De novo SNP Discovery in the Spotted Hyena (*Crocuta crocuta*)

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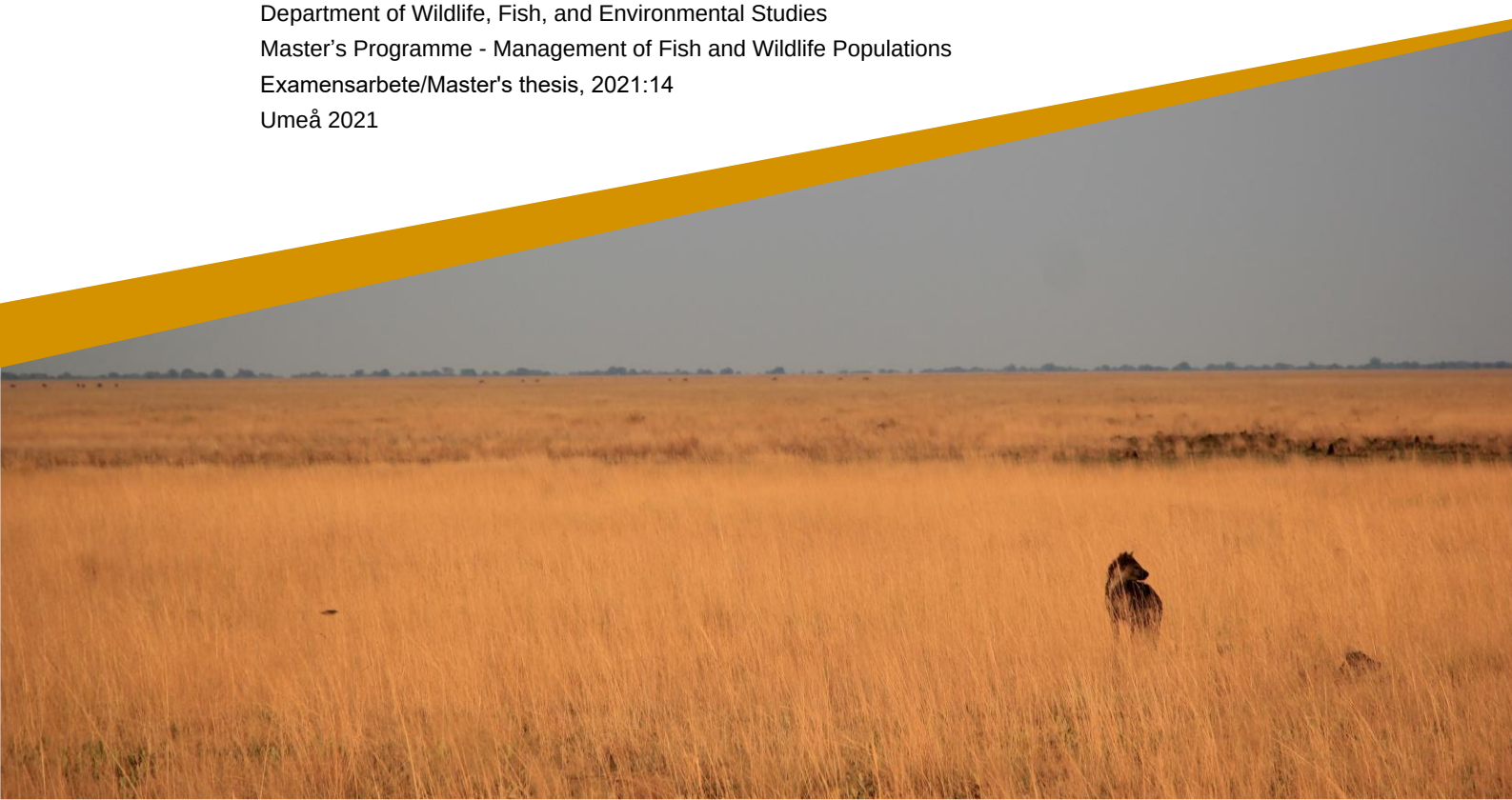

