

Assessment of dietary patterns for cheetah and brown hyena in Iona National Park, Angola, using DNA metabarcoding

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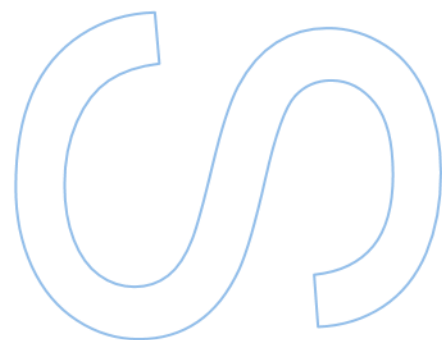
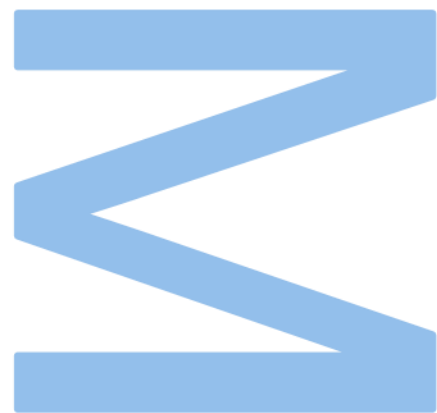
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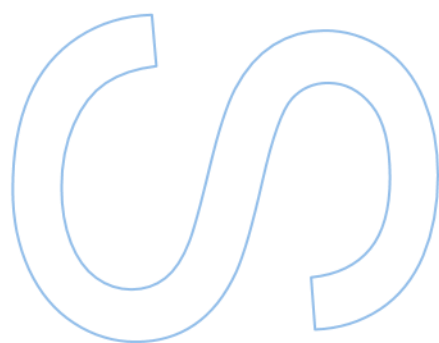
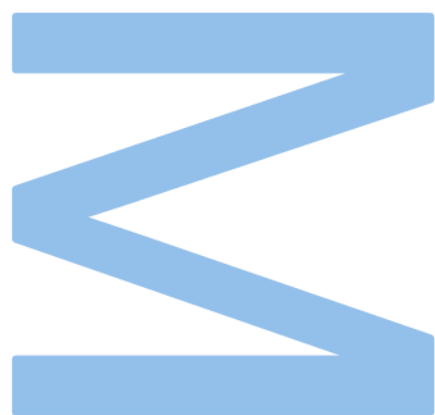
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Resumo

Os grandes carnívoros e necrófagos são essenciais para o funcionamento dos ecossistemas, pois desempenham papéis críticos na regulação das populações de presas, na manutenção do equilíbrio ecológico e na reciclagem de carcaças. No entanto, estas espécies enfrentam diversos perigos, incluindo a perda e fragmentação de habitat, perda de presas e conflitos com seres humanos, intensificados pelo rápido aumento da população humana. Deste modo, é necessário entender a ecologia e comportamento destas espécies para que medidas de conservação possam ser aplicadas.

Dada a natureza elusiva de muitos destes carnívoros, o uso de técnicas que envolvem observar ou manusear as espécies tornam-se desafiadoras, e podem causar muita perturbação aos animais. A utilização de métodos não invasivos, como a análise de excrementos, tem sido usada como uma abordagem eficaz para estudar a ecologia de espécies em comparação com métodos invasivos tradicionais. Através dos excrementos é possível extrair o DNA não só da espécie em estudo como o DNA das espécies presa consumidas.

Nesta tese excrementos de duas espécies, a chita (*Acinonyx jubatus*) e a hiena castanha (*Parahyaena brunnea*) foram recolhidos no Parque Nacional do Iona, durante os anos de 2021 e 2022. O DNA extraído foi usado para identificação individual das espécies e estudo das relações entre os indivíduos da população. Adicionalmente foi utilizada a técnica de *DNA metabarcoding* combinada com sequenciação de alto rendimento que permitiu não só confirmar a espécie do predador como também a identificação das espécies consumidas, permitindo o estudo da dieta das chitas e hienas castanhas.

Após a extração do DNA, foi feita a identificação individual das espécies usando o gene ATP6. Em seguida, usando um painel de microssatélites para cada uma das espécies, identificaram-se os indivíduos. Esta análise identificou nove indivíduos de chitas, todos eles machos, e 15 indivíduos de hiena castanha, 4 fêmeas e 11 machos. A análise de relações de parentesco entre os indivíduos identificou relações de irmãos verdadeiros entre alguns indivíduos de chita, e de irmãos verdadeiros e maternidade entre indivíduos de hiena castanha. Analisaram-se ainda o uso do espaço nestas duas espécies.

No que diz respeito à dieta, usou-se a técnica de *DNA metabarcoding*. Inicialmente, amplificou-se o gene 12S do DNA mitocondrial e construíram-se bibliotecas de DNA indexadas individualmente para cada amostra, seguido de sequenciação de alto rendimento numa plataforma MiSeq da Illumina. Foi seguido um pipeline bioinformático

para processar e filtrar as sequências, identificando-as por meio de uma base de dados do NCBI. Os resultados revelaram que, nas chitas, os ungulados foram o grupo mais frequentemente consumido. As espécies predominantes foram a cabra-de-leque (*Antidorcas marsupialis*) e a lebre-do-cabo (*Lepus capensis*), mas também espécies de aves e um mesocarnívoro fizeram parte da dieta da chita. Encontrou-se um total de sete espécies diferentes na dieta das chitas e não foram encontradas espécies de gado doméstico. Por outro lado, na dieta das hienas castanhas foi possível encontrar 29 espécies de presas diferentes, reforçando o comportamento alimentar oportunista da espécie. Assim, como nas chitas, o grupo mais frequentemente consumido foi o dos ungulados, seguido de aves e de mamíferos marinhos. As hienas castanhas mostraram um consumo elevado da mesma espécie que as chitas, a cabra-de-leque, seguido de Orix-do-cabo (*Oryx gazella*) e de lobo-marinho do cabo (*Arctocephalus pusillus*). Além disso foram identificados lagomorfos, mesocarnívoros, e outros pequenos mamíferos. Na dieta das hienas castanhas foi encontrado gado doméstico, embora em baixas percentagens. Verificou-se ainda indivíduos que se alimentavam exclusivamente de lobo-marinho do cabo e cujo uso do espaço se concentrava mais junto à costa.

Este estudo contribui para a compreensão da ecologia e comportamento das chitas e das hienas castanhas no Parque Nacional do Iona, destacando a importância dos métodos não invasivos e do *DNA metabarcoding* na investigação dessas espécies no seu habitat natural. Além disso, reforça a necessidade contínua de conservação desses grandes carnívoros e necrófagos para a preservação dos ecossistemas.

Palavras-chave: DNA metabarcoding, *Acinonyx jubatus*, *Parahyena brunnea*, Parque Nacional do Iona, Grandes Carnívoros, Necrófagos, Frequência de Ocorrência, Amostras não invasivas, Dieta, Padrões de Distribuição, Relações de parentesco, Microssatélites, Conservação de espécies.

Abstract

Large carnivores and scavengers play critical roles in ecosystem functioning by regulating prey populations, maintaining ecological balance, and recycling carcasses. However, these species face various threats, including habitat loss and fragmentation, prey depletion, and human-wildlife conflicts, intensified by the rapid increase of human population. Therefore, a better understanding of their ecology and behaviour is required so conservation measures can be taken.

Given the elusive nature of many of these carnivores, traditional observational and handling techniques can be challenging and potentially stressful for the animals. The use of non-invasive methods, such as faecal analysis, has proven to be an effective approach to studying species compared to invasive traditional methods. Through faecal samples, it is possible to extract DNA not only from the target species but also from consumed prey species.

In this thesis, faecal samples from two species, cheetah (*Acinonyx jubatus*) and brown hyena (*Parahyaena brunnea*) were collected in Iona National Park during the years of 2021 and 2022. DNA extracted from these samples was used for individual species identification and the study of population relationships. Furthermore, DNA metabarcoding, combined with high-throughput sequencing, allowed not only the confirmation of the predator species but also the identification of consumed species, enabling the study of the diet of cheetahs and brown hyenas.

Following DNA extraction, individual species identification was performed using the ATP6 gene. Subsequently, microsatellite panels were used for each species to identify individuals. This approach allowed to identify nine individuals of cheetahs, all males, and 15 individuals of brown hyenas, 4 females and 11 males. Genetic relatedness analysis revealed full-sibling relationships among cheetah individuals and both full-sibling and maternity relationships among brown hyena individuals. Additionally, space use by these species were analysed.

Regarding diet analysis, a DNA metabarcoding approach was employed. Initially, the mitochondrial 12S gene was amplified by PCR and individually indexed DNA libraries were constructed for each sample, followed by high-throughput sequencing on the Illumina MiSeq platform. A bioinformatics pipeline was applied to process and filter sequences, identifying them through the NCBI database. Results revealed that in cheetahs, ungulates were the most frequent prey group. The predominant species were the springbok

(*Antidorcas marsupialis*) and cape hare (*Lepus capensis*), but also some bird species and a mesocarnivore were part of the diet. A total of seven different species were found in the cheetah diet, with no domestic livestock detected. On the other hand, the diet of brown hyenas consisted of 29 different prey species, reflecting their opportunistic feeding behaviour. Like cheetahs, ungulates were the most frequent group, followed by birds and marine mammals. Brown hyenas exhibited high consumption of some of the same species as cheetahs, like springbok, followed by gemsbok (*Oryx gazella*), but also high consumption of species that are not used by cheetah, like the Cape fur seal (*Arctocephalus pusillus*). Additionally, lagomorphs, mesocarnivores, and other small mammals were identified. Domestic cattle were found in brown hyenas' diet, although in low percentage. Moreover, some individuals were found to exclusively feed on Cape fur seal and concentrated more along the coastline.

This study contributes to the understanding of the ecology and behaviour of cheetahs and brown hyenas, emphasizing the importance of non-invasive methods and DNA metabarcoding in researching these species in their natural habitat. Furthermore, it underscores the ongoing need for the conservation of these large carnivores and scavengers for ecosystem preservation.

Keywords: DNA metabarcoding, *Acinonyx jubatus*, *Parahyena brunnea*, Iona National Park, Large Carnivores, Scavengers, Frequency of Occurrence, Non-invasive samples, Dietary Patterns, Space Use Patterns, Parental Relationships, Microsatellites, Species Conservation.

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List of Abbreviations

bp	Base pairs
CIBIO	Research Centre in Biodiversity and Genetic Resources
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CMS	Convention on Migratory Species
DNA	Desoxyribonucleic acid
FOO	Frequency of Occurrence
<i>H</i>	Shannon's diversity index
HTS	High-Throughput Sequencing
INP	Iona National Park
IUCN	International Union for Conservation of Natural Resources
Kg	Kilograms
Km	Kilometers
m	Meters
ml	Milliliters
mm	Millimeters
MOTUs	Molecular operational taxonomic units
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
PI	Probability of identity
Rnase	Ribonuclease
rRNA	Ribosomal RNA
Secs	Seconds
SSR	Simple Sequence Repeats
UV	Ultraviolet
µL	Microliters

1. Introduction

1.1 General introduction

Large carnivores play a crucial role in ecosystems by occupying high trophic positions and controlling lower trophic levels through a cascade effect (Welch et al., 2016). They affect prey communities such as mesopredators and herbivores, through predation and competition, influencing their densities and indirectly affecting other species (Hoeks et al., 2020; Wolf & Ripple, 2016). They can also influence prey behaviour and distribution by forcing them to shift home ranges to avoid predation (Hoeks et al., 2020). Therefore, a decline of large carnivores allows the growth of mesopredator and herbivore communities, which can have serious impacts on the plant communities and other species at lower trophic levels (Hoeks et al., 2020). The impact of large carnivores in ecosystems goes beyond interactions such as predation or competition. The food remains they leave behind can serve as a resource for scavengers, detritivores, and microorganisms (Marker et al., 2003), which greatly benefit from this coexistence (Prugh & Sivy, 2020).

Scavenging carnivores also play an important role as they are able to remove waste from the environment, through the consumption of carrion and carcasses. This contributes to nutrient recycling, decomposition processes while reducing the risk of disease transmission (Probst et al., 2019; Welch et al., 2016). Thus, both large predators and scavengers play a key role in the ecosystems, and their decline or extinction can have drastic consequences (O'Bryan et al., 2018).

However, due to their low population densities, slow population growth rates, and large spatial needs, large carnivores are the ones that suffer the most from population and range decline (Comley, 2016). In fact, it has been documented a rapid decline in the total number of large carnivores' population over the past two centuries (Comley, 2016).

One of the greatest threats to large carnivores is the rapid growth of human populations that causes habitat loss and brings human communities into closer proximity to wildlife (Hoeks et al., 2020). Large carnivores often have a negative reputation among human population due to their potential danger to livestock, human lives, and pets as well as their role in disease transmission (O'Bryan et al., 2018; Wolf & Ripple, 2016). Prey abundance is also a critical factor in the survival of large carnivores, and a lack of sufficient prey can lead to large declines in predator populations (Wolf & Ripple, 2016). As an alternative to wild prey, predators may seek domestic species. These two factors combined results in the conflict between large carnivores and human communities. Consequently, they often

suffer from persecution by humans, which is a major factor in their decline (Hoeks et al., 2020).

Large carnivores are considered “umbrella species”, meaning that their conservation implies the conservation of other species in the ecosystem. This increases their importance (Comley, 2016) and highlights the need for implementing conservation measures (Hoeks et al., 2020). It is believed that the successful conservation of these species greatly benefits from the implementation of measures away from areas where human population live (van Eeden et al., 2018). However, humans and wildlife can coexist, as has been demonstrated in some areas of Europe and Asia (van Eeden et al., 2018). For that, it is necessary to improve people's understanding of predatory behaviour and promote collaborations with local communities and stakeholders (Ashish et al., 2022). Measures like using fences or livestock guarding dogs to protect livelihoods or improving wild prey availability are often highlighted as crucial to mitigate this conflict (van Eeden et al., 2018; Wolf & Ripple, 2016).

1.2 Social structure

The social structure of species provides valuable insights into species behaviour, foraging habits, and social interactions, while highlighting variations between individuals and species (Di Fiore, 2003). It also provides information on genetic aspects such as mating systems, genetic drift, and inbreeding depression. This study is crucial for understanding evolutionary adaptations and interactions with the environment (Parreira & Chikhi, 2015).

Direct observation is a fundamental approach to studying the behaviour, ecology, and social organisation of species. However, some information remains difficult or impossible to obtain using only this method. For example, information on patterns of dispersal, genetic relationships between individuals, gene transfer, impact of genetic relatedness on social interactions and reproductive success are aspects that can be elusive (Di Fiore, 2003).

Over the past few decades, the development of non-invasive DNA based molecular techniques has provided an alternative for exploring these issues. Among the many molecular markers available for genetic studies, microsatellites are the most widely used (Reed et al., 1997).

Microsatellites or Simple Sequence Repeats (SSRs) are ideal for this type of study due to their polymorphism (Pajuelo et al., 2015), high abundance, distribution throughout the genome, the fact that they occur mostly in non-coding regions (Grover et al., 2012; Xiao et al., 2016), and are inherited in codominance (Struss & Plieske, 1998). Furthermore, the

flanking region of microsatellites may be sufficiently conserved to allow the use of primers designed for one species, to be used in species from closely related taxa (de Souza et al., 2018; Xiao et al., 2016).

Microsatellites are a powerful tool that can be used in genetic diversity studies, allowing the identification of individuals, and tracing the genetic fingerprint of an organism. They also facilitate the study of the evolutionary history of species through phylogenetic analysis, as well as the study of population structure and genetic relationships within a population (Akemi et al., 2012).

1.3 Diet assessment

Diet assessment is crucial to understand the ecology, evolution and conservation needs of species (Shehzad et al., 2012). It allows the study of the dynamics between prey and predator (Wachter et al., 2012), and consequently the impact of predators on prey populations (Andheria et al., 2007). For large carnivores, such as cheetahs and hyenas, which have an important role in the ecosystems and are the top predators, diet assessment provides a representation of interactions with the other species, but it also helps to understand the structure of the whole trophic webs (Bonin et al., 2020). Therefore, understanding the dietary patterns of these species is crucial to implement conservation measures in the ecosystem (Monterroso et al., 2019).

Furthermore, dietary analyses allow to mitigate one of the main problems faced by carnivores, the wildlife-human conflict, which has significantly contributed to their decline (Monterroso et al., 2019). However due to the nocturnal activity and the low population densities present by large carnivores, it is difficult to study feeding habits by direct observations of predation events (Kaunda & Skinner, 2003).

Alternatively, faecal samples are largely used as a non-invasive method to study the diet of these species (Ando et al., 2020). The traditional method of analysing diet from faecal samples is the observation of undigested remains. In this approach, the identification of prey depends on the presence of remains in the scats, such as hair, bones, hooves, or teeth (Shi et al., 2021) and different preys are differently digested what could induce errors in the analyses (Havmøller et al., 2021). Furthermore, it is common to assume the species that a given scat belongs based only on its morphology (Monterroso et al., 2019). However, errors in the identification of the target species are common, especially in the case of sympatric species, with similar body weights and diets (Haag et al., 2009; Monterroso et al., 2019).

An alternative method that helps overcome these two most common errors associated with the traditional methodology is the implementation of molecular approaches (Ando et al., 2020). Until 2009, molecular analysis to study diet were conducted using traditional sequencing methods, involving a laborious time-consuming and expensive procedure (Shehzad et al., 2012). However, new approaches using high throughput sequencing, known as DNA metabarcoding, are now routinely used for diet analysis (Shi et al., 2021). DNA metabarcoding uses universal primers to amplify specific DNA regions that are a priori known, and then the sequencing of these PCR products and the identification of all species whose DNA was present in the sample by comparison to reference databases (Shi et al., 2021). An important benefit of using this molecular approach is the possibility of analysing numerous samples at the same time (Shi et al., 2021). This method also allows to identify the predator and the prey consumed simultaneously (Monterroso et al., 2019), even when the extracted DNA is low in quantity or is degraded (Ando et al., 2020). Its accuracy allows to detect prey previously missed by traditional methods (Hardy et al., 2017), while providing a better taxonomic resolution to the species level and being time and cost saving as it allows the sequencing of millions of DNA fragments simultaneously (de Sousa et al., 2019).

The region commonly used to identify vertebrate species is a 100 bp fragment of the 12S rRNA mitochondrial gene (Riaz et al., 2011). Although it's a small region, it is highly suitable as a DNA barcode as it provides high taxonomic resolution while also having conserved primer binding sites, which helps avoid bias in the amplification process (Kocher et al., 2017; Valsecchi et al., 2020).

1.3.1 DNA Metabarcoding limitations

DNA metabarcoding is considered the most powerful tool to study trophic networks and ecological interactions, providing better results than traditional morphological techniques (de Sousa et al., 2019). However, it has some limitations. One of them is related to the use of universal primers in amplification as it can also amplify predator DNA, potentially leading to the amplification of only predator DNA when prey DNA is scarce (Shehzad et al., 2012). Furthermore, DNA metabarcoding does not provide information on prey age or size (Havmøller et al., 2021), and cannot distinguish between active predation and scavenging (Shi et al., 2021).

In addition, this technique is prone to contamination, which can lead to misidentification of prey, and it is unable to distinguish between the direct prey of the species under study and

prey of its own prey, known as secondary predation (Brassea-Pérez et al., 2019). Despite these limitations, DNA metabarcoding remains a highly useful approach.

1.4 Model Species – *Acinonyx jubatus*

Cheetah (*Acinonyx jubatus*) belongs to the Felidae family and is the only species belonging to the *Acinonyx* genus (IUCN, 2022).

This species was once widespread across the African and Asian continents; however, it is estimated that the species no longer inhabits 76% of its original distribution (Fusari et al., 2010; Khalatbari et al., 2022). In Eastern Africa, cheetah is found across the boundaries of northern Tanzania and southern Kenya; in the southern of Ethiopia; there is a population in South Sudan that possibly extends to northern Kenya and can also be found in northern Uganda. In southern Africa, it is possible to find populations in Namibia, Botswana, southern and central Angola, northern South Africa, south-western and central Mozambique, southern and central Zambia, and central Zimbabwe (Durant et al., 2015; IUCN, 2022; Monterroso et al., 2020; RWCP & IUCN/SSC, 2015; Weise et al., 2017). In Asia, cheetahs are currently found only in Iran, and their numbers are quite low, being classified as Critically Endangered (Figure 1; IUCN, 2022). The largest estimated population in the region is believed to have fewer than 50 individuals (Khalatbari et al., 2023). Cheetah population is estimated at 7500 individuals in the known areas, if we consider the areas where the presence of this species is not certain, the population can reach 10 000 individuals (Fusari et al., 2010). Nevertheless, the decline is severe, as in 1970 the population was estimated to be around 15 000 (Fusari et al., 2010). Nowadays, cheetahs are one of the most threatened species (Khalatbari et al., 2022) and are classified as vulnerable according to the Red List of Threatened Species by the International Union for Conservation of Nature (Fusari et al., 2010).

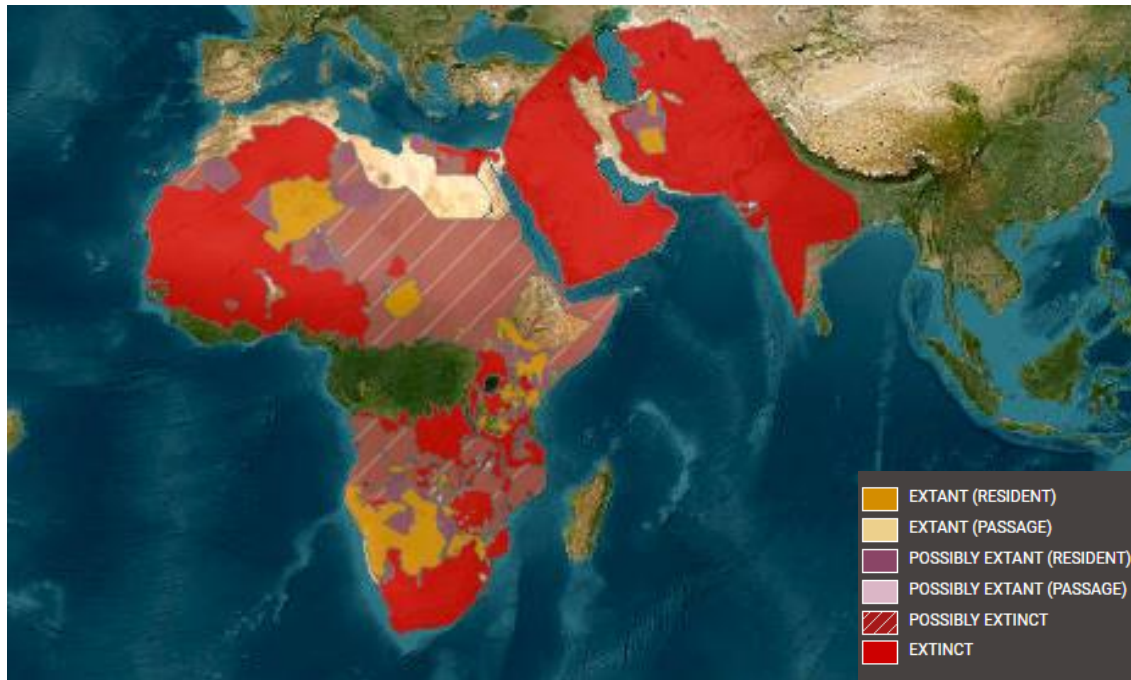


Figure 1: Distribution of cheetah, *Acinonyx jubatus* –Distribution of extinct and extant cheetah populations among its whole range in Africa and Asia. Figure retrieved from IUCN (2022).

In Africa, cheetah occurs in different habitats, from savannas, shrubland, grassland, wetlands, rocky areas, and deserts (Broomhall et al., 2003; IUCN, 2022). In Iran cheetahs have preference for deserts with an annual precipitation less than 100 mm (Khalatbari, 2018).

Cheetahs are known as the world's fastest land mammal and have adaptations to achieve high speed over short distances (Ewer, 1998; Meachen et al., 2018). These adaptations include a lightweight skeleton, long limbs, and a flexible spine. Additionally, they have enlarged heart and lung capacity facilitating enhanced oxygen intake during high-speed chases (Marker, 2002). They have a slender body, a small head and a yellow or tan coat colour with solid black dots (Woolf et al., 2003). There is notable sexual dimorphism among cheetahs with males being larger than females. In adults, weights range approximately from 20 kg to 60kg (Figure 2; Marker & Dickman, 2003).

They are mostly diurnal, which may be a strategy to avoid other competitors (Caro, 1994). However, some cheetahs' populations can have nocturnal habits which is possibly associated with 1) low competition with other carnivores or 2) avoidance of the contact with humans due to high human activity during the day (Belbachir et al., 2015; IUCN, 2022; Marnewick et al., 2006).



Figure 2: Cheetah running. Cheetah photography retrieved from CMS (2020)

Cheetah populations face severe threats, with historical declines attributed to habitat loss and fragmentation due to human expansion, land use for agriculture, and road construction (Marnewick et al., 2007; Mossotti, 2010). However, the most significant factor contributing to this decline was the illegal trade, where cheetahs were hunted and killed (Mossotti, 2010). As a result, cheetahs have been listed under Appendix I of CITES since 1975 (CITES, 2023; Marnewick et al., 2007).

Similar to other carnivores, cheetahs require extensive territories and often travel beyond protected areas, with the majority of the cheetah population living outside these zones (Mutoro et al., 2022; Thuo et al., 2020). In these areas, they contact more frequently with human populations and therefore habitat, species abundance, and community composition are affected by human activities (Mutoro et al., 2022). Additionally, the likelihood of encounters with livestock increases. With more domestic preys available than wild preys, cheetahs may be more prone to preying on domestic animals (Thuo et al., 2020). This leads to conflicts between wildlife and human populations, often resulting in human retaliation, with the persecution and deaths of cheetahs further contributing to carnivore population declines (Boast et al., 2016; Thuo et al., 2020).

Cheetahs are diurnal and easily spotted by humans (Thuo et al., 2020) which, combined with their proximity to livestock, makes them convenient targets for blame in cases of livestock deaths. Even in the absence of direct evidence, cheetahs are often accused of being the responsible (Thuo et al., 2020). Lastly, cheetahs are highly influenced by prey

availability, and the reduction of prey populations is another significant threat to their survival (Khalatbari et al., 2022).

1.4.1 Cheetah social organization

Cheetahs are mostly solitary. Females can be seen alongside their cubs, while males have been observed forming coalitions usually with their male siblings (Bailey & Caro, 1997). Within these coalitions, male cheetahs cooperate in hunting and share resources, enabling them to target larger prey than females are capable of (IUCN, 2022; Mills et al., 2004). Males can either exhibit territorial behaviour, defending small areas, or employ roaming tactics (Wachter et al., 2018). Opportunities for mating arise when female territories overlap with those of males. Both males and females use marking trees and specific locations for defecation to communicate their presence (Woolf et al., 2003). These interactions between males and females are temporary with the purpose of reproduction only, with males not providing parental care (Gottelli et al., 2007; Woolf et al., 2003). The mating system of cheetahs is complex and can vary. Males seek to mate with as many females as possible, thereby enhancing their reproductive success. Females have been found to be promiscuous, displaying a high rate of multiple paternity (Gottelli et al., 2007). While cheetah females generally mate with unrelated males (Gottelli et al., 2007), inbreeding can also occur, especially in smaller populations (Meer et al., 2021). Females give birth to 2 to 6 cubs and hide them during the first two months. Cub mortality is generally high, as they are frequently preyed upon by lion and spotted hyenas (Wachter et al., 2018).

1.4.2 Cheetah diet

The diet of cheetahs consists of a wide variety of species, including small rodents, birds, hares, and large ungulates, but they prefer small and medium-sized ungulates (Khalatbari et al., 2022). Livestock can be part of their diet, but cheetahs always prefer wild prey (Khalatbari et al., 2022; Marker et al., 2003).

A study using DNA metabarcoding to analyse cheetah scats in Iran found that the most consumed prey by cheetahs were mouflon (*Ovis vignei*), ibex (*Capra aegagrus*), cape hare (*Lepus capensis*), goitered gazelle (*Gazella subgutturosa*), wild boar (*Sus scrofa*), and dromedary camel (*Camelus dromedarius*, Khalatbari et al., 2022). Livestock were not found in the diet, contrary to what was previously found in the same region and suggested as livestock being an important part of cheetah diet (Khalatbari et al., 2022). However, another study in Kenya also using DNA metabarcoding found birds, wild and domestic prey (Thuo et al., 2020). The percentage of livestock was high compared to other studies,

representing 12,8% of the cheetah's diet (Thuo et al., 2020). The authors associated it with the rapid increase of human population expanding to cheetah habitat, resulting in higher availability of domestic animals and lower of wild prey (Thuo et al., 2020). These authors found a pattern of one prey per sample, and the most common prey was the Thomson's gazelle (*Eudorcas thomsoni*) followed by impala (*Aepyceros melampus*) and wildebeest (*Connochaetes taurinus*) (Thuo et al., 2020).

Another study using DNA metabarcoding found two species of primates in the diet, yellow baboon (*Papio cynocephalus*), and vervet monkey (*Chlorocebus pygerythrus*), that have not been documented before in cheetah diet. Furthermore, the Grant's gazelle (*Nanger granti*) was the prey with the highest consumption and domestic animals were pointed as potential prey for cheetahs in the region, suggesting that cheetahs may target opportunistically livestock as a food source (Mutoro et al., 2022)

Their diet is influenced by the presence of other carnivores, such as lions or spotted hyenas. In areas where they coexist cheetahs can lose their preys to kleptoparasitism (Hunter et al., 2007; IUCN, 2022). This is indicated as a reason for their high consumption of small to medium sized ungulates. This allows cheetahs to consume their prey rapidly, minimizing the losses for other predators (Marker et al., 2018).

1.5 Model Species – *Parahyaena brunnea*

The brown hyena (*Parahyaena brunnea*) belongs to the Hyaenidae family, along with other three species, striped hyena (*Hyaena hyaena*), spotted hyena (*Crocuta crocuta*) and Aardwolf (*Proteles cristata*; Comley, 2016).

Brown hyena is the rarest of the four species and since the 18th century its populations have suffered a significant decline in the range (IUCN, 2015), having an estimated population size below 10,000 mature individuals (Edwards et al., 2019). They are listed as Near Threatened by the International Union for Conservation of Nature (IUCN)

Brown hyenas are endemic to southern Africa, only occurring in Angola, Botswana, Namibia, South Africa, and Zimbabwe (Figure 3; IUCN, 2015). The species' range was once larger, with fossils discovered in Kenya. The species is known to prefer arid and semiarid environments with rainfall less than 1,000 mm per year (IUCN, 2015).

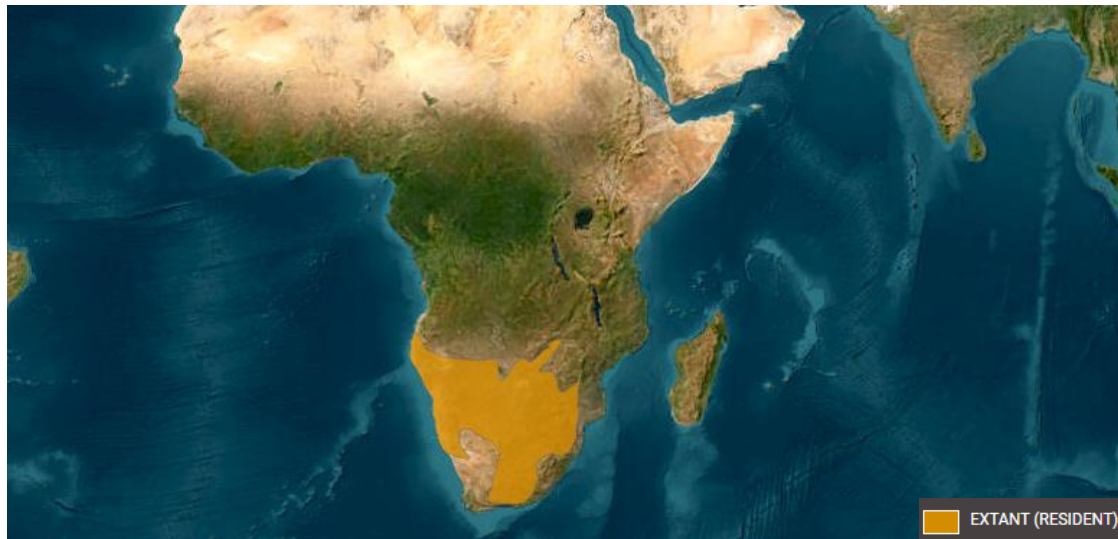


Figure 3: Distribution of brown hyena, *Parahyaena brunnea* –Distribution of brown hyena in Africa. Figure retrieved from IUCN (2015)

This species is characterised by its long and dark brown hair in most of the body and lighter hair around the neck. Brown hyenas have large, pointed ears and legs with black stripes (Figure 4; Wiesel, 2006). As adults, they can weigh between 34kg-43kg, they reach sexual maturity at 2-3 years, have gestations of 92-100 days, and give birth to 1 to 5 cubs (Miller & Fowler, 2014). Like other hyenas, this species has a strong jaw that enhances the bite allowing it to crush bones of large prey (Miller & Fowler, 2014). Furthermore forelimbs, shoulders and neck are well developed, making them capable of dismembering animals larger than themselves (Miller & Fowler, 2014). Hyenas communicate primarily through marking trees and for that purpose they have well developed anal glands that segregate pheromones (Miller & Fowler, 2014). They use it to define territory, signalling potential mates, and identifying conspecifics individuals (Miller & Fowler, 2014). They are mostly active at the night, can survive in urban areas (IUCN, 2015), are solitary by nature and have a small size what makes difficult to find them in the wild and consequently their study (Miller & Fowler, 2014).



Figure 4: Brown hyena figure. Brown hyena photograph retrieved from IUCN (2015)

The main threat to this species is the conflict between brown hyena and humans, as brown hyena is believed to be responsible for livestock losses (IUCN, 2015). As a result, humans persecute, shoot and poison hyenas even though hyenas rarely prey on livestock (IUCN, 2015). The human population growth and consequently the occupancy of areas for activities like agriculture had led to a fragmentation of hyena habitat, resulting in less connectivity among populations, representing therefore a threat to this species (Westbury et al., 2017). Road kills are also presented as a significant threat to brown hyenas (Welch et al., 2016)

Studies suggest that the population decrease experienced by the species is responsible for its genetic diversity when compared with other mammalian species. Besides this, there is no evidence for inbreeding within populations of brown hyena (Westbury et al., 2017). This fact added to their small range and the threats they face, makes them vulnerable to extinction (Comley, 2016).

1.5.1 Brown hyena social organization

Brown hyena social organization was extensively studied in the Kalahari Desert (Mills, 1982a, 1982b, 1983, 1990; Owens & Owens, 1996). The species lives in small clans with four to 14 members (Mills, 1983; Westbury et al., 2018). Notably, there is no sexual dimorphism within brown hyenas (Knowles et al., 2009), and typically within a clan, there is a dominant pair female and male of equal status, who are not considered to be related (Owens & Owens, 1996). The clan may also include other females, subadults, and

juveniles. Despite living in a clan, individuals from the same clan usually hunt and feed alone (Owens & Owens, 1996). Females live in the same clan the whole life, unlike the males, who typically live in their natal clan until a certain age, when they leave it to integrate a new clan or become nomadic and look for reproductive opportunities (Mills, 1990; Westbury et al., 2018). Females often mate with the dominant male but may also breed with a nomadic male, resulting in multiple paternity (Mills, 1982b; Owens & Owens, 1996). The members of the same clan have dens as meeting points where cubs are raised, being fed by all members that bring food back to the den. Furthermore, females may nurse cubs that are not their own (Mills, 1983, 1990). As expected, it has been observed that females within a clan are more related with one another than with the males or with females from different clans (Knowles et al., 2009).

1.5.2 Brown hyena diet

Despite their importance, brown hyenas are poorly understood. Their elusive and nocturnal behaviour difficult diet studies, and as a result, information on their feeding ecology is scarce (Slater & Muller, 2014).

Brown hyenas are primarily scavengers feeding mostly on large vertebrate carrion, therefore having an important role in the nutrient cycle and in controlling diseases (Westbury et al. 2018). However, their diet can be diversified, by consuming insects, reptiles, birds, eggs, and plants (Slater & Muller, 2014). Hunting is rare among brown hyenas, which are inefficient predators (Slater & Muller, 2014). When hunting, hyenas prefer small prey (Kuhn et al., 2008). Although some studies have found large mammals in brown hyenas' scats, this was associated with scavenging episodes and not to hunting (Slater & Muller, 2014). For example, in South Africa, a study using traditional diet analysis to analyse scats found a large proportion of large mammals' remains, specifically kudu (*Tragelaphus strepsiceros*), blesbok (*Damaliscus pygargus*), bushbuck (*Tragelaphus scriptus*), plains zebra (*Equus quagga*) and warthog (*Phacochoerus africanus*), which was associated with scavenging (Slater & Muller, 2014).

The diet of brown hyenas may also vary depending on the presence of other species such as lions, leopards, cheetahs, and caracals (Faure et al., 2019). In one study, different sizes of hyena prey were found. When only the cheetah was present, the detected prey of hyena's diet was smaller when compared to when the lions were released in the area, which may mean that brown hyenas switch between cheetah prey and lion prey (Comley, 2016).

The diet of the brown hyena can vary according to the habitat. Coastal populations, for example on the Namib Desert Coast, feed mainly on Cape fur seals, especially during seal pup season (Kuhn et al., 2008; Slater & Muller, 2014). In winter, when seals spend mostly the time at sea, brown hyenas seek alternative food sources (Kuhn et al., 2008), like small mammals, reptiles, cetaceans, mustelids, and fish. In this area, the species is also active during the day, showing variation in the circadian rhythm within the species (Kuhn et al., 2008).

One of the biggest threats to brown hyena is the human persecution caused by the belief that the species prey on livestock. However, brown hyenas are known for their scavenging behaviour and livestock make up a small portion of the hyena's diet. In Zingela Game Reserve, South Africa, hair analysis in scats found no livestock (Faure et al., 2019).

1.6 Objectives and Working Hypotheses

The focus of this thesis is to investigate the diet and the social structure of cheetahs and brown hyenas in Iona National Park, Angola, and infer their potential interactions with livestock. Three aims have been established:

1. Investigate the minimum number of brown hyena clans and cheetah individuals dwelling in the sampling area in an area of the Park, and their familiar relationships and relatedness.
2. Analyse the individual patterns of faeces deposition at latrines and relate them to potential families found to understand social organization and dynamics.
3. Assess the diet of these two species by DNA metabarcoding and identify the primary prey species. Additionally, investigate whether there is livestock consumption and its importance in the diet of the species in order to contribute for the mitigation of human-wildlife conflicts.

Accordingly, the hypotheses presented in this thesis are:

1. Genetic relatedness found among individuals indicate the occurrence of familial bonds.
2. Brown hyena individuals belonging to the same family share the same latrines. Cheetah individuals deposit faeces primarily for territory marking, reflecting a more independent social structure.
3. Cheetahs have a diet primarily consisting of small to medium-sized ungulates, while brown hyenas, being opportunistic, are likely to have a wider range of prey

but the proximity to the coast allows a considerable use of marine resources. Both species also consume livestock given the availability of domestic animals in close villages.

With this research we intend to contribute for a better understanding of the ecology of these two species in the Iona National Park and increase the path towards their conservation.

2. Methodology

2.1 Study Area

Angola has the highest diversity of biomes compared to other African countries, but despite this, its biodiversity is understudied (Huntley & Ferrand, 2019). However, since 2002, when the civil war ended, there have been some collaborations that have allowed for some biodiversity research (Huntley & Ferrand, 2019). Angola has 13 protected areas, of which six are National Parks, one Regional Park and six Reserves (MINAMB, 2016).

One of the National Park is the Iona National Park (INP), which is situated in south-western Angola in the Namib Desert (Lobón-Rovira et al., 2022). It was established as a game reserve in 1937 and later, in 1964, was upgraded to National Park (African Parks, 2023b). INP covers an area of approximately 15 150 km² and is one of the largest protected areas in Angola (African Parks, 2023b). It is adjacent to Skeleton Coast National Park in Namibia, forming the Iona-Skeleton Coast Transfrontier Conservation Area, one of the largest conservation areas in Africa (Lobón-Rovira et al., 2022).

The park is bordered by the Atlantic Ocean to the west, by the Cunene River to the south and the Curoca River drainage to the north and east (Lobón-Rovira et al., 2022). Due to its topography, the INP has a great variety of habitats, which increases the diversity of fauna and flora in the park (Lobón-Rovira et al., 2022; Morais et al., 2018). It can be divided into four main zones: i) a coastal zone limited by the Atlantic Ocean to the west and extending to where the dunes begin; ii) desert dunes, the driest zone in sub-Saharan Africa; iii) a gravel and sand plain punctuated by waterways and steep slopes, which covers the central part of the park, with extremely low rainfall and little surface water, and dry valleys; and finally iv) the mountains, hills and valleys. In the latter, fresh water is often available and is therefore densely populated by local people and their livestock (Figure 5). Habitat degradation is evident in some areas due to livestock overpopulation (MINAMB, 2016).

The civil war in Angola that lasted almost four decades (from 1975 to 2002), had several consequences for mammals in the park. Species such as black rhino (*Diceros bicornis*), elephant (*Loxodonta africana*), lion (*Panthera leo*), plains zebra (*Equus quagga*) and black-faced impala (*Aepyceros melampus petersi*) were eradicated, but some populations of mountain zebra (*Equus zebra hartmannae*), gemsbok (*Oryx gazella*) and springbok (*Antidorcas marsupialis*), resisted and persist today. Populations of large carnivores such as cheetah, leopard (*Panthera pardus*) and brown hyena are also present in the park,

although in small numbers and it is generally difficult to spot them. It is also possible to find aardwolf (*Proteles cristatus*), meerkat (*Suricata suricatta*), dik-dik (*Madoqua kirkii*), and klipspringer (*Oreotragus oreotragus*) (African Parks, 2023b; Laia et al., 2021).

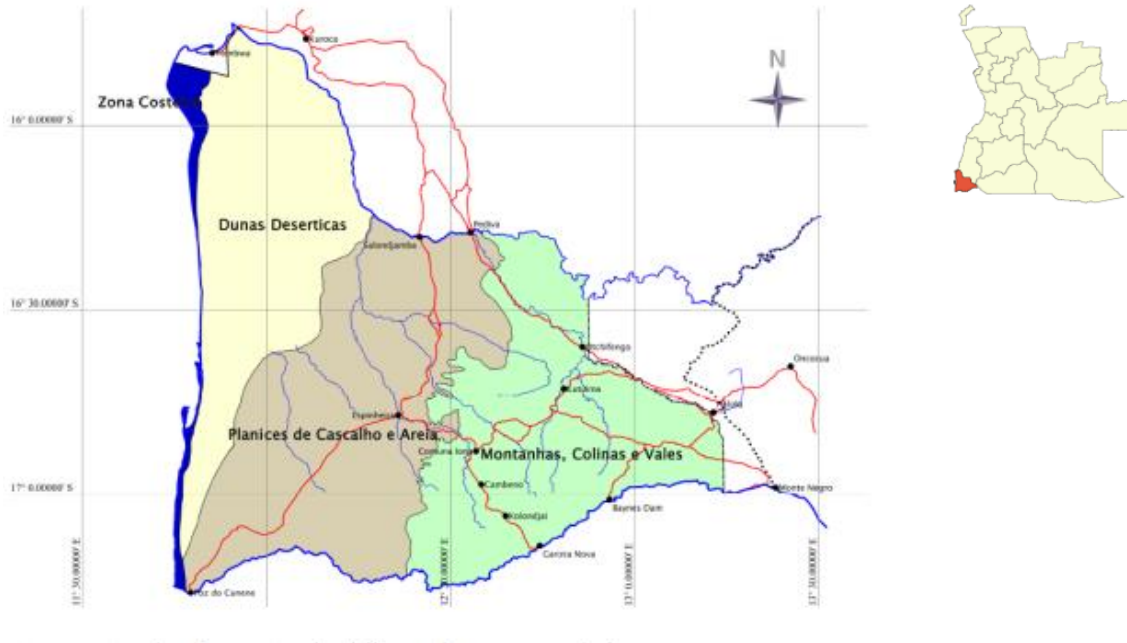


Figure 5: Map of Iona National Park: Location of Iona National Park in Angola and map of the four main zones highlighted by different colours. Figure retrieved from National Biodiversity: Conservation of Iona National Park (UNDP, 2018)

There are at least 63 species of reptiles, eight of which are endemic and well adapted to the arid environment of the park, and around 250 species of birds, such as the ostrich (*Struthio camelus*), Herero chat (*Namibornis herero*), Monteiro's hornbill (*Tockus monteiri*), and Damara tern (*Sternula balaenarum*). In the marine ecosystems there are colonies of Cape fur seals (*Arctocephalus pusillus*), humpback whales (*Megaptera novaeangliae*), green turtles (*Chelonia mydas*), sunfish (*Mola mola*) and some species of sharks (*Selachimorpha*) (African Parks, 2023b).

In Angola, the rainy season begins in October and lasts until April, but in the Namib Desert, the rainy season is more likely to last from January to March (Laia et al., 2021). The climate is characterized as arid, with an average annual temperature just above 18°C (MINAMB, 2016).

Due to the arid environment, the human settlement is limited and is concentrated in the south-eastern part of the park. Here wildlife coexists with people and livestock. One of the biggest threats to the park is the large growth of the human population and the pressure it puts on the park. The number of pastoralists living in the villages within the park

boundaries and its surroundings increased from 200 in 1971 with 3000 cattle and goats (Laia et al., 2021) to 3300 people in 2018 with 15 000 cattle and 31 800 goats (Rocha et al., 2022). Not only do people encroach on wildlife territory, but livestock compete with wildlife, people hunt more, and because the population is concentrated in areas where there is access to water, wildlife movement around this area is restricted (MINAMB, 2016).

2.2 Scat Collection and DNA Extraction

Scats were collected in Iona National Park over an area of approximately 1,500 km², and conserved in silica beads up to its use at CIBIO, the Research Centre in Biodiversity and Genetic Resources (CIBIO). Two batches of samples were collected, the first during March 2021 consisting of 42 scats, and the second during November 2022, consisting of 94 scats.

DNA extraction was performed on all samples according to the two batches, and during this thesis, only the second batch was extracted for DNA. DNA extraction was performed in a dedicated room with positive air pressure and UV lights to avoid contamination, following Frantz et al. (2003) using the GuSCN/silica procedure (Boom et al., 1990). Negative controls of extraction were included to monitor for putative contaminations.

2.3 Identification of carnivore species

Species identification was performed by polymerase chain reaction (PCR) amplification of the mitochondrial ATP6 gene for all samples in a dedicated room under sterile conditions. PCR mixes were prepared by combining QIAGEN PCR Mix containing Taq DNA Polymerase, RNase inhibitor, dNTP's reaction buffer, Mg²⁺ and stabilising agents, ATP6 forward and reverse primer and water. The resulting mix was distributed into the wells of a PCR plate, and 2.5 µL of extracted DNA was added to each well (Table_SI 3; Thermocycling conditions in Table_SI 4). The PCR products were analysed by electrophoresis on a 2% agarose gel. Some samples were diluted (5 µL of DNA mixed with 15 µL of H₂O) and repeated using 2 µL of DNA. For samples that did not have a band on the gel the PCR reaction was repeated using 4 µL of concentrated DNA. Successful PCR products were cleaned using ExoSap enzymatic reaction in a thermocycler for 30 minutes, and then prepared for sequencing. For the sequencing reaction, a mix was prepared containing water, TRR buffer, primer and TRR mix (Table_SI 5). Nine µL of the mix was distributed into the wells of a sequencing plate and 0,8-1 µL of the ExoSap-cleaned PCR product was added to each well. The sequencing reaction was performed at an annealing temperature of 52°C. The resulting sequencing products were cleaned with Sephadex, dried at 95°C, hydrated with formamide, and separated by capillary

electrophoresis in an automatic sequencer (Applied Biosystems). Finally, the resulting sequences were aligned using MEGA11 software and compared to a reference database using the BLAST tool (REF NCBI).

2. 4 Individual Identification and sex determination

2.4.1 Microsatellite primer selection

Individual identification was performed in a dedicated room under sterile conditions. We selected a panel of 16 microsatellites developed in previous studies for spotted hyena (Horn et al., 2004; Libants et al., 2000; Wilhelm et al., 2003). Markers were optimised for amplification and grouped into 4 different multiplexes (Table_SI 12). For cheetahs, the selected microsatellites were developed in domestic cat (*Felis catus*; Menotti-Raymond et al., 1999) and previously used for leopards (Dutta et al., 2012) and cheetahs (Charruau et al., 2011; Khalatbari et al., 2022). A total of 22 microsatellites were used and grouped into 4 multiplexes (Table_SI 11). Markers were selected based on the number of alleles and the heterozygosity observed in the optimization process.

2.4.2 Sorting and genotyping

Prior to genotyping, each sample was tested using only one of the four multiplexes. For this purpose, Mix 1 was used for brown hyenas and Mix 3 for cheetahs. A two-step PCR (pre and post) was carried out with four replicates for each mix, always including negative controls to detect possible contamination. All pre-PCR procedures were performed in a dedicated room for low-quality DNA samples, exposed to UV light before use. PCR mixes were prepared using QIAGEN PCR Mix, primer mix, and water. After distributing the mix into each well, the DNA was added (Table_SI 6). The PCR conditions for both species included a pre-denaturation, followed by 5 or 9 cycles (brown hyena and cheetah, respectively), of denaturation, annealing with a decrease of 0.5°C per each cycle, and extension. A second denaturation, annealing and extension were performed with 11 or 15 cycles (cheetah and brown hyena, respectively) and a final extension (detailed conditions for time and temperature at each step are available in Table_SI 7 and 9). A second PCR was performed with the product (1.2 µL) of the first PCR reaction (Table_SI 8 and 10).

PCR products were prepared for capillary electrophoresis in an automated sequencer (Applied Biosystems). For that, 10 µL of a 10% solution of size standard (Liz 500) mixed with formamide was distributed into each well of the plate and combined with 1 µL of DNA per sample. GeneMapper software was used to inspect the peaks of the alleles. Inconclusive samples were removed from the process while the additional three mixes were used for the samples that showed good amplifications for the first mix. Procedures

followed the same protocols as for the first multiplex. Markers with amplification issues for some samples were repeated using single primers instead of a multiplex.

2.4.3 Sex identification

Sex identification was performed differently for the two species. For cheetahs, the ZFX marker was included in the Mix 2 and amplifications were performed together with the other markers in this multiplex. In this case, the marker amplifies only in males and one fragment of approximately 465 bp is generated, while females do not amplify any fragment. Additionally, it was also used the amelogenin marker to confirm sex identification in cheetahs. For brown hyenas, two markers for sex identification were used, CCrA3 and SRY, and the amplification was performed individually. These two markers amplify only for males, while no fragments are produced for females.

2.5 NGS Library Preparation and sequencing for metabarcoding

All steps on this procedure were performed in a dedicated room specific for low quality DNA and exposed to UV light prior to use. All samples identified as cheetah and brown hyena were used for diet analysis, regardless of being individually identified. Library preparation was performed in a two-step PCR protocol, following Illumina procedures (Bourlat et al., 2016). The first step was set to amplify the V5 region of the mitochondrial 12S gene using the 12S-V5 forward/ reverse pair primer. The same PCR conditions were used for cheetah and hyena, and two replicates per sample were amplified for each species. Negative controls were always included for each PCR. Mixes were prepared together for both species to a final volume of 8 µL in each well with 5 µL of MM, 0.3 µL of primer 12s forward diluted 1:10, 0.3 µL of primer 12s reverse diluted 1:10 and 2.4 µL of water. Then, 2.5 µL of extracted DNA was added (Table_SI 13; Thermocycling conditions in Table_SI 14).

The PCR amplicons were visualised on a 2% agarose gel, and dilutions of 1:2, 1:3 and 1:4 were made for all samples based on the amplification results.

In the second step, named index PCR, P5/P7 Illumina adapters and indexes for individual identification of samples after pooling were incorporated to the diluted PCR products from the first step. A mix with 7 µL of Kappa HiFi Hot Start enzyme and 2.8 µL of water was prepared and distributed into the plate. Additionally, 1.4 µL of the index was also added to the respective well associated with a single sample. This way each sample had a different label for later identification. Then, 2.8 µL of the first PCR product was added (Table_SI 15). The thermal cycling conditions included an initial step of 3 minutes at 95°C, followed

by 8 cycles (10 cycles for some samples with a weaker band on the gel) of denaturation, 30 secs at 95°C, annealing, 30 secs at 55°C and extension, 30 secs at 72°C. Finally, the temperature was maintained at 72°C for 5 minutes (Table_SI 16). To check if the index had been incorporated, an agarose gel was prepared with the products of the first PCR and the products of the index PCR. The expected difference in size informs about the success in the index incorporation.

Throughout the analysis of the agarose gel, it is also possible to determine the approximate size of the target fragment, which allows determining the correct ratio of beads to be used in the following PCR clean-up step. This step is necessary to remove unwanted DNA fragments (free primers and primer dimer) and purify the target fragment. For the PCR clean-up step, we used AMPure XP Beads following the TruSeq DNA Nano protocol from Illumina. A ratio of 0,85 was used for 10 µL of Index PCR products, which allows to eliminate fragments shorter than 100bp.

Quantification of libraries was performed using an Epoch spectrophotometer. The libraries, pooled per replicate and quantified using a Qubit fluorometer, and were then grouped in a single pool. The final pool was checked for integrity using TapeStation High Sensitivity DNA 5000 and was prepared for sequencing on the MiSeq platform (Illumina).

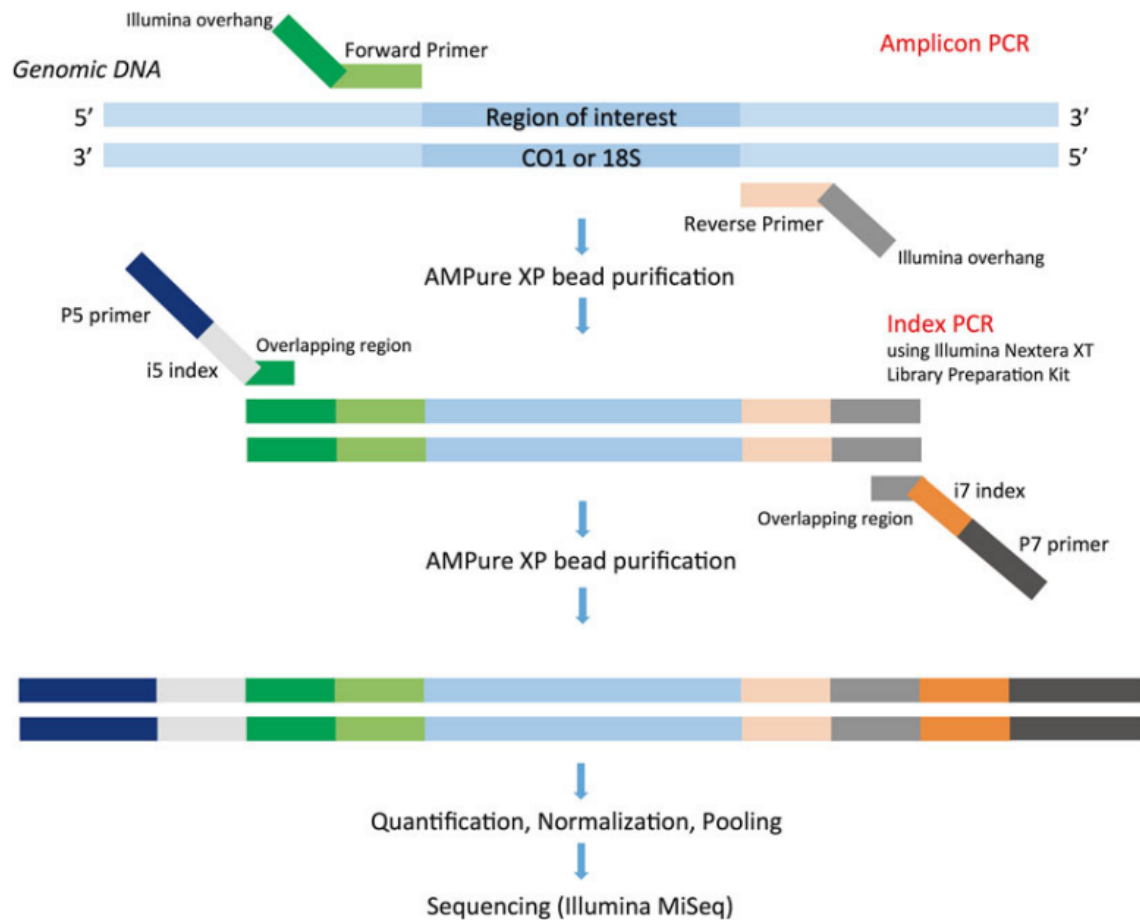


Figure 6: Schema for Illumina MiSeq library preparation using a two-step PCR approach – The first PCR uses amplicon-specific primers (12S) including Illumina adapter overhangs (Amplicons PCR). The second PCR allows the incorporation of Illumina index adapters i5 and i7 (Index PCR). Bead purifications are carried out after each step. Quantification, normalization, and pooling are carried out before sequencing on Illumina MiSeq. Figure retrieved and adapted from Bourlat et al., 2016.

2.6 Data Analysis of Genotyping

2.6.1 Individual identification of cheetah and brown hyena

After construction of a genetic profile per replicate for the microsatellites used in each species, it was established a consensus genotype based on the four replicates per marker for each sample and following the three criteria: i) homozygous genotypes were considered if they were amplified three or more times in different replicates; ii) in cases where homozygous genotypes were amplified in only two replicates, the observed allele was considered but missing data was set to the second allele to leave open the possibility of a different allele; iii) heterozygous genotypes were considered only when they were amplified at least twice in different replicates. After building the consensus genotype for

all samples, analyses were performed on GenAlEx software (Peakall & Smouse, 2006). Individuals with the same genotype were identified using the multilocus matches tool. The missing data was ignored for the analysis. A maximum value of three possible differences between genotypes was allowed. The final genotypes for all individuals were used to calculate the probability of identity (PI) and the Probability of Identity between sibling (PID_{sib}) per species using the "Probability of Identity" tool. The PI represents the probability of two different individuals being considered the same, while "PI_{sibs}" is the PI in case of siblings. The lower the PI value, the lower is the probability of wrongly identifying different individuals as the same, which gives an indication of the informativeness of the set of markers being used for individual identification. It was also calculated the Hardy-Weinberg equilibrium using the "HWE" tool, and the agreement between the allelic frequencies expected based on the Hardy-Weinberg equilibrium and the allelic frequencies observed was tested using a Chi-square test. The same software was also used to calculate genetic diversity measures (observed and expected heterozygosity for each locus and overall) for both species. The software Gimlet (Valière, 2002) was used to calculate genotyping errors, namely allele dropout (caused by the non-amplification of one of the alleles in heterozygous genotypes) and false alleles (when spurious PCR products or amplification errors are interpreted as alleles that in fact do not exist).

2.6.2 Social organization of cheetah and hyena

First the CoAncestry software (Wang, 2011) was used to analyse pairwise relatedness between individuals. This was done following a first simulation of the best relatedness estimator from the six available in the software (TrioML, Wang, LychLi, LynchRd, Ritland, QuellerGt, DyadML) to apply for each of our species. The selected estimator was then used to estimate relatedness using the observed genotypes of cheetah and brown hyena. To a better understanding of the social structure in each case, and to achieve parental and sibling probabilities, the Colony software (Jones & Wang, 2010) was also implemented using the allele frequencies estimated in the previous software.

In both species, different parameters were experimented, and multiple tests conducted. In brown hyenas, inbreeding was not taken into account, both sexes were considered monogamous, and the probability of a parent within the sample was set at 0.75.

In cheetahs, the mating system was considered polygamous for both males and females, and the possibility of inbreeding within the population was also not considered. The probability of a parent within the sample was set at 0.5 just for males due to the absence of females.

With the results from both software programs, possible family groupings were established for cheetahs and hyenas.

2.6.2 Space use for cheetah and hyena

The coordinates of individually identified samples were plotted into a map of the INP using the QGIS program (Sherman et al., 2004). Two maps representing the data from the two years of sample collection were generated for each species. Additionally, individual maps were created to represent only each of the defined families.

2.7 Data Analysis of Diet

Diet analyses were conducted using OBITools package (Boyer et al., 2016). An internal CIBIO pipeline optimised for this analysis was followed. First, sequences obtained from Illumina MiSeq were loaded into the server in a fastq format. Each sample comprises four sequences, representing different replicates (replicate a and b) and different primers (reverse, R2, and forward, R1).

Steps of the analysis include: i) pairwise alignment followed by filtering using a defined score of <50 (assemblies with a score lower than 50 were considered “bad” and were discarded, while those with a score higher than 50 were considered “good” as used to continue the analysis); ii) demultiplexing data into multiple samples. For this, a ngsfilter file with the information of each sample was built, including name, replicate, primers used and the position of the library in the plate before pooling. This step splits reads not assigned to any sample (“bad”) and the ones assigned to each of the samples in the whole dataset (“good”). All the “good” files were then combined into one file; iii) remove unaligned sequences and get count statistics for each sample; iv) identical reads were then clustered into a unique sequence, and sequences with less than 10 reads were excluded; v) filtering by size to exclude sequences with lengths < 90bp and > 110bp; vi) cleaning for PCR and sequencing errors; vii) combining results in a tab delimited file; and viii) compare final sequences obtained (which are known as MOTUs – molecular operational taxonomic units) with sequences on a reference database using BLAST.

Results from ObiTools were then imported to RStudio software (Allaire, 2012) after converting the blast document to text format. In Rstudio, the use of Taxonomizr package allowed to obtain a taxonomic level attributed to each MOTU. The taxonomic category was given based on the probability of identity from the blast result (pident). Sequences with at least 98% of pident, were assigned to species level, sequences from 95% to 98% of pident were assigned to genus level and those from 92% to 95% to the family level.

We used the number of reads in the controls, possibly indicating human contamination or cross-contamination, to discard MOTUs with the same or fewer number of reads, in both cases discarding all samples with less than 50 reads. MOTUs with more than 50 reads were checked on the NCBI platform using BLAST. Subsequently, sequences identified as human or other contaminations were removed from the results. The outcomes of this filtering process were organised by presence of prey species per sample.

2.8 Cheetahs and Brown Hyenas diet

To analyse the diet of the two species, it was calculated the frequency of occurrence (FOO) for each prey item. The FOO is the number of scats with a particular prey item present over the total number of scats with any prey item present for the predator. Sample not containing any prey item did not count for FOO.

Shannon's diversity index (H), which takes into account the number of species and their relative abundance, was also calculated to describe species diversity present in the diet. Values equal to or close to 0 mean that the predator feeds on only one prey or a few different prey items, and as the value increases, the diversity of the prey consumed is greater.

It was also calculated the Jaccard similarity index (Jaccard, 1901) that measures the similarity between the two diet compositions. This index range between 0 and 1, with 0 meaning no species shared and 1 meaning that, in this case, the preys in both diets are the same (Dick, 2021).

Finally, Pianka's index was calculated to infer the niche overlap between cheetahs and hyenas and to understand resource sharing. This index ranges between 0 and 1, with 0 meaning no species in common and 1 when species are shared and have the same abundance. Contrary to the first index this one considers not only shared species but also their relative abundance (Pianka, 1974). The Shannon and the Pianka indices were calculated using R software and the Vegan package.

3. Results

The first batch of samples resulted in the identification of 12 samples of cheetahs and 28 samples of brown hyenas. The second batch of samples resulted in a final set of 30 samples of cheetah and 55 of hyena. From the latter batch, nine samples were eliminated because were wrongly identified in the field as belonging to the target species, while three samples originally classified as brown hyena were identified molecularly as cheetah.

3.1 Individual identification and sex determination

In cheetah, all microsatellites were found to be polymorphic, ranging from a minimum of 2 alleles in the FCA97 and FCA205 markers to a maximum of 5 alleles in 8 different markers (Table_SI 11). Out of the 42 samples of cheetah, 27 (64,3%) were individually identified. After genotyping, these 28 samples corresponded to 9 different individuals, meaning on average 1,5 samples per individual. The most common was one sample per individual, but for one cheetah (AJU04) we found 10 samples. Regarding sex, all cheetahs were identified as males (Table_SI 1).

In case of brown hyenas, two microsatellites, Ccr11 and Ccr15, were found to be monomorphic. The polymorphism ranged from a minimum of 2 alleles in 9 microsatellites to 6 alleles in the Ccroc07 marker (Table_SI 12). Out of the 83 samples of brown hyena, it was possible to individually identify 51 (61.4%) samples that corresponded to 15 different individuals, 4 females (26.7%) and 11 (73.3%) males. This corresponds to two samples per individual on average. Like cheetahs, the most common was 1 sample per individual, while 2 individuals, BH2 and BH03, stood out for having 10 and 11 samples, respectively (Table_SI 2).

3.1.1 Probability of identity and HW equilibrium

In terms of probability of identity for cheetahs, the FCA224 marker presented the lower PI and PIsibs values, being the most informative for this species. The least informative marker was the FCA045 with the highest PI value. For brown hyenas, the most informative marker was the Ccroc07 with the lower PI and PIsibs values, while the least informative marker was Ccr11. As expected, in both species the PI and PIsibs values decreased with the addition of markers, however in cheetahs this trend was more pronounced, making the addition of markers more important for differentiating individuals (Figures 7 and 8).

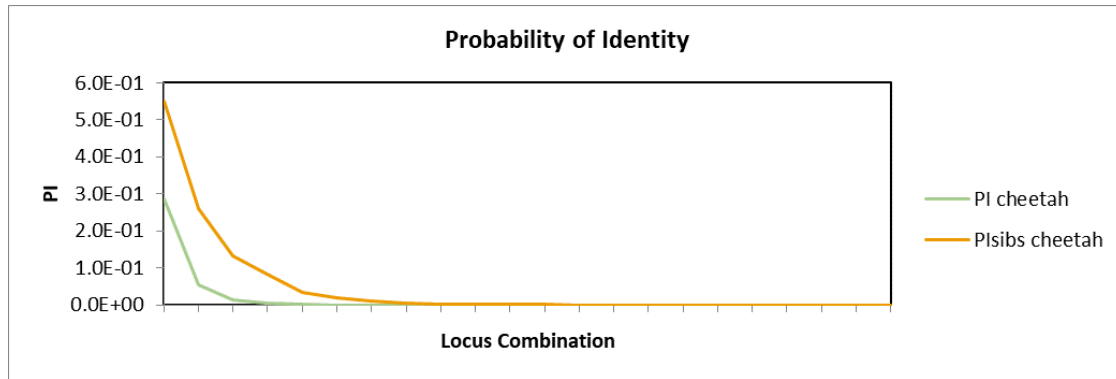


Figure 7: Probability of identity for cheetah, with the addition of markers up to a total of 22.

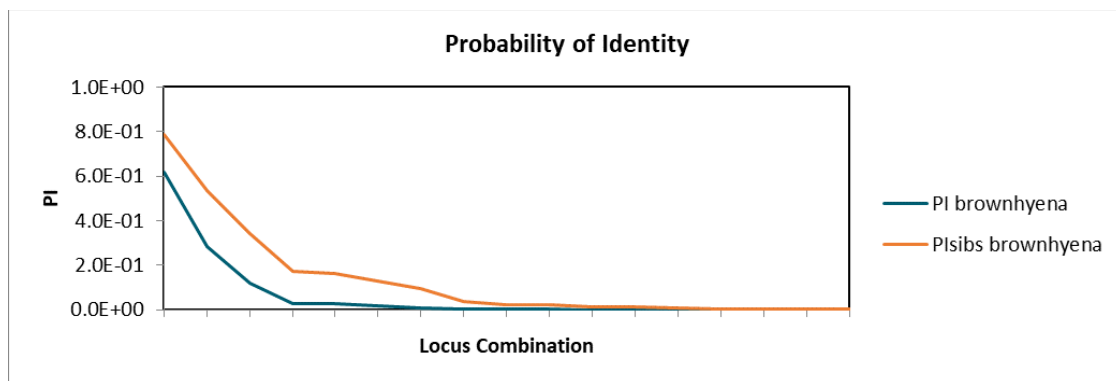


Figure 8: Probability of identity for brown hyenas, with the addition of markers up to 16.

The test for Hardy-Weinberg equilibrium in the cheetah dataset identified four markers in disequilibrium, FCA07, FCA008, FCA230 and FCA045. In brown hyenas, two microsatellites were monomorphic so the test could not be applied to those markers. For the others, only one microsatellite was found in disequilibrium, Ccroc02.

3.1.2 Amplification errors

In cheetahs, markers exhibited allele dropout values ranging from 0 to 0.222, while values of false alleles ranged from 0 to 0.250 (Table 1). In brown hyenas, the allele dropout observed for the amplification of microsatellites ranged from 0 to 0.208 and the false alleles ranged from 0 to 0.019 (Table 2).

Table 1: Amplification errors in cheetah samples – Allele dropout and false alleles calculated from 22 different microsatellites amplified in non-invasive samples of cheetah.

Locus	Dropout	False allele	Locus2	Dropout	False allele
FCA075	0.092	0	FCA133	0.044	0
FCA171	0.027	0	FCA14	0.134	0
FCA310	0.028	0	FCA212	0	0

FCA97	0.033	0	FCA247	0.061	0
FCA008	0.078	0	FCA85	0.161	0
FCA005	0.068	0	FCA026	0.115	0
FCA126	0.040	0.250	FCA045	0.023	0
FCA205	0	0	FCA105	0.156	0
FCA224	0.208	0	FCA132	0.052	0
FCA230	0.222	0	FCA214	0.133	0
FCA096	0.197	0	FCA69	0.031	0

Table 2: Amplification errors in brown hyena samples – Allele dropout and false alleles calculated from 16 different microsatellites amplified in non-invasive samples of brown hyena.

Locus	Dropout	False allele	Locus	Dropout	False allele
Ccr12	0.083	0.015	Ccr11	0	0
Ccroc04	0.064	0	Ccr13	0.132	0
Ccroc08	0.038	0.019	Ccr15	0	0
Ccr16	0.667	0	Ccroc01	0.033	0
Ccr17	0.019	0	Ccroc02	0	0
Ccroc03	0	0	Ccr05	0.017	0
Ccroc07	0.068	0	Ccr07	0.063	0
Ccroc09	0.036	0	Ccr14	0.208	0

3.2 Individuals space use

Cheetah samples of both years were gathered within an area of over 409.000 km² and were gathered mainly in the gravel and sand plains area (Figure 9). Individuals AJU04 and AJU05 were found in both years, the remaining individuals were just present in the sampling in one of the two years (Figure 10).

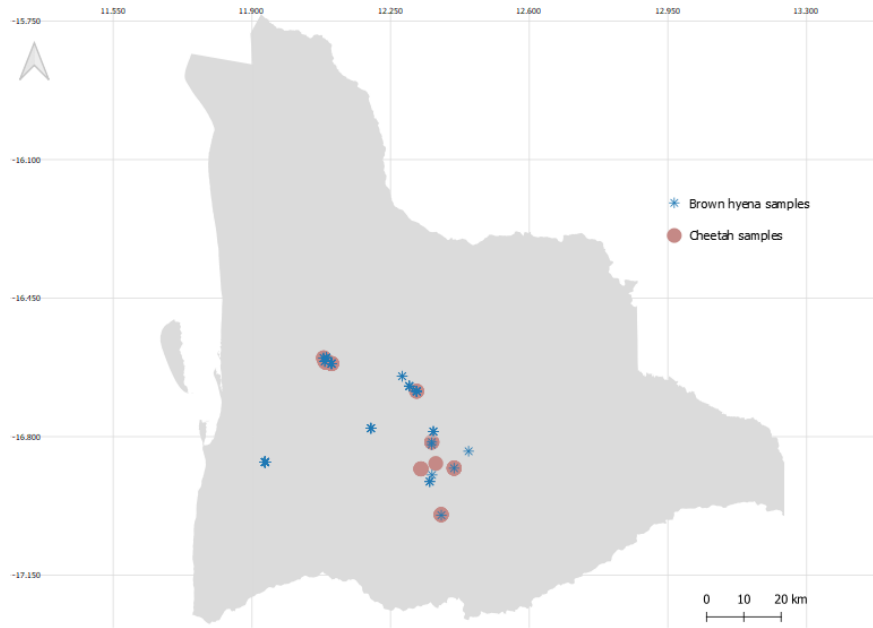


Figure 9: Location of marking trees of cheetah and latrines of brown hyena. Location of all samples identified as cheetahs collected in marking trees during the years of 2021 and 2022 and location of all samples identified as brown hyenas collected in latrines during the years of 2021 and 2022.

The distribution of cheetah individuals is represented in Figure 10, where individuals AJU04 and AJU05 are the ones presenting larger home ranges according to our sampling, possibly also reflecting the higher number of samples for AJU04. The observed distribution does not clearly show clusters of individuals nor geographic segregation.

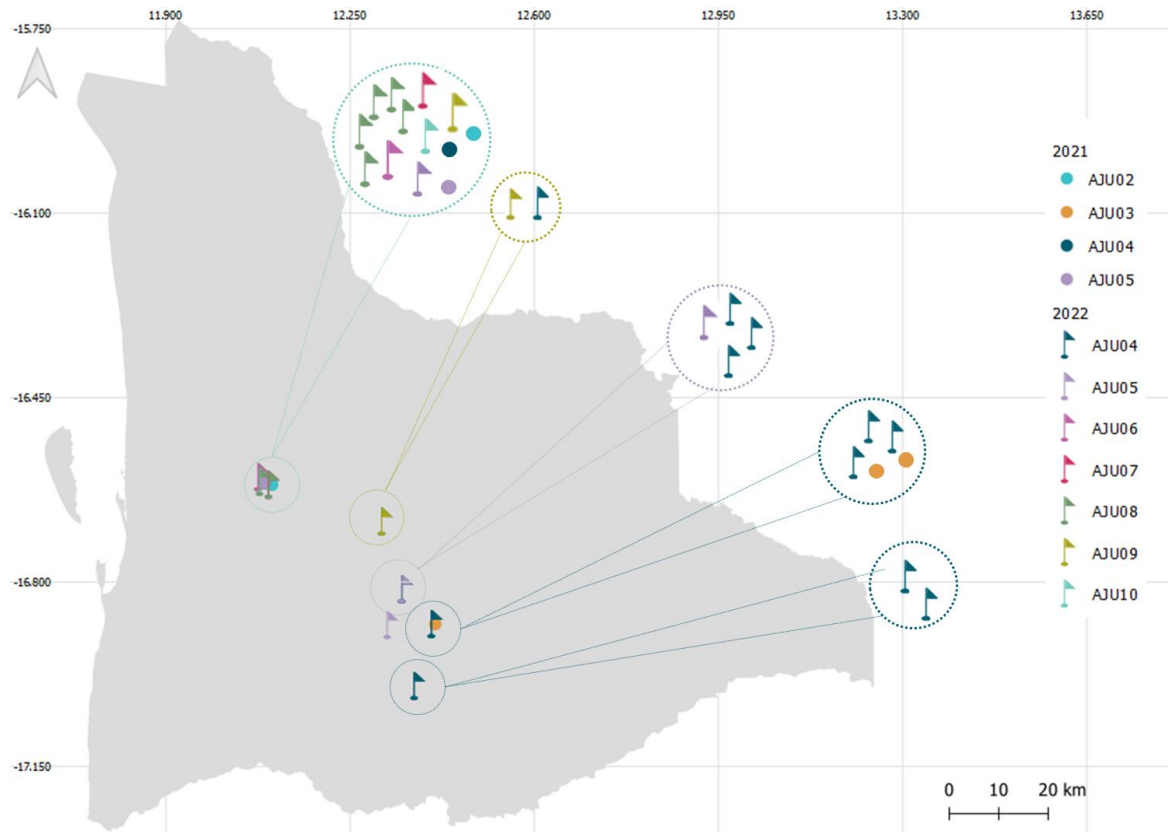


Figure 10: Distribution of cheetah samples in 2021 and 2022- Each individual is represented by a different colour, and when multiple samples overlap, the number of collected samples at that location is represented within the circles. Samples collected from individuals AJU02, AJU03, AJU04 and AJU05 in 2021 are represented by small circles, samples collected from individuals AJU04, AJU05, AJU06, AJU07, AJU08, AJU09 and AJU10 in 2022 2022 are represented by small flags

Brown hyena samples were collected across an approximate area 1060.000 km² and like cheetahs, many samples were collected in the gravel and sand plain area. However, a large part of the samples was collected from the dunes closer to the coast (Figure 9). Individuals BH02, BH03 and BH09 were sampled in both years, while the remaining were only found in one year (Figure 11).

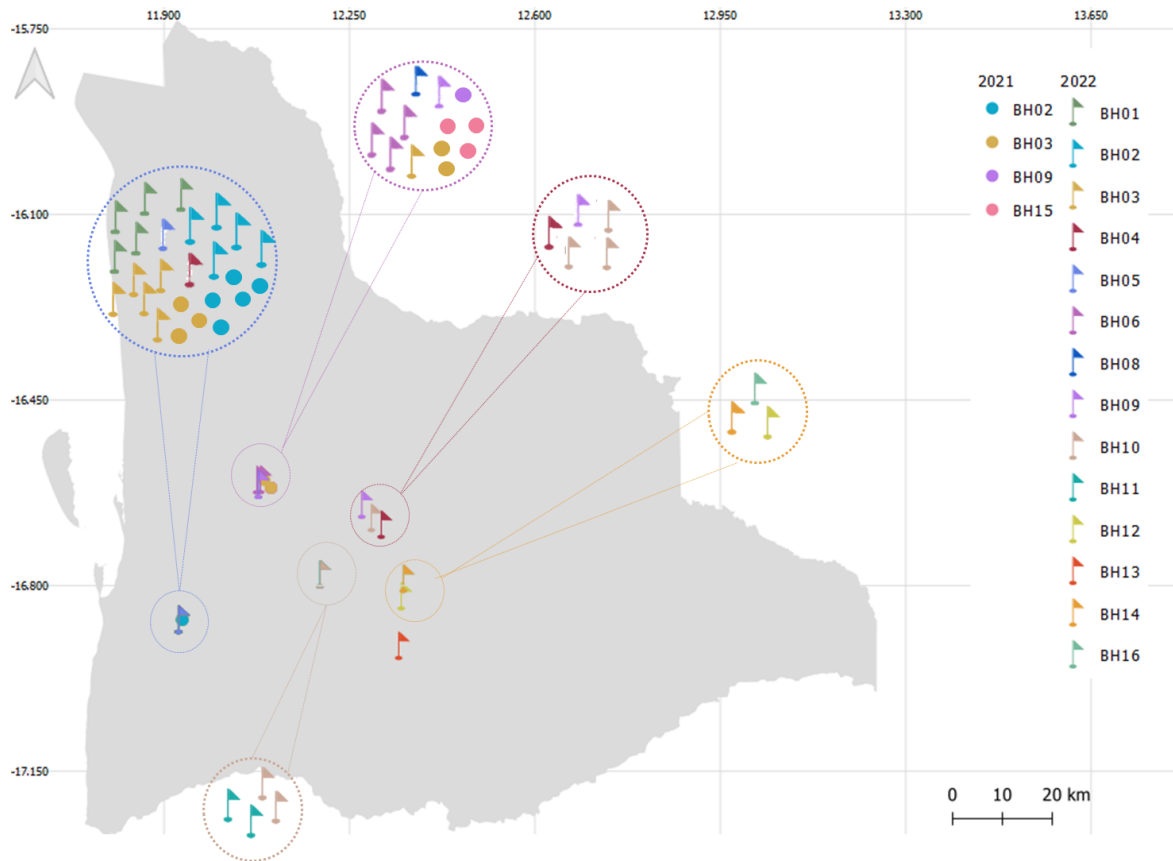


Figure 11: Distribution of brown hyena samples in 2021 and 2022- Each individual is represented by a different colour, and when multiple samples overlap, the number of collected samples at that location is represented within the circles. Samples collected from individuals BH02, BH03, BH09 and BH15 in 2021 are represented by small circles, samples collected from individuals BH01, BH02, BH03, BH04, BH05, BH06, BH08, BH09, BH10, BH11, BH12, BH13, BH14 and BH16 in 2022 are represented by small flags.

3.3 Social organisation

Simulations testing for different relatedness estimators for cheetah in Iona have shown that the most suitable estimator to apply in this population is TrioML. We used it to calculate pairwise relatedness for the nine male cheetahs observed in the area and achieved results between 0 and 0.784 (Table_SI 17). These results, combined with parentage and sibling relationships calculated in Colony, allowed to establish three families of cheetah's full siblings involving a total of eight individuals (Figure 12). One individual (AJU03) did not present any supported familial relationship with the other cheetahs in the area.

Cheetah Family 1 is composed by three full siblings (AJU02, AJU04 and AJU10) that exhibited an average pairwise relatedness of 0.528 (Figure 12a). Cheetah Family 2 is also composed by three male siblings (AJU05, AJU06 and AJU09), with an average

relatedness of 0.544 (Figure 12b). The last pair of full siblings was named Cheetah Family 3 and was formed by AJU07 and AJU08, with a pairwise relatedness of 0.334 (Figure 12c).

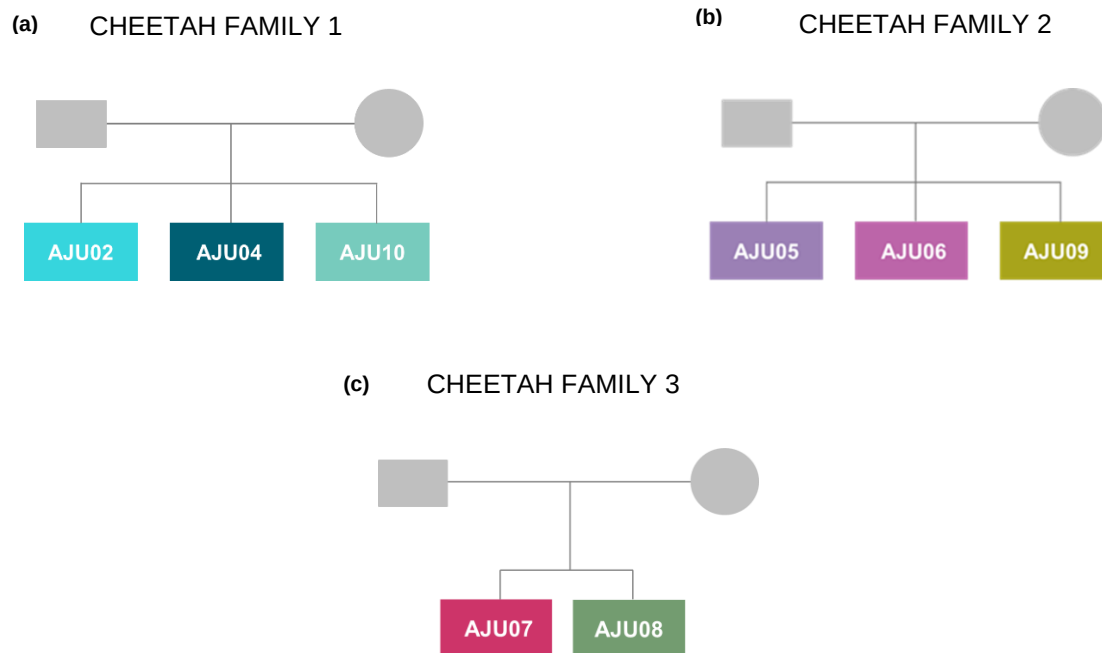


Figure 12: Genealogical trees with the inferred cheetah's families in the INP – (a) Family 1 formed by individuals AJU02, AJU04 and AJU10; (b) Family 2 formed by individuals AJU05, AJU06 and AJU09; (c) Family 3 formed by individuals AJU07 and AJU08.

Samples collected for cheetah family 1 came from five different marking trees, covering a maximum distance between them of approximately of 53 km. However, individuals AJU02 and AJU10 were only sampled in one marking trees, while AJU04 was sampled in the five sites (Figure 13a). For cheetah family 2, the three siblings were found in one marking trees, while AJU05 and AJU09 were also observed in other two and one marking trees, respectively (Figure 13b). These sites were the same used by siblings of family 1. The two males of cheetah family 3 were only collected in a single marking trees (Figure 13c). This site was the only one used by the three families.

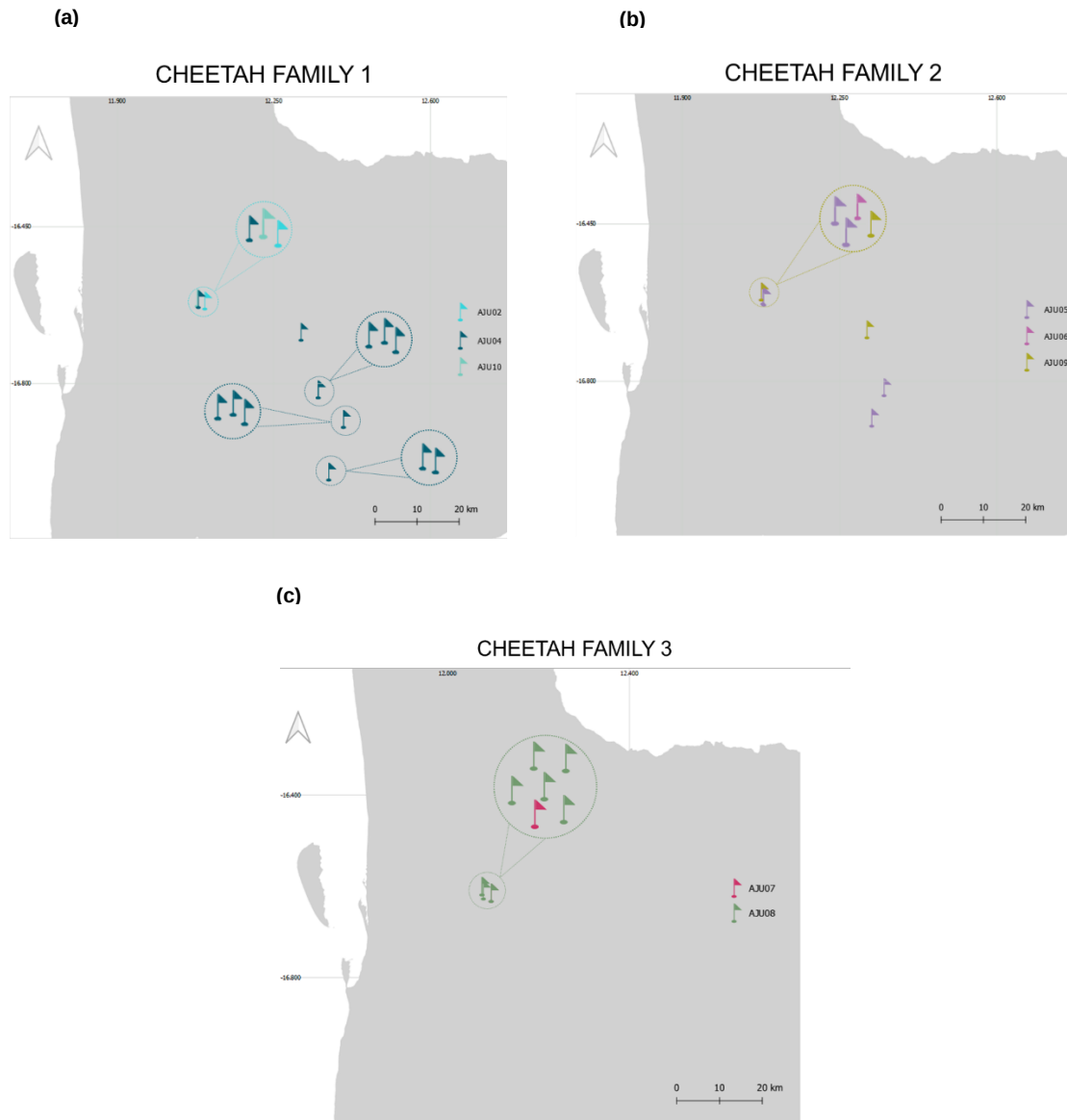
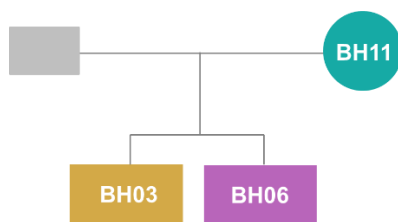


Figure 13: Distribution of samples from individuals of cheetah families – (a) Distribution of samples from individuals AJU02, AJU04 and AJU10, which constitute cheetah family 1; (b) Distribution of samples from individuals AJU05, AJU06 and AJU09, which constitute cheetah family 2; (c) Distribution of samples from individuals AJU07 and AJU08, which constitute cheetah family 3.

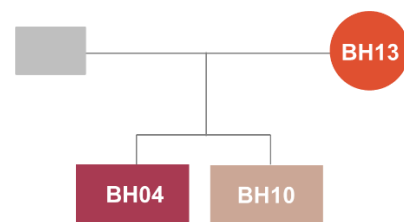
In the case of brown hyenas, the relatedness estimator that exhibited the highest correlation with the true value of the population was DyadML. Consequently, this was selected to calculate pairwise relatedness between all brown hyena individuals and achieved results between 0 and 0.0774 (Table_SI 18). Relatedness and parentage analysis allowed to infer four different families with high probabilities. Brown hyena family 1 was formed by three individuals (BH03, BH06 and BH11), including two full sibling males, with an estimated relatedness of $r=0.832$, and their maternal progenitor (Figure 14a). Both siblings presented a pairwise relatedness with her mother of $r=0.500$. Brown

hyena family 2 presented the same genealogy observed for family 1, namely two male siblings (BH04 and BH10) with a pairwise relatedness of $r = 0.617$ and their maternal progenitor (BH13), with whom have relatedness estimates of $r = 0.500$ and $r = 0.774$, respectively (Figure 14b). Brown hyena family 3 represents a pair of mother-offspring (individuals BH02 and BH05, respectively) with a pairwise relatedness of $r = 0.646$ (Figure 14c). Finally, brown hyena family 4 is constituted by a pair of male full siblings (BH08 and BH12), with a pairwise relatedness of $r = 0.506$ (Figure 14d).

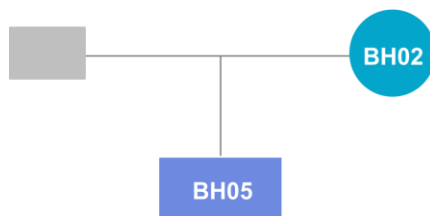
(a) BROWN HYENA FAMILY 1



(b) BROWN HYENA FAMILY 2



(c) BROWN HYENA FAMILY 3



(d) BROWN HYENA FAMILY 4

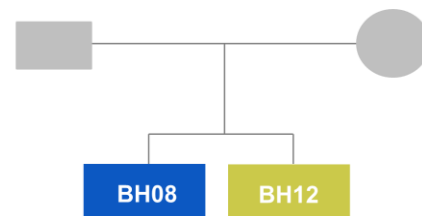


Figure 14: Genealogical trees with the inferred brown hyena's families in the INP – (a) Family 1 formed by individuals BH03, BH06 and BH11; (b) Family 2 formed by individuals BH04, BH10 and BH13; (c) Family 3 formed by individuals BH05 and BH02; (d) Family 4 formed by individuals Bh08 and Bh12.

Regarding localization, samples from brown hyena family 1 were collected in three latrines, one shared by the two siblings, and two where only samples of one sibling or the progenitor were found (Figure 15a). A very similar scenario is observed for the location of samples from brown hyena family 2, collected from four latrines. In a single site, there were samples from the two siblings, while in three latrines there were only samples from one of each individual of the family (Figure 15b). For brown hyena family 3, samples were

only observed in a site for both the mother and the offspring (Figure 15c). Finally, for family 4, samples of the two siblings were collected in different latrines (Figure 15d).

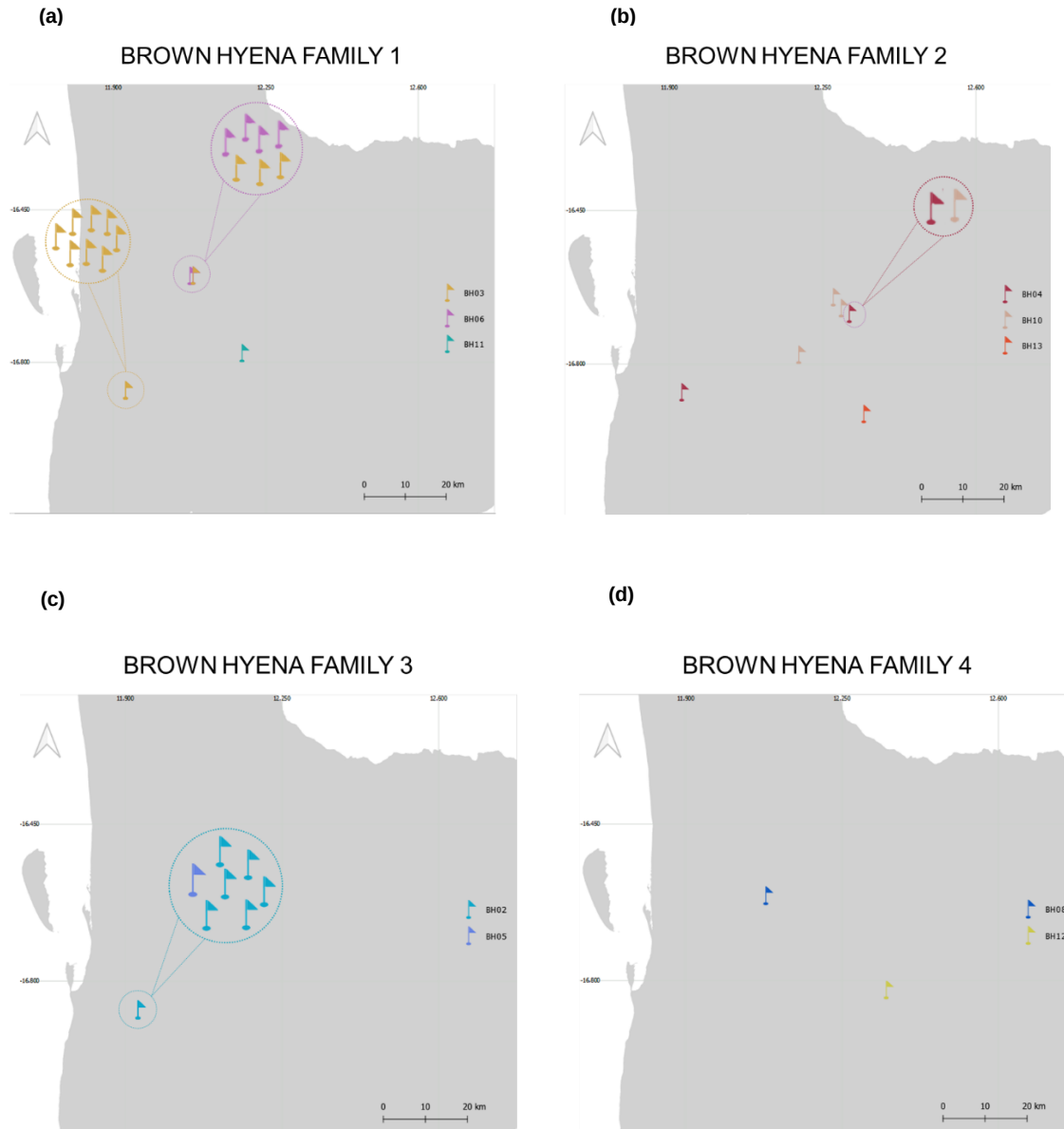


Figure 15: Distribution of samples from individuals of brown hyena families – (a) Distribution of samples from individuals BH03, BH06 and BH11, which constitute brown hyena family 1; (b) Distribution of samples from individuals BH04, BH10 and BH13, which constitute brown hyena family 2; (c) Distribution of samples from individuals BH05 and BH02, which constitute brown hyena family 3; (d) Distribution of samples from individuals BH08 and Bh12, which constitute brown hyena family 4.

3.4 Diet analysis

3.4.1 Metabarcoding taxonomic resolution

For the dietary analysis, two sequencing runs were conducted, for the two batches of samples.

In the first batch of cheetah samples produced a total of 347 182 reads, corresponding to 21 MOTUs after filtering out MOTUs below the threshold of 50 reads. In the second batch, 815 650 reads corresponding to 25 MOTUs were obtained for cheetahs. Data merge of the two runs resulted in 1 162 832 reads and 46 MOTUs. Among the 46 MOTUs, those corresponding to human sequences, sequences with no taxonomic correspondence in NCBI database (e.g., chimerae) or sequences corresponding to species not present in the study area (possible laboratory contamination) were excluded (23 MOTUs). Finally, among the remaining MOTUs, seven were identified as sequences from cheetah origin. The resulting 16 MOTUs, corresponding to 212 062 reads, were used for the assessment of taxonomic identification of prey items. MOTUs were mostly identified to the species level (N=15), while one was identified at the genus level. This resulted in the identification of seven different taxa of prey items.

Furthermore, the filtering steps reduced the sample size from 42 initial cheetah samples down to 19 cheetah samples (45,2%) with informative metabarcoding data, which were included in the following analyses.

Regarding the first batch of brown hyena, samples produced a total of 800 263 reads, corresponