genome mapping

DNA extractions of molecular weight >100 kb were sequenced on an Illumina HiSeq 2500 platform by a commercial provider using paired-end (2 × 150 bp) chemistry following the manufacturer's instruction. We targeted 10X depth of coverage and generated whole-genome sequencing data for four leopards that each have unique traits. The paired-ends reads of the 4 individuals and whole genome sequence data from a deeply sequenced Indian leopard (Fusca) (PREJB43565; Paijmans et al., 2021) were mapped to an Amur leopard reference genome (*P. p. orientalis*; ASM2436296; Wei et al., 2011) using BWA-mem2 v2.1.1 (Li, 2013) to generate a BAM file for each of the 5 individuals. Read duplicates were removed and all BAM files were merged, sorted and indexed with SAMtools v.1.9 (Li et al., 2009). Mapping statistics were obtained using Qualimap (García-Alcalde et al., 2012) (Supplementary Table S2).

Variant calling

For population-based variant calling, we used a pipeline by Lensing (2023) with FreeBayes v1.3.7 (Garrison & Marth, 2012) to identify SNPs, insertions and deletions (indels), which provide insight into the genetic variation underlying phenotypic differences between individuals. We used the following settings: min-base-quality 10, min-alternate-fraction 0.2, haplotype-length 0, ploidy 2 and min-alternate-count 2. Variants were filtered based on a minimum quality score of 20 using vcffilter of VCFlib v1.0.0-rc1 (Garrison et al., 2022). SnpEffect v5.2c (Cingolani et al., 2012) was used for variant annotation. The following variant classes were considered as high-impact candidate variants by SNPeff: exon_loss_variant,

frameshift_variant, feature_ablation,splice_acceptor_variant, splice_donor_variant, stop_lost, start_lost, stop_gained, and transcript_variant. Principal component analysis (PCA) was performed using PLINK on the filtered vcf file (and plotted using the default R plotting utilities).

SV calling

We used a pipeline by CarolinaPB (github/CarolinaPB/population-structural-var-calling=smoove) to perform SV calling with Smoove v0.2.8 (Pedersen & Quinlan, 2019). The Smoove pipeline uses "Lumpy" v0.2.14 to call SVs in each sample relative to the reference genome, using signals from split reads and discordant paired end reads to predict breakpoints of deletions, duplications, and inversions and annotates all variants (Brandler et al., 2016; Layer et al., 2014). SVtools v0.4.0 was used to combine all SV calls into one single variant call format file (Larson et al., 2019). All SVs were genotyped using SVTyper v0.7.0 (Chiang et al., 2015) and annotated with read coverage using Duphold v0.2.3 (Pedersen & Quinlan, 2019). We looked for SVs in the variant call format output file from Smoove, where we applied strict filters for an SV deletion DHFFC_0/1 < 0.7, and a SV duplication DHBFC_0/1 > 1.3. We sorted them by length and used JBrowse (Buels et al., 2016) to manually inspect the SVs. In this study, we only identified deletions, duplications, and inversions, as other SV types, such as translocations and insertions, cannot be detected with Lumpy.

Genomic diversity

We estimated nucleotide diversity (π) along the genome in non-overlapping windows using VCFtools v0.1.15 (Danecek et al., 2011). Parameters to measure π of all samples were adapted from Yuan et al., (2023): window-pi 50,000--window-pi-step 20,000--maf 0.05 --max-missing 0.90. Boxplots and histograms for overall nucleotide diversity per individual were made with the package snpden_plot (Figueiró, 2021) and ggplot2 (Wilkinson, 2011) in R. We also created plots for each scaffold per individual based on a script from Bursell et al., (2022). To identify ROHs on all autosomes of the four sequenced individuals, we used BCFTools v1.9 (Danecek & McCarthy, 2017), with adjusted parameters: roh -G30 --AF-dflt 0.4. We also measured Froh, which is the inbreeding coefficient (F) derived from the ROHs in the genome, retrieved by dividing the total length of the genome covered in ROHs (in bp) by the full length of the genome (in bp). We used pairwise sequentially Markovian coalescent (PSMC) plots (Li & Durbin, 2011) to infer the population demographic history through changes in effective population size (Ne), applying default settings. We followed the procedure for generating a diploid sequence per individual using BCFtools (Danecek & McCarthy, 2017) and -c for calling genotypes. For scaling, we assumed a mutation rate of 1.43e-08 mutations per site per generation, and a generation time of 5 years, based on a previous study (Kim et al., 2016).

Genomic divergence

We quantified the proportion of the genome that is IBD between the leopard pairs using the PIHAT function in PLINK v1.07 (Chang et al., 2015; Purcell et al., 2007). The data was pruned for linkage disequilibrium using a 1000-kb window size, 100-kb step size and r 2 threshold of 0.2 (second-degree relative) in order to remove any false signals of population structure caused by related individuals clustering as distinct groups. The IBD ratio represents the observed proportion of shared alleles divided by the expected proportion of alleles shared under the assumption of random mating and no population substructure (Zheng & Weir, 2016). This can be used as a proxy for genetic differentiation between individuals. We also quantified the proportion of shared alleles across the genome between each leopard pair using the Dst scores. Genetic distance (D) between individuals was derived as 1-Dst. To see how our leopard samples fit in with the rest of Africa, we mapped and variant called (both small polymorphisms and SVs) our samples and the previously published genomes by Pečnerová et al. (2021) (ENA: PRJEB41230) to PanPar1.0 (GenBank: GCF_001857705.1) using the same methods as described above. We excluded Indian leopard genomes to avoid the potential strong polarizing influence of a distant outgroup. We also removed samples that were excluded by Pečnerová et al. (2021) due to either low DNA quality, species mis-labeling, or sample duplication (Supplementary Table S3), resulting in 39 African leopard samples, to which the red leopard, and wildtype South African leopards (CA and SA) were added. Two PCA plots based on SNVs obtained with FreeBayes and SVs obtained with Lumpy were created. We also applied the clustering software Admixture v1.3.0 (Alexander et al., 2009) to look further into population structure, using PLINK formatted files as input and maximum likelihood-based models to infer underlying ancestry for unrelated individuals. In order to infer the most likely value of population clusters (K), we ran maximum likelihood analysis with K = 2–5. The best number of K clusters was based on the cv errors, which is the value with the lowest cross-validation. Results were plotted in CLUMPAK (Kopelman et al., 2015). With the dataset provided by Pečnerová et al. (2021), we also ran NGSadmix (Skotte et al., 2013), based on genotype likelihoods, to include low coverage-data. To avoid the polarizing effect of an outgroup, we ran this analysis with African leopards only. Values of K = 2–6 were run.

Candidate genes

The mapped reads of ASIP (NW_026526821.1, 24798051-24869882) and TYRP1 (NW_026526766.1, 37906523-37927005) were retrieved using samtools-cat. The protein structure of the mutated genes was predicted with RoseTTAFold (Baek et al., 2021), after which we searched for variants that were either homozygous (1/1), heterozygous (0/1 or 1/0) or absent (0/0) in the color morphs and wildtypes. In the SV output file, we also searched for 171 pigmentation-associated genes that have been identified in mice (Baxter et al., 2019), which was updated in 2022 (https://www.ifpcs.org/colorgenes/).

Results

The number of mapped reads averaged 99.6% (SD 0.004) in NSA leopards (belonging to red leopard, "gold" CA leopard, and "green" SA leopard) and 80.71% in black leopards, with an average sequencing depth of 8.41 (SD 19.98) across the genome, and an average mapping quality of 36.51 (SD 0.26). The average GC content was 42.87% (SD 0.006). Quality filtering resulted in a final dataset of 16,335,294 small polymorphisms, of which 13,776,069 were SNPs and 2,494,259 were indels (short insertions and deletions); see Supplementary Table S2 for global statistics of *Panthera pardus* genomes. After SV calling, we found 27,213 deletions, 1,101 duplications, and 102 inversions.

Genomic diversity

Among the 13,776,069 filtered SNPs, an average of 2,944,210 SNPs were heterozygous, which varied from 1.2 to 2.1 million heterozygote sites in Indian leopards to 3.7–3.9 million in leopards from NSA. When estimating the proportion of heterozygous sites per sample, we inferred an average of 0.12 (SD 0.05) heterozygous sites for Indian leopards and 0.28 (SD 0.01) for NSA leopards. Leopards with the highest ROH coverage were wildtype Indian (Fusca) and black leopards (Figure 2A), with over 20,000 ROHs distributed among all autosomal chromosomes. NSA leopards had much higher levels of genetic diversity (Kruskal–Wallis chi-squared = 109.16, *df* = 5, *p*-value < 0.001), with less than 15,000 ROHs. The individual with the lowest inbreeding coefficient (Froh) was green (0.33), followed by gold (0.40), and finally red leopards (0.45). The estimated nucleotide diversity (π) across chromosomes showed large blocks of low diversity in the black leopard, but only several short regions of low diversity in NSA leopards (Figure 2B; Supplementary Figure S1). We estimated lower autosome-wide diversity in the black leopard (0.0007) compared to the Indian (Fusca; 0.0011), and that of South Africa leopards (red: 0.0011, gold: 0.0019, and green: 0.0019) (Kruskal–Wallis chi-squared = 40,102, *df* = 4, *p*-value < 0.001). See Supplementary Table S4 for all summarized diversity statistics.

Genomic divergence

The PIHAT scores by PLINK indicated that all individuals were unrelated (0) and could thus be kept for subsequent analyses. Our Identity By Descent (IBD) results illustrated that a large proportion of alleles in NSA leopards are shared by IBD (0.95–1.13), suggesting that they are part of the same substructure

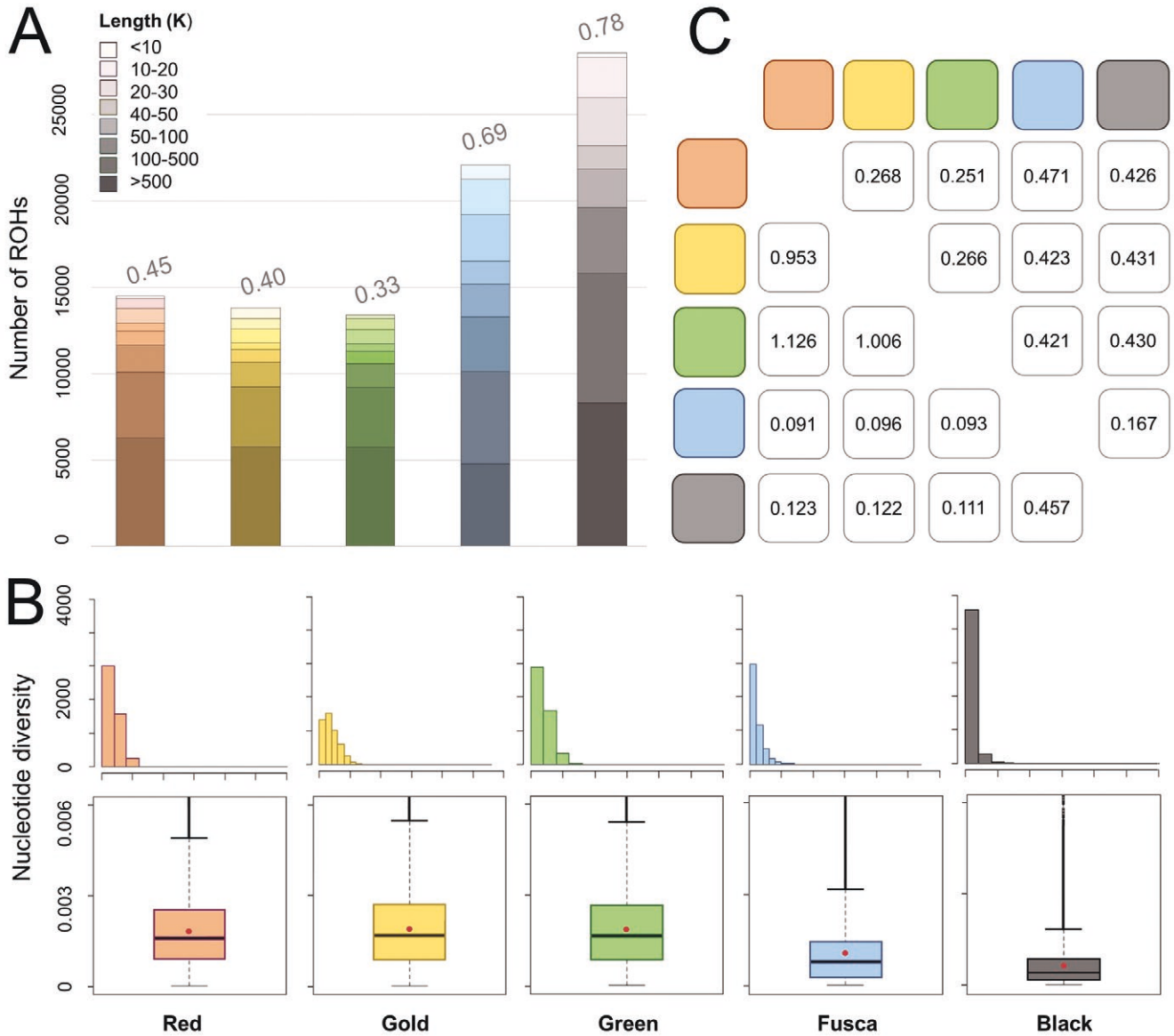

Figure 2. Genomic diversity of leopards from northern South Africa. (A) Runs of homozygosity, binned by their length (*K* = 1,000 base pairs) of five leopards: "red" color morph leopard, wildtype "gold" (mitochondrial CA clade) and wildtype "green" (mitochondrial SA clade), wildtype Indian leopard (Fusca) and black (Indian) leopard. The Froh measurements indicated on each bar showing how inbred individuals are, ranging from 0 (lowest) to 1 (highest). (B) Histograms and boxplots of nucleotide diversity (π) in the five leopards. (C) Relatedness among five leopards based on genetic distance (*D*) (top right) and Identity By Descent (IBD) ratios (bottom left). Only polymorphic SNPs were used that were pruned with PLINK.

(Figure 2C). Indian leopards share much less alleles with NSA leopards (0.091–0.096) and also a relatively large position of alleles amongst themselves (0.457). Genetic distance (D) showed a close relation within NSA leopards (D = 0.261, SD 0.009) and Indian leopards (D = 0.167), but a much larger D between both groups (D = 0.434, SD 0.019). Within the South African, PCAs of 39 previously published African leopard genomes captured a north-to-south cline (PC1), in which NSA leopards are closely related to Zambian ones (Figure 3A; Supplementary Figure S2). The northern part of Africa, in this case the geographic location of Ghana, is an outlier, and the other sampling locations are not discretely clustered along the PC axes (Figure 3A). The admixture plot showed genetic structure among our samples, where the highest likelihood was retrieved for K = 2, separating Indian and African leopards (Figure 3B). When including samples from Pečnerová et al. (2021), we found the highest K = 4, separating Ghana, SA (Namibia, South Africa), eastern Africa (Zambia and Tanzania), and northern Tanzania. NSA leopards showed admixed ancestry between southern and eastern Africa (Figure 3B). The NSA leopards and Indian leopards went through the same demographic history, which experienced a reduced Ne around 800 thousand years ago (kya), and the divergence between NSA and Indian leopards appeared around 300 kya (Figure 3C). Minor differences in population demographic history were also apparent between wildtype and black Indian leopards.

SVs

To identify genomic polymorphisms that could be of evolutionary significance or adaptive value, we screened the genomes for SVs (Supplementary Dataset S1). Of all 28,416 SVs, a total of 27,213 were deletions, 1,101 duplications, and 102 inversions. We found 14,030 SVs in red leopards, 15,061 in gold, and 14,933 in green leopards from South Africa. Of these, 242 (0.74%), 303 (0.93%) and 314 (0.97%) SVs were homozygous and occurred in red, gold and green leopards exclusively. In Indian leopards, we found 20,804 SVs in wildtype and 18,202 in black leopards, of which respectively 761 (2.34%) and 508 (1.56%) SVs were homozygous and unique to those leopards. The only profound adaptive differences appear to be between Indian and African leopards, in which respectively 4,101 (13%) and 3,864 (12%) of SVs occurred in all individuals of these subspecies, whether in homozygous or heterozygous state. When looking at the PCA using only SVs, in which NSA leopards were compared to the rest of the continent, a slightly different pattern emerges compared to SNVs (Figure 3A), in which the first and second principal components capture genetic variation in two clusters (explaining 5.76% of the variation), with admixed individuals being inconsistent with their geographic location. Overall, the structural PCA shows a lack of isolation by distance or genomic divergence between NSA leopards and the

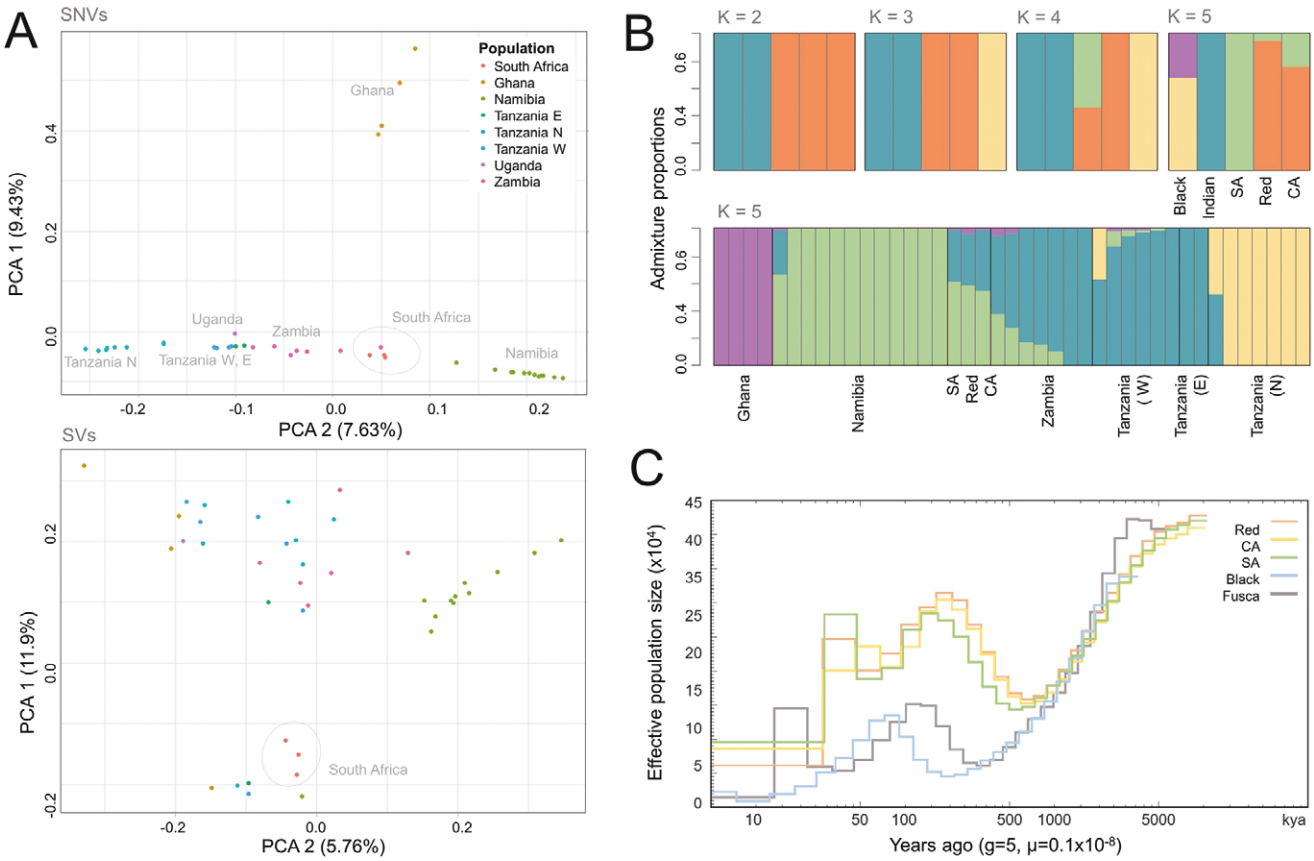

Figure 3. Genomic divergence of leopards from northern South Africa (NSA). (A) PCA of all genome wide SNPs and indels (top) and structural variants (bottom) retrieved by using PLINK. All individuals were colored by sampling location. For the structural PCA, we left out outliers that had low mapping coverage and a sample that was predicted to have a higher contamination rate. (B) Population admixture based on ancestry proportions for the number of discrete populations (K), where NSA and Indian leopards were compared (high coverage) and NSA leopard to genomes available from the rest of Africa (low coverage), provided by Pečnerová et al. (2021). (C) Effective population sizes (Ne) reconstructed by using pairwise sequentially Markovian coalescent assuming a mutation rate of 1.43e-08 and a generation time of 5 years.

rest of Africa for all SVs across the genomes (Supplementary Table S5).

After applying strict SV filters, we were left with 13,209 deletions (ranging from 18 to 47,384,918 base pairs in length) and 321 duplications (130 to 836,337 bp). Of the deletions, 3,488 were unique to NSA leopards, and 9,654 to Indian leopards (Figure 4A). Of the duplications, 64 were unique to NSA leopards, and 257 to Indian leopards. What is also striking is that Indian leopards had a much higher number of homozygous SVs compared to NSA leopards (Figure 4B). Furthermore, the total number of SVs unique to black leopards was much smaller than that of wildtype Indian leopards (775 vs. 2,551), which was also observed when applying strict filters (8 vs. 81). The green leopard had the smallest number of deletions and duplications and shared most SVs with red leopard, even though they belonged to a different mitochondrial clade, indicating that the overall impact of mitochondrial divergence on protein-coding genes is low. We also found 102 inversions (ranging in length from 28 to 84,902,811 bp), of which 40 were unique to Indian leopards and 20 to NSA leopards. Of the latter, five inversions were unique to the red leopard, and four to gold and green leopards. Inversions that stood out were one of 61,299 bp in green leopard and one of 140,323 bp in gold leopard. When searching for the gene function of the inversions, we found no obvious links that could relate to local adaptation.

High-impact mutations

To find gene variants that were in close proximity (genetically linked) to the causative mutations related to the color morphs, we filtered SNPs and single base-pair indels (SNVs) that had a high impact on protein-coding genes (i.e., variants that were homozygous and caused exon loss, a frameshift/transcript variant, feature ablation, splice acceptor/donor, or stop codon loss/start), which we refer to as HIMs (Supplementary Dataset S2). We found a total number (N) of 2,065 SNVs predicted to have a high impact (Supplemetnary Table S5). Of all HIMs, 208 (10%) were shared among NSA

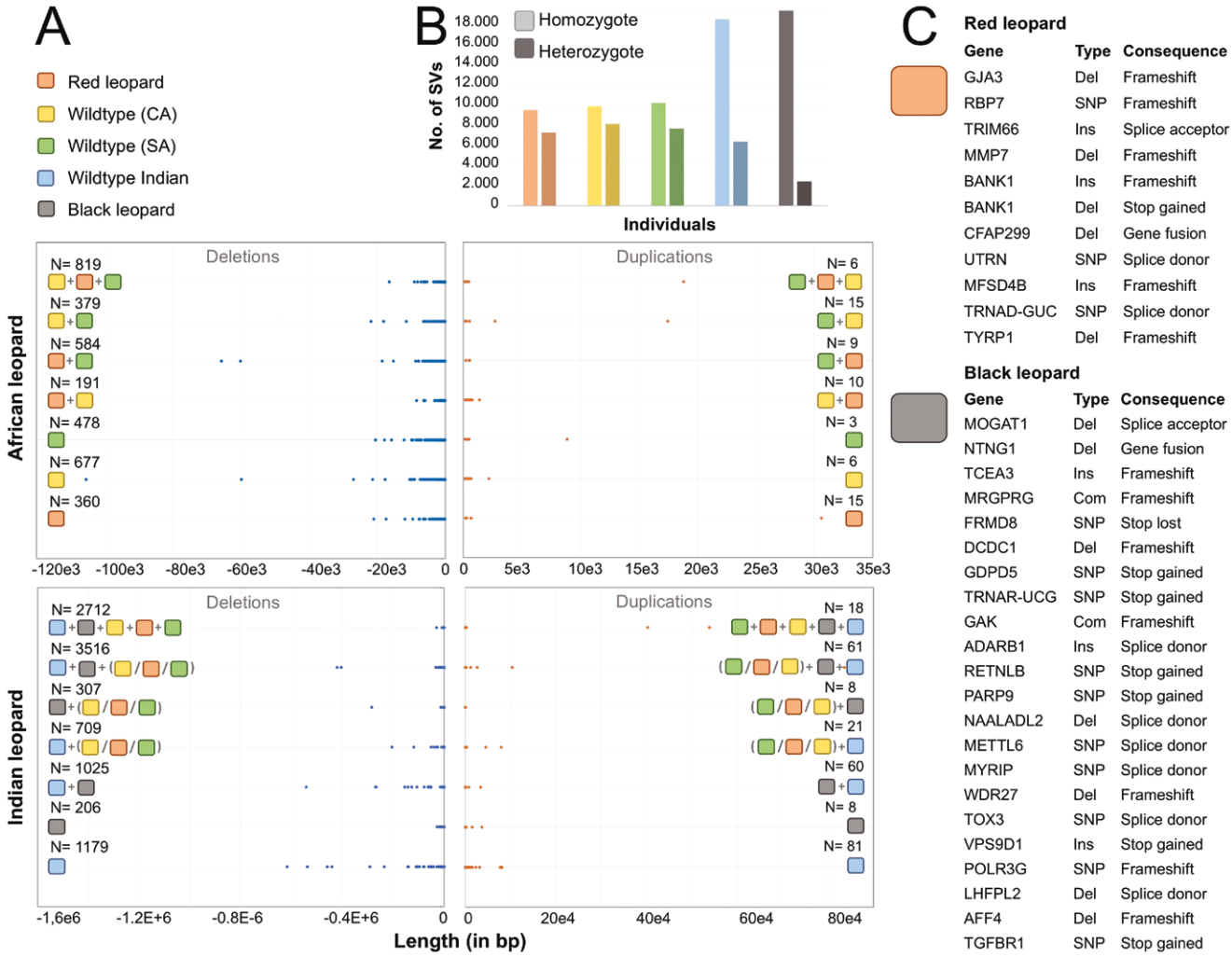

Figure 4. Single nucleotide variants (SNVs), including SNPs and single base-pair indels, and SVs across five leopards. (A) Strictly filtered SVs, of deletions (DHFFC_0/1 < 0.7) and duplications (DHBFC_0/1 > 1.3) of five leopards: "red" color morph leopard, wildtype "gold" (mitochondrial CA clade) and wildtype "green" (mitochondrial SA clade), black (Indian) leopard and wildtype Indian leopard (*P. p. fusca*). Outliers were removed from the figure: a deletion of 437,358 base pairs (bp) found in the CA "gold" leopard and of 7,112,657 bp in Fusca, and a deletion of 47,384,918 bp in Black/Fusca. (B) Histogram of the number of filtered SVs per leopard. (C) SNVs that were predicted to be HIMs that were homozygous in red or black leopards and absent in other leopards (Supplementary Table S6). SNP = missense mutation, Del = deletion, Ins = insertion, Com = complex, e.g., a frameshift variant.

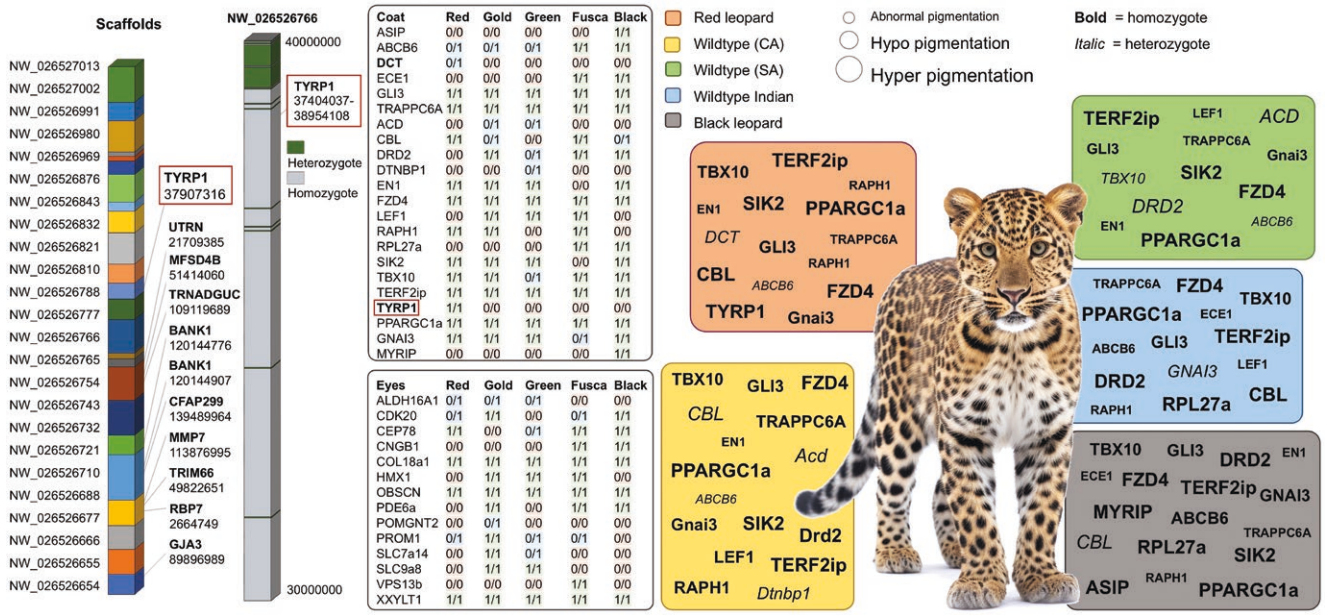

Figure 5. Genes that relate to coat color or eye pigmentation in *Panthera pardus*. The tables show all genes relating to either coat (top) and eye (bottom) pigmentation in five leopard samples: a "red" color morph leopard, wildtype of the mitochondrial CA clade (from South Africa; *P. p. pardus*), wildtype of the mitochondrial SA clade, a black leopard (Indian; *P. p. fusca*), and wildtype Indian leopard. Mutations are either homozygote (1/1), heterozygote (0/1), or absent (0/0) (Supplementary Table S7). Only coat color or pattern-related genes were visualized in the figure. Abnormal pigmentation could entail patches or irregularities, hypo pigmentation causes a diluted coat color, and hyper pigmentation a darkened pigmentation. The picture license (user55626824) was granted through www.freepik.com).

leopards (red, gold and green), and 142 (6.8%) among Indian leopards (black and wildtype). Only a small proportion of HIMs were homozygote (1/1) in either red leopard ($N = 48$; 2.3%), gold leopard ($N = 50$; 2.3%), green leopard ($N = 42$; 2%) or black leopards ($N = 82$; 4%), and heterozygote (1/0 or 0/1) or absent (0/0) in other leopards. Of these, 17 HIMs (0.8%) exclusively (1/1) occurred in red leopards and were absent in other leopards (0/0). The total number of HIMs was 14 (0.7%) for gold leopards (CA clade), and 9 (0.5%) for green leopards (SA clade), indicating that, overall, the impact on protein-coding genes is low. In red leopards, 11 HIMs belonged to classified genes (Figure 4C), including TYRP1. When extracting the TYRP1 gene, we found a total of 7 SNPs that were unique to red leopards as well as the causative deletion (c.188delG). We also searched for HIMs that were unique to black leopards, which totaled 39 mutations, of which 22 belonged to classified genes. Unexpectedly, none of these occurred in the ASIP gene or other genes involved in the melanin pathway. When extracting ASIP from the alignment, we found that the causative mutation in our sampled individual was the same as *P. p. delacouri* (c.333C>A). We found another missense mutation (c.106C>A) that was homozygote in black leopards and absent in other leopards. Overall, the number of homozygote HIMs across the genome appeared to be higher in black leopards compared to red leopards (1099 vs. 883), and our results illustrate that many HIMs that we found in black leopards may be associated with health deficits (Supplementary Table S6).

Coat color-related genes

To identify potential causative HIMs related to color polymorphism, we searched for 171 known pigmentation-associated genes that relate to skin and eye phenotypes, including abnormal hair growth or pigmentation in mice (https://www.ifpcs.org/colorgenes/). We identified 36 HIMs in five polymorphic leopard samples (Figure 5). We found that DCT was unique to red leopards, which is a gene known to cause diluted coat color and abnormal iris color in mice (Supplementary Table S7). Although TYRP1 and DCT were not located in the same genomic region, we found that both were located in a genomic bin that had a long run of homozygosity (TYRP1: scaffold NW_026526766, position 37404037-38954108, and DCT: scaffold NW_026526654, position 1903631-2744041). An amino acid change in MYRIP was unique to black leopards, which is a gene that regulates melanosome motility. When screening for eye-specific pigmentation genes, we found that HMX1 was missing in red and black leopards, which relates to eye and auricular developmental abnormalities in mice, such as developmental retinal layer disorganization, RPE increased thickness, and microphthalmia. Of all 36 pigmentation-related genes we found in leopards, 12 were fixed in all five leopards (present as either homozygote or heterozygote). Of these, GLI3, TRAPPC6A, FZD4, TERF2ip, PPARGC1a, COL18a1, OBSCN and XXYLT1 occurred in homozygosity in all leopards, suggesting a critical importance for their appearance. In mice, they are (respectively) known to relate to belly spots, irregular coat pigmentation, light black or silver agouti background color, hyperpigmentation, pigmentation abnormalities, abnormal iris stromal pigmentation, and abnormal retinal pigmentation (Baxter et al., 2019).

Discussion

In this study, we applied comparative genomics to investigate genome-level diversity and divergence in two color morphs and two mitochondrial clades that occur in South Africa. The red leopard is a native, wild phenotype, whereas the black leopard only occurs in captivity and is of Indian descent. As a

result of keeping the black phenotype alive in captivity, generations of inbreeding have left their marks, which were primarily visible through long ROHs, low nucleotide diversity across the genome, and a high number of deleterious mutations in homozygous state. The number of SVs unique to black leopards was low, in stark contrast to the wildtype Indian leopard, which may indicate that hybridization among subspecies has occurred in captivity. Some admixture of the black leopard was observed at $K = 5$, although this was not caused by NSA alleles. We saw no such pattern for the red leopard, although ROHs were slightly longer compared to wildtype leopards, with seemingly some deleterious mutations relating to its phenotype as a result. This was expected, given the fact that the red leopard belongs to the same population as the wildtypes, and that the mutation causing the phenotype is recessive and segregates at low frequency, which means that inbreeding increases the chance of this allele becoming homozygous and thus expressed. The two wildtype leopards belonged to two deeply diverged mitochondrial clades, yet no substantial differences were observed at a nuclear level. This clearly illustrates the homogenizing effect of recombination and introgression, and highlights that although NSA leopards may be phenotypically and historically unique, they are not genetically different. This was confirmed with PCAs, which showed no divergence between leopards from NSA and the rest of Africa when comparing SNVs and SVs.

Wild cats display a large diversity in coat color and pattern, likely because they are more reliant on camouflage compared to other terrestrial carnivores (Darul et al., 2022; Schneider et al., 2012). Because phenotypic variation is a strong driver of speciation (Heuer et al., 2024), it has been suggested that the Felidae may contain more protein polymorphisms that are associated with pigmentation genes (Tensen & Fischer, 2024). When we screened the leopard genomes for pigment-type switching genes found in mice (Baxter et al., 2019), we located high-impact amino acid substitutions in 36 genes. A previous study on clouded leopards (*Neofelis nebulosa*), which is a medium size wild cat with similar coat markings as leopards (Yuan et al., 2023), found variants in MYSM1 and GOLGB1 genes that are closely associated with skin phenotypes, including abnormal hair follicle patterning and pigmentation (Yuan et al., 2023), different to the ones found in our study. Among our findings, GLI3 and TRAAPC6A may relate to the patchy coat pattern of leopards instead, based on their gene functions in mice (Gwynn et al., 2006; Sun et al., 2023). For future research, it would be interesting to see which of the coat and eye color related genes are under positive selection (Yuan et al., 2023). Our results also showed a DCT mutation in red leopards that was absent in any other leopard, which could be linked to the tyrosinase (TYR) pathway as well (Garrido et al., 2021; Kaelin et al., 2012). In mice, a slaty-light phenotype was caused by a TYRP2/DCT association (Budd & Jackson, 1995). Because genes rarely act alone and are known to be linked (Hensch et al., 2019), our results may suggest that a similar mechanism occurs in red leopards, for a TYRP1/DCT association. In black leopards, we found a MARIP mutation that may be of interest, as it was absent in any other leopard and known to be linked to melanosomes in mice (Klomp et al., 2007). We also found a LEF1 amino acid change to be missing in red and black leopards, which is known to regulate TYR gene expression, with an important role in regulating melanocyte development in cats (Kaelin et al., 2021; Wang et al., 2015).

When screening the red leopard genome for HIMs, we identified the TYRP1 mutation that was previously published (c.188delG) (Tensen et al., 2022), alongside with ten other HIMs. Among these was a stop gained in BLANK1, which is a gene that is associated with a skin disease in mice (Kozyrev et al., 2012), and a lupus-like disease in dogs (Wilbe et al. 2010). These were not located in the same genomic region and thus not directly linked through potential hitch-hiking. A correlation between diluted coat color mutations, such a leucism and albinism, and skin diseases has been made before, although the topic remains heavily understudied (Lucati & López-Baucells, 2017). We also found a deletion and frameshift in the GJA3 gene, which has been associated with the eye defect congenital cataract (Li et al., 2016). As mentioned before, an important aspect of the red leopard phenotype is its light eyes, which range from light brown to bright blue, possibly depending on whether mutations occur in homozygosity or heterozygosity (Tensen et al., 2022). In albinistic animals, light blue eyes have been related to compromised vision (Caro, 2005; Laikre et al., 1996) and deafness (Strain, 2007), which would indicate that the red leopard may also suffer some fitness defects. Interestingly, we found a 3,663 base pair deletion in the TMTC4 gene in the red leopard, which in mice was associated with hearing loss (Li et al., 2018). Furthermore, mutations in the KIT gene have been associated with deafness in mice and cats (Strain, 2015), and we found a deletion in this gene that was homozygous in red leopards (and heterozygous in black and Indian leopards). Nonetheless, a specific gene mutation responsible for deafness in white, blue-eyed cats has not yet been confirmed, and the condition of the sampled red leopard is unknown. That this phenotype is still increasing in numbers either suggests that the defects are not deleterious to its survival, and thus reproductive success (Power et al., 2024), or that genetic drift may be locally overruling natural selection (Tensen & Fischer, 2024). If a recessive mutation is not or only mildly deleterious, it may not be purged out of a population that suffers from genetic drift (Wellenreuther et al., 2014), which would be supported by longer ROHs and lower nucleotide diversity in red leopards than the other two NSA leopards. Something similar has occurred in pseudo-melanistic tigers in a small and isolated population in India, where the recessive phenotypic trait is reaching near fixation due to drift and the lack of a selective disadvantage (Sagar et al., 2021).

In the black leopard, we found an ASIP gene mutation on the c.333A>C position, causing a stop codon, similar to the Indochinese leopard (*P. p. delacouri*), and thus different from the Sri Lankan leopard (*P. p. fusca*), which has a missense mutation on c.353C>A (Schneider et al., 2012; Sooriyabandara et al., 2023). A different causal mutation would have been a possibility, since melanism occurred at least four times independently in the cat family (Eizirik et al., 2010). We found another missense mutation at position c.106C>A that is either absent in other black leopards or has been previously overlooked, which may have a phenotypic consequence as well. Melanism is typically not associated with health deficits, but may even confer a selective benefit under certain ecological circumstances (Majerus & Mundy, 2003). In our case, however, we found many HIMs in the black leopard, likely due to the effect of enforced inbreeding in captivity to sustain the black phenotype (Warrick, 2010; Willoughby et al., 2015). To give some illustrations: MRGPRG mutations are associated with a neurogenic disease causing chronic itch

(Liu & Dong, 2015), FRMD8 mutations can cause inflammatory diseases and colon cancer growth (Künzel et al., 2018), and mice with POLR3G knock out mutations had impaired development and often died prematurely (Wang et al., 2020). In combination with the exceptionally low nucleotide diversity and long ROHs we found in the black leopard, we conclude that inbreeding has likely negatively affected the individual's health, generally referred to as inbreeding depression (Charlesworth & Willis, 2009). Unfortunately, we have no information about the physical health of the individual from which the sample was retrieved.

Although SNPs are typically considered to make up the majority of genetic variation, evidence is growing that SVs represent a more significant source of diversity, related to genotype–phenotype associations, with a major role in local adaptation and speciation (Mérot et al., 2020; Wellenreuther et al., 2019). When we searched for SVs, we observed less diversity (by number of large deletions and duplications) in the black leopard, and an exceptionally high number of SVs unique to wildtype Indian leopards, *P. p. fusca*. This large difference may indicate that hybridization in captivity between Indian and NSA leopards has occurred (Zhang et al., 2021). Subspecies hybridization often occurs in captivity (Powell, 2023), including in privately owned reserves in South Africa, such as Sebaka Game Reserve (Benjamin-Fink & Reilly,). Our Admixture plot showed that at $K = 5$ the black leopard had traces of admixture. However, the risk remains that this is an artifact of long ROHs, which may bias cluster assignment due to deviation from Hardy–Weinberg expectations (Pritchard et al., 2000). Detecting introgression through hybridization remains difficult with only few individuals and no parental genomes (Randi, 2008). Furthermore, SVs are known to suppress recombination and preserve linkage between alleles even in the face of admixture (Tigano & Friesen, 2016). Large inversions are most frequently associated with the maintenance of population differentiation and adaptive phenotypes (Faria et al., 2019; Mérot et al., 2020), of which we found 102 in our leopard samples. However, we found no obvious link between our phenotypes and large SVs, either through large inversions or gene duplications that were unique to NSA leopards, despite their varying demographic history.

The mitochondrial CA and SA clades that were compared, according to their location in the northern and southern part of the African continent (Anco et al., 2018), likely originated during the Mid-Pleistocene when savanna habitat retracted into isolated islands (Tensen et al., 2024). Our PSMC results suggest that all leopards went through similar demographic histories, with a main divergence event between NSA and Indian leopards, likely resulting from an out-of-Africa dispersal event (Pečnerová et al., 2021). Divergence between black and wildtype Indian leopards was also noticeable but may be uninformative, because PSMC outputs become unreliable after only few generations when species experienced artificial selection (Li et al., 2021). An overall strong reduction in Ne was observed around 800 kya, which was detected in previous studies but dated earlier (500 kya), likely because of differences in set generation time (Paijmans et al., 2021; Pečnerová et al., 2021). All leopards went through a mtDNA divergence event during this time as well, likely resulting from Mid-Pleistocene climatic cycles (Pečnerová et al., 2021; Tensen et al., 2024). Therefore, our results may support the hypothesis that CA and SA once formed separate populations with small effective populations, which is known to speed up divergence events due to the stochastic nature of genetic drift (Charlesworth, 2009), but have since come into secondary contact.

Currently, and likely for the past ten-thousand years when geological barriers were no longer present (Harrington et al., 2018), dispersal is predicted to occur across SA (Fattebert et al., 2013). In South Africa, long-distance leopard dispersal (>100 km^2) has been illustrated through radio-collaring and camera-trapping data (Greyling et al., 2023; McManus et al., 2022), although some effects of anthropogenic habitat fragmentation on genetic diversity occurs (McManus et al., 2015; Naude et al., 2020). This has only been tested for neutral genes, such as microsatellites and mtDNA so far, which are not always linked to adaptive, protein-coding genes (Mittell et al., 2015; Väli et al., 2008; Zimmerman et al., 2020). With regards to ancient divergence, as evidenced through the mitogenomes, secondary contact after allopatric speciation is considered to alleviate genetic diversity in species (Dong et al., 2020). Indeed, our leopard samples had a much higher genetic diversity than the Asiatic leopards, as found previously (Paijmans et al., 2021). The previous study included one sample from South Africa and found exceptionally high heterozygosity compared to other leopards in Africa, possibly indicating hybridization (Ellegren & Galtier, 2016), and admixture of ancient clades (Tensen et al., 2024). However, we found no indications of subspecies-level differentiation of leopards in South Africa when looking at genome-wide SNVs or SVs, which is an issue that was recently raised as a result of the deep divergence on mtDNA genes (Morris et al., 2024).

Our results illustrate that mtDNA is not suitable for revealing recent patterns of population substructure (Hurst & Jiggins, 2005), nor candidate genes that relate to selective pressures or phenotypes (Dong et al., 2021; Springer et al., 2001). Mitochondrial DNA also reflects only the female lineage and neglects male-biased dispersal, which is especially profound in leopards (Fattebert et al., 2015). A lack of genetic differentiation among African leopards was also found in previous studies (Miththapala et al., 1996; Mochales-Riaño et al., 2023; Paijmans et al., 2021; Pečnerová et al., 2021; Uphyrkina et al., 2001), which suggested that leopard populations remained genetically connected after the Pleistocene due to long-distance gene flow. However, our study is the first to include South African leopards to the continental comparison. Nonetheless, our samples only originated from the Mpumalanga province, where the two mtDNA clades are known to be admixed (Ropiquet et al., 2015). In the Eastern and Western Cape of South Africa, some genetic substructuring was found when using nuclear DNA (McManus et al., 2015). Therefore, an extended study into the southern provinces may be of interest. A cryptic subspecies has been suggested to occur there, previously referred to as the "Cape leopard," based on the smaller body size (Martins & Martins, 2006). However, this notion was not supported by microsatellite and mitochondrial analyses (McManus et al., 2015; Ropiquet et al., 2015). Genetic population designation has become very important for species conservation and has been included in the guidelines for reintroductions and other translocations by the International Union for Conservation of Nature (IUCN/SSC, 2013). Leopard translocations, which are very common in South Africa for human-wildlife conflict mitigation (McManus et al., 2022; Power et al., 2021), are generally guided by the suggestions of Ropiquet et al. (2015), who concluded that the population structure of leopards

in South Africa is driven by isolation by distance and that translocations should be restricted to 82 km, even though leopards disperse over significantly larger distances (>300 km; Fattebert et al., 2013). Consequently, when leopards are translocated over short distances, they often exhibit homing behavior, returning to their original ranges. Therefore, translocations tend to be unsuccessful when conducted within distances below approximately 150 km (McManus et al., 2022). Integrating insights from spatial ecology, resource selection functions, conservation biology, and genetics is imperative when creating pragmatic and efficacious conservation strategies. We here recommend a genome-wide study of leopard populations in other areas of South Africa, and particularly Western Cape, Eastern Cape, and North West, Northern Cape, to see whether this pattern is due to recent habitat fragmentation or evolutionary drivers instead. Our results offer a good baseline for such a study.

In conclusion, we provide some interesting insights into evolutionary mechanisms that drive genetic and phenotypic diversity in species. We conclude that the red leopard phenotype likely relates to genetic drift, and possibly to health defects such as compromised vision, illustrated by longer ROHs than other leopards and HIMs in candidate genes. The black leopard showed many more deleterious alleles, likely relating to decades of selective breeding in captivity and possibly leading to inbreeding depression. Despite the color polymorphism and mitochondrial variation in leopards from NSA, they are genomically diverse and not differentiated from each other nor the rest of Africa. PCAs further illustrated that an isolation by distance pattern can be observed when looking at all SNVs, which completely disappears when looking at SVs only. This suggests the presence of one connected metapopulation across the African continent, with no strong adaptive difference between populations. The results could form a baseline for future population genomic studies for leopards in South Africa.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

The raw sequence data (fq.gz) of the red and black leopard are available on dryad (DOI): https://doi.org/10.5061/dryad.0000000c4. The code used for analyses in this project is available on: https://git.wur.nl/kim.lensing/population-mapping-version2 (mapping), https://git.wur.nl/kim.lensing/population-variant-calling-version2 (variant calling), https://git.wur.nl/kim.lensing/population-structural-var-calling-smoove-version2 (structural variant calling).

Ethical statement

Ethical clearance was granted by the University of Johannesburg (2023-02-03/Tensen).

Author contributions

The study conception and design were done by L.T. and M.B. Sample collection was performed by G.C. Data collection was performed by K.F. and J.M. Data analysis was done by L.E. and K.L. The first draft of the manuscript was written by L.T. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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