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Abstract

Relatedness studies of wild populations using reliable high-quality genetic markers enhance our understanding of a species' behavior and ecology. Spotted hyenas (*Crocuta crocuta*) have a matriarchal social structure, unique amongst carnivores. However, genetically based relatedness studies on this species have been limited, but SNPs now provide an opportunity to study kinship patterns in better detail. We have developed a panel of 74 SNP markers suitable for genotyping spotted hyenas in Zambia. High-throughput *de novo* sequencing of reduced representation libraries of 27 individuals generated millions of read pairs. Aligned reads were used as input for the Stacks pipeline, resulting in the discovery of 261,694 putative SNPs. Strict filtering criteria were applied to find high-quality SNP markers informative for individual identification and population level analyses. Validation by genotyping a set of 80 individuals resulted in the selection of 74 autosomal SNPs (77%). To obtain a fully functional panel, we recommend an additional targeted approach for the discovery of Y-chromosome and mitochondrial markers. This SNP panel is a promising tool to infer relatedness in spotted hyena populations and has the potential to yield valuable information to support management and conservation efforts.

Keywords: Single Nucleotide Polymorphism, SNP, Spotted Hyena, *Crocuta crocuta*, Relatedness, Genetic monitoring

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Glossary

Autosomal	Pertaining to a non-sex chromosome
Genetic marker	DNA sequence with a known location on a chromosome that can be used to identify individuals or species
High-throughput sequencing	Automated sequencing of multiple DNA molecules in parallel, so that large scale repetition becomes feasible
Minor allele frequency	Frequency at which the second most common allele occurs in a given population; differentiates between common and rare variants
Point mutation	A mutation that only affects a single nucleotide, most commonly involves the substitution of one base for another
Read	A sequence of nucleotides; a DNA fragment
Reduced representation library sequencing	Sequencing a representative set of DNA, rather than a whole genome, to reduce the sequencing effort
SNP	Single Nucleotide Polymorphism: a genetic marker, occurs in DNA where a base pair has variable nucleotides between individuals of a population or species
SNP-chip	A set of SNP markers; a SNP panel

1. Introduction

Genetic data are increasingly applied to support research and management of wild populations. Amongst many uses, they can be used to determine relatedness between individuals, either to validate suspected relatedness or *de novo* when prior knowledge is absent. Patterns of genetic structure and relatedness also allow for a wide range of inferences about behavior and demography. This includes, amongst others, mating systems, paternity, reproductive success, genetic diversity, geneflow, and dispersal patterns. The possibility to establish kinship and reconstruct pedigrees therefore provides a powerful tool for understanding population dynamics and individual behavior, but relies on the access to reliable high-quality genetic markers.

Single nucleotide polymorphisms (SNPs) have been shown to be highly informative and reliable genetic markers in many wild non-model species (e.g. [1-5]). A SNP is derived from an ancestral point mutation and occurs when a particular base pair has variable alleles that are found between individuals of a population or species (Fig. 1). When a point mutation (i.e., mutation of a single nucleotide) has a beneficial effect, it is often prone to selection and can therefore be maintained and passed through subsequent generations and spread throughout populations. SNPs are attractive markers as they are the most abundant form of genetic diversity within a species [6] and distributed at relatively high densities across the entire genome [7, 8]. By comparing SNP genotypes of individuals, we can extract valuable information, such as relatedness patterns.

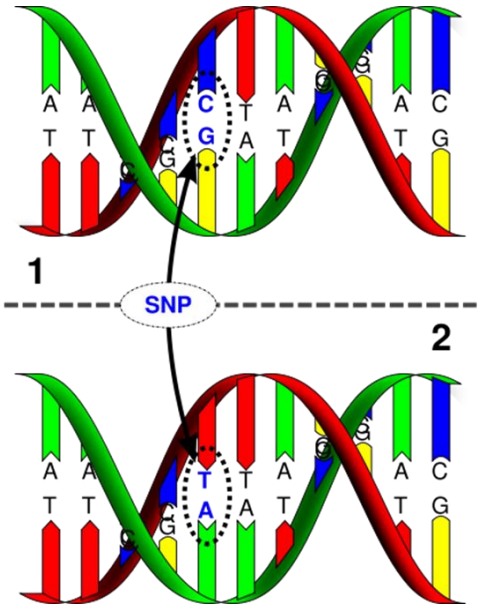

Figure 1. ***SNP marker.*** *A SNP location that consists of different alleles between two individuals (1 and 2).*

While microsatellite markers have been the markers of choice in genetic studies for the past decades [9, 10], in more recent years SNPs have been presented as a superior alternative [11]. Compared to microsatellites, their attraction lays in the lower mutation rates [12] and the bi-allelic nature of SNPs allows for more simplified genotyping that is less erroneous [7]. Contrary to microsatellites, SNP-based assays are automatically standardized across laboratories as the genotyped polymorphisms are displayed directly on the DNA sequence, allowing for a high repeatability [13, 14]. Additionally, SNPs are the more economical choice than fragment-based analyses as more loci can be genotyped at comparable cost, providing higher resolution genotypes for more confident individual assignments [3]. Another major advantage is that SNPs are much more suitable for providing high-quality genotypes from degraded or highly fragmented DNA as shorter intact target sequences are required for successful amplification of SNPs (50-70 bp) than for microsatellites (80-300 bp) [15-17]. This makes SNPs a convenient genetic marker for non-invasively collected samples of relatively low quality, such as feces [1, 18]. However, a lower resolution and statistical power can be expected per marker for bi-allelic SNPs when compared to multi-allelic microsatellites [19]. Yet, this can easily be compensated by increasing the number of SNP markers used in the identification panel [20].

The spotted hyena (*Crocuta crocuta*) is the largest living hyena species with a range throughout sub-Saharan Africa in savannas, woodlands and grasslands [21]. There are three other living species of hyena – striped hyena (*Hyaena hyaena*), brown hyena (*Hyaena brunnea*) and aardwolf (*Proteles cristata*). Even though these large carnivores are mostly known as scavengers, the diet of spotted hyenas consists in large part of prey that was killed by hunting in packs [21-27]. This hyena species has a few particularities related to their reproductive biology, most notably the presence of a pseudo-penis in females [28-32] and a matriarchal social structure, unique among carnivores, with larger dominant females leading the clan [21, 33]. These gregarious predators form clans that are generally composed of multiple matrilines of related female individuals, their offspring and immigrant males, where the latter occupies the lowest spot in the hierarchical ladder [21, 34]. In these linear dominance hierarchies, an individual's rank position is maternally inherited and determines its priority of access to food resources [31].

Genetically based relatedness studies have been conducted on several large carnivore species (e.g., [5, 35]), but similar studies on spotted hyenas have been sparse. While observational data exists for the species, the corresponding genetic data to infer relatedness has been limited to studies using microsatellites (e.g., [36, 37]). Recently however, the first draft genome assembly of a male spotted hyena has been published [38] and a mitochondrial genome of the species is also available [39, 40]. SNP-based studies of spotted hyena relatedness provide a new approach

to test our current knowledge about social structure and behavior, potentially uncovering new patterns of relatedness. Development of a SNP panel for spotted hyena would contribute greatly to this end (Fig. 2).

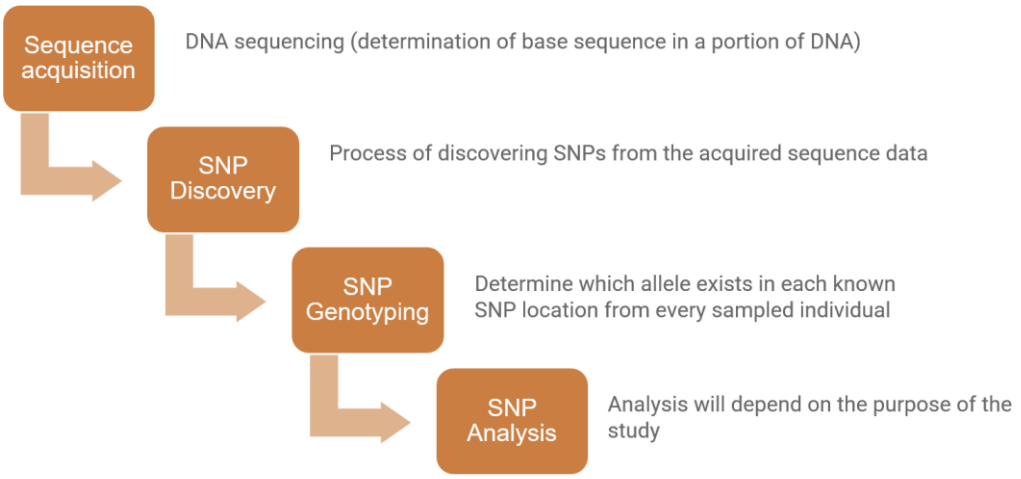

Figure 2. ***SNP Process.*** *Schematic overview of the general process of SNP-based studies. The first two steps are only necessary in absence of prior SNP information and can be avoided once a SNP panel has been developed for a certain species. Newly collected samples can then be genotyped immediately based on the existing SNP panel and used for analysis.*

The aim of this project was to develop a novel SNP panel to facilitate relatedness studies of spotted hyena. We applied a reduced representation sequencing approach for *de novo* SNP discovery. Here we present an initial set of 74 autosomal SNP markers that have been validated with an ascertainment panel of 80 spotted hyenas from three national parks in Zambia. The current SNP panel allows for reliable individual identification and relatedness estimates, but we aim to extend it with Y-chromosome sex-determination markers and mitochondrial SNPs for parentage assignment. Among many uses, this set of high-quality SNPs will be a valuable tool to study a wide array of relatedness-related topics, including population structure, dispersal, and mating patterns.

2. Materials and Methods

2.1. Sample collection & DNA extraction

We collected samples as part of long-term field studies conducted by the Zambian Carnivore Programme (ZCP), of which 27 were included for SNP discovery. Samples were collected between 2009 and 2017 in three Zambian national parks (NP); Kafue, Liuwa Plain and South Luangwa (Fig. 1). The samples that were included for the SNP discovery are tissue biopsies collected from individuals that were anesthetized in the field for the purpose of equipping them with radio collars or during snare removal interventions. These biopsy samples were collected by licensed wildlife veterinarians and immediately transferred to ethanol for storage prior to DNA extraction. All activities were performed under relevant permits from local authorities.

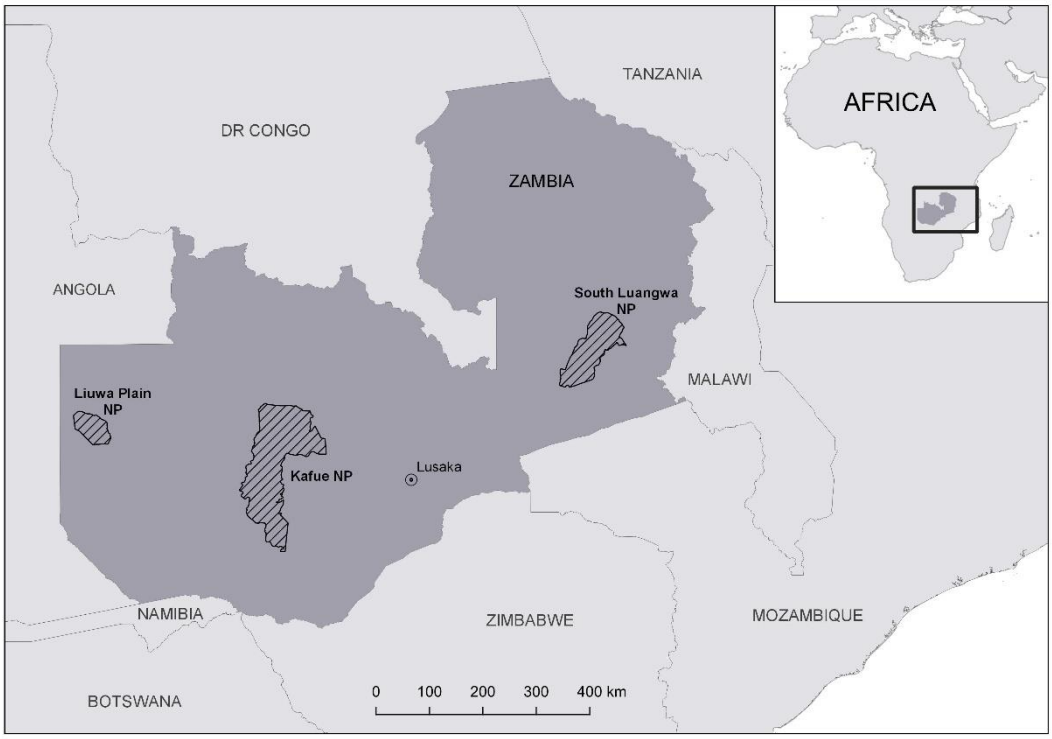

Figure 3. ***Sampling locations.*** *Spotted hyena samples were collected in three Zambian national parks (NP); Liuwa Plain, Kafue and South Luangwa.*

DNA was extracted manually at the Luangwa field site using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Nucleotide quantity and purity were assessed with spectrophotometry (NanoDrop, Thermo Fisher Scientific) in our lab in Umeå, Sweden. DNA extract quality used for sequencing was screened visually by gel electrophoresis using the Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company).

2.2. DNA Sequencing

We followed an anonymous sequencing approach involving high-throughput sequencing of reduced representation libraries [41, 42]. This next-generation sequencing method has shown to be promising for *de novo* sequencing of wild species because it is not only applicable to model species with a readily available reference genome, but also to non-model species with no prior existing genomic data [1, 3, 5, 43]. We digested 0.5µg of each DNA extract with the restriction enzyme EcoRI (Thermo Fisher Scientific) consistent with manufacturer's instructions. The activated enzyme was removed using the MinElute Reaction Cleanup kit (Qiagen) after which the digestion quality was visualized by gel electrophoresis. We selected digested DNA fragments of 400-700 bp to be sent to the National Genomics Infrastructure (NGI, SciLifeLab, Stockholm) for paired-end library construction (2 x 150bp) and RAD-sequencing on an Illumina NovaSeq 6000 platform (S4 flowcell).

2.3. SNP Detection

Sequenced Illumina reads were delivered demultiplexed. Sequence quality was assessed using FastQC (0.11.9, [44]). Reads were trimmed and adapters were removed with Trimmomatic (0.39, [45]) in paired-end mode. Only reads with length 140 bp after trimming and cropping were selected for further processing. These remaining sequences were used as input for SNP detection using Stacks (2.55, [46, 47]), running the individual components of the pipeline manually. Initially, we ran the *process_radtags* feature for pre-processing of the reads with the setting *disable_rad_check*, since we had trimmed the restriction cut site. Then, we ran *ustacks* with default parameter settings m 3 and M 2, which assembles loci *de novo* in each individual. Next, we executed *cstacks* with setting n 2, which creates a catalog of assembled loci, followed by *sstacks* to match the samples against the newly created catalog. Then, we ran *tsv2bam* which transposes the data to be stored per locus instead of per sample, followed by assembling and merging the paired-end contigs through *gstacks*. Finally, the feature *populations* was run to

filter the data, calculate basic statistics and to export the data for further analysis, which was aggregated into a report using MultiQC (1.10, [48]).

The putative SNPs that resulted from running Stacks were filtered to remove ones of low quality using customized R scripts [49]. Since our aim was to develop a 96-well SNP-chip, we could afford to be stringent with our filtering and only retain the highest quality SNPs. First, we chose to continue only with the SNPs that were the only one on their respective read. This was to ensure not to select for SNPs in hypervariable regions and to reduce the possibility of selecting pseudo SNPs that resulted from sequencing error. Additionally, we required the SNP to be located in the middle of the read to ensure adequate flanking regions of minimum 40 bp up- and downstream of the SNP. Next, we selected for SNPs that were present in at least 23 of the 27 individuals with a minor allele frequency (MAF) > 0.35. Finally, the retained SNP were required to contain all three genotypes (i.e., xx, xy and yy) among the sequenced individuals, which was true for the SNPs with an observed heterozygosity below 0.8. Of the remaining high-quality SNPs, a total of 150 SNPs were randomly selected for assay development (Fluidigm) and used to genotype 80 spotted hyena samples using the Fluidigm Biomark.

2.4. SNP Validation

The SNP assays were validated by genotyping a set of 80 spotted hyena samples, including the initial samples used for SNP detection. We included both positive (duplication of 7 samples) and negative controls. We used two types of negative control, five in total; for three “no template controls” (NTCs), DNA was replaced by water and run through PCR, while for the other two controls the PCR was preceded by 14 preamplification cycles. We evaluated the call rate of the DNA samples and the performance of the assayed SNPs at successfully assigning a genotype to the samples. Every SNP was visually assessed on the allele clustering of the DNA samples in the Biomark scatterplot. SNPs were invalidated if the controls did not work as expected, if the different allele combinations (i.e., xx, xy and yy) clustered too near to each other or if not all allele combinations were present. The remaining validated SNPs were selected for the final SNP-chip.

To assess the performance of the final SNP-chip as markers for identifying population structure, we conducted a Principal Component Analysis (PCA). We used the R package *SNPRelate* (1.24.0) to infer relatedness and detect genetic separation within the 80 genotyped samples.

3. Results

High-throughput sequencing was successful for all 27 individuals. The sequence quality was satisfying with a mean sequence quality (Phred quality score, Illumina) exceeding 30 throughout all the sequences. A total of 261,694 SNPs were identified using the Stacks software, out of which 76% did not pass the first filtering criterium of one SNP per read, reducing the amount of putative SNPs to 62,360 (Fig. 4). Out of these, 351 SNPs were retained after applying the remaining filtering criteria: SNP located in the middle of the read with flanking sequences of 40 bp on each side; present in at least 23 individuals; all three allele combinations present; and with high minor allele frequencies (MAF > 0.35, Fig. 4). Out of the remaining 351 high quality candidates we randomly selected 150 SNPs for assay design of which 139 passed the *in silico* assay design.

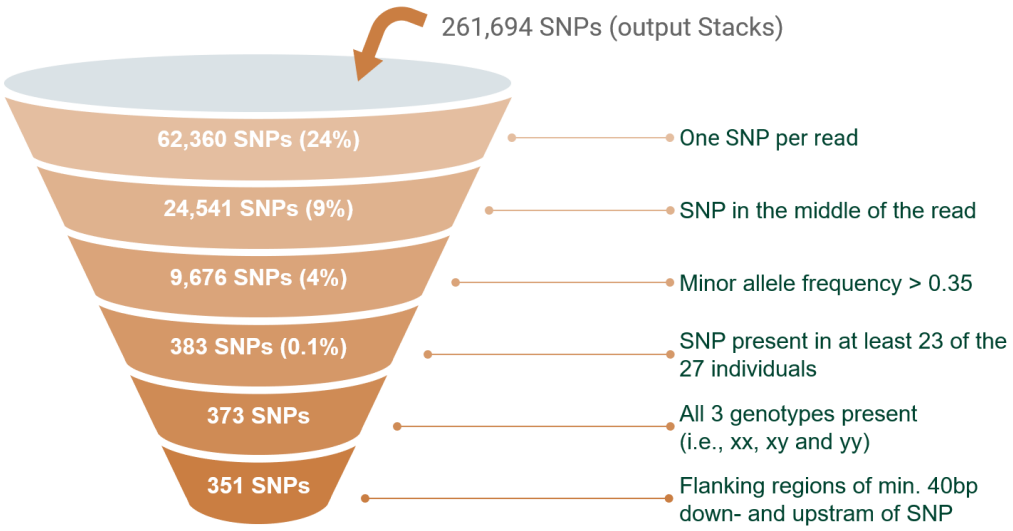

Figure 4. ***SNP Quality Filtering.*** *Schematic overview of the SNP selection process, starting with a high number of putative SNPs detected by the Stacks software and resulting in the selection of 351 high-quality SNPs.*

Validation by genotyping a first set of 96 SNPs resulted in 74 SNPs (77%) that produced good results (Figure 5a&b). Of the 22 SNPs that were removed from further analysis, ten did not have the three different allele combinations (Figure 5c), eight had indistinct allele clusters (Figure 5d), three passed but the controls (NTC) did not work, and one gave no signal. Except for the latter one, all SNPs had a high call rate (successfully genotyped samples) of at least 84%. The 74 SNPs passed all control checks and only a single mismatching genotype occurred for one of the seven duplicates (error rate < 0.001). Two DNA samples did not produce results and were removed from further analysis. More potential SNPs can be found when the remaining 43 SNPs are validated.

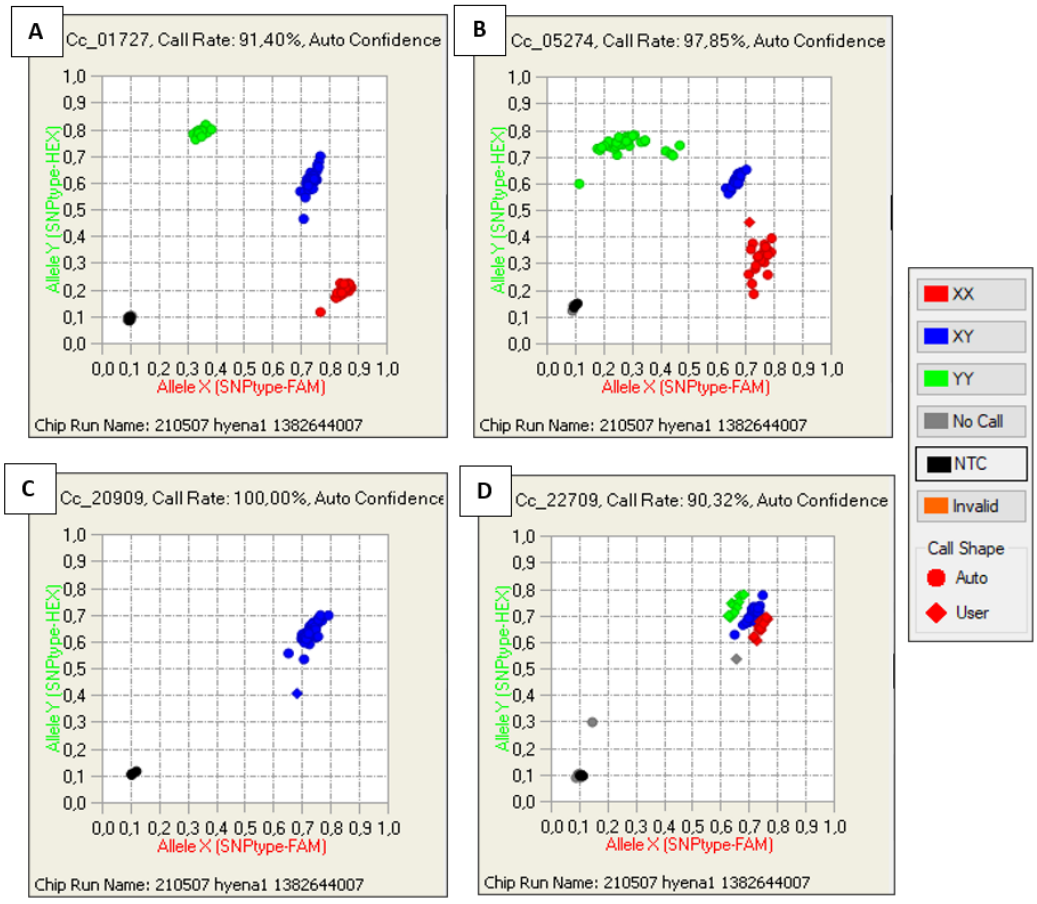

Figure 5. ***SNP Scatterplots.*** *Scatterplots showing allelic clustering per SNP based on fluorescence. Figures A and B are representative of successful SNPs, while C and D were unsuccessful, respectively because of the absence of all three allelic combinations (C) and indistinct allele clusters (D). Scatterplots were generated by the Fluidigm Biomark system.*

The Principal Component Analysis (PCA) allowed for the distinction of two clusters (14.2% EV1, 7.8% EV2). The clustering separated samples originating from Liuwa Plain NP, while samples from Kafue NP and South Luangwa NP clustered together (Fig. 6).

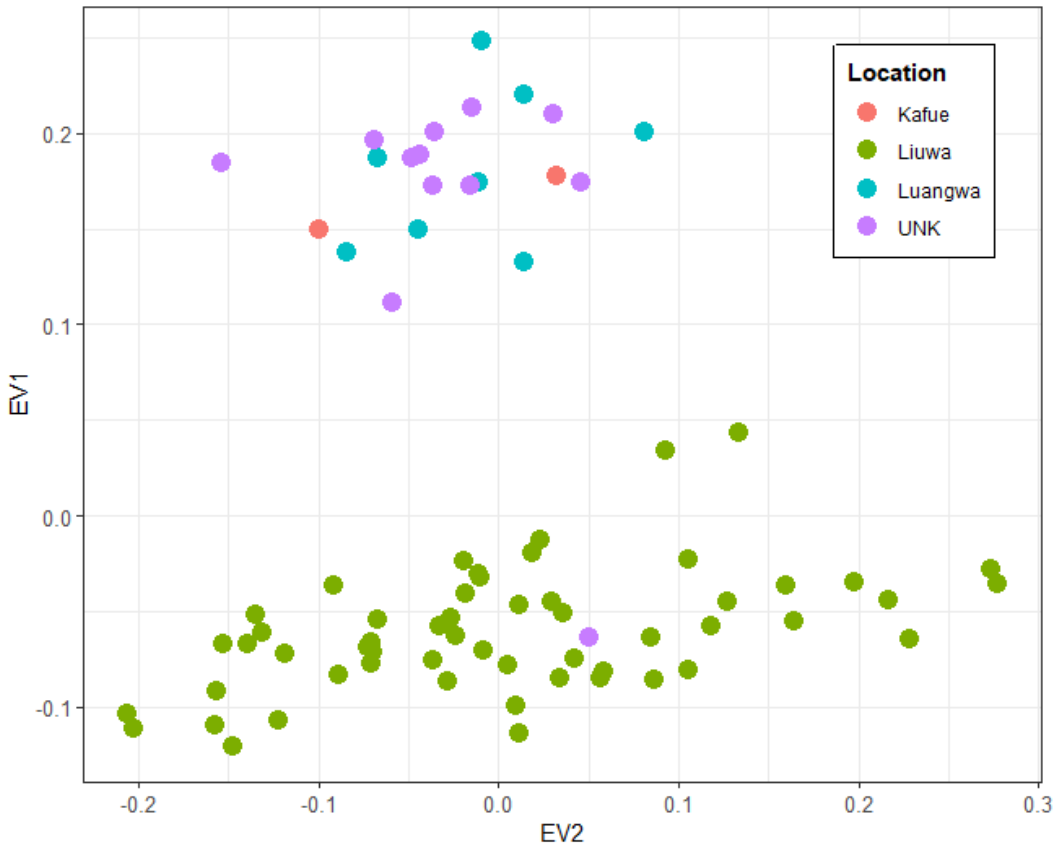

Figure 6. ***Population PCA.*** *Population structure and genetic separation of 80 genotyped samples based on Principal Component Analysis (PCA). The axes represent Eigenvalues 1 and 2. Colors indicate sampling locations; Kafue NP, Liuwa Plain NP and South Luangwa NP. Samples with unknown location (UNK) were included in the analysis.*

4. Discussion

We have developed a novel panel of 74 high-quality SNP markers for individual identification of spotted hyena (*Crocuta crocuta*), an ecologically important top-predator in sub-Saharan Africa. This novel panel is in line with previous SNP discovery studies in non-model species, such as sun parakeet [1], beaver [2], moose [3], white rhino [4], and brown bear [5]. In a first test to identify population structure within 80 Zambian spotted hyena samples, genetic separation could be observed between individuals from Liuwa Plain NP and the two other NPs. While Kafue NP and Liuwa Plain NP are geographically located closer, the capital city Lusaka is located between Kafue NP and South Luangwa NP, creating an assumed anthropogenic border for the movement of the species. The result that individuals from Kafue seem to be genetically similar to Luangwa individuals, while significantly differing from Liuwa individuals is therefore unexpected. A possible explanation could be the presence of a natural border, the Zambezi river, separating the Liuwa population from the rest of Zambia. Further analysis using this newly developed SNP panel can shed more light onto the population structure and can therefore be a valuable tool for monitoring and management of the species.

The current SNP panel was obtained through an anonymous sequencing approach and is therefore likely to contain mainly autosomal SNPs. It is possible that X-chromosome markers have been retained, which can be tested by identifying if all male samples are homozygous for that SNP marker. The panel does not yet include Y-chromosome sex-determination markers or mitochondrial markers informative of maternal lineage. Currently, this has not been an issue as the samples used for SNP discovery were biopsy tissue samples collected when the individual was anesthetized and the sex could be recorded. However, when using non-invasively collected samples for analysis, the need to genetically identify the sex of the sampled individual will be more important. This can be solved with an additional targeted approach to identify these markers *de novo* by designing primers based on published mitochondrial [39, 40] and Y-chromosome genomes [50].

Further development of a 96-well SNP-chip of autosomal, mitochondrial and Y-chromosome markers is needed to obtain a fully functional panel. Following the success rate of our first validation test (77%), we can expect to obtain approximately 33 more autosomal SNPs from the remaining non-validated

candidate SNPs. Final evaluation of the panel will also include quality testing on low-quality samples, such as feces. Some chosen SNPs might not perform as well on these non-invasively collected samples and will eventually have to be dropped from the panel. Our target for the final 96-well SNP-chip is to include approximately ten Y-chromosome and mitochondrial markers and the remaining amount of autosomal SNPs, informative for multiple types and qualities of samples.

There are many potential applications of this SNP panel including estimating population size, survival and population structure through pedigree reconstruction and kinship analysis (e.g., [51, 52]). It could also make use of the relatedness estimates to monitor mating strategies, reproductive success, genetic diversity and to track individual and dispersal movements (e.g., [18]). For example, it could potentially reveal the impact of a matriarchal social structure on the genetic structure of the clan and population; the main factors that influence a male's reproductive success in a female-dominated environment; and whether paternity in a clan is shared. Moreover, this SNP-chip could also function as a tool against illegal wildlife trade and poaching by providing an easy genetic verification of the individual and its origin, and to deliver forensic evidence to wildlife crime investigators and enforcement. Ideally, SNP-based studies on spotted hyena could improve our knowledge and understanding about the species and therefore benefit its management and conservation status.

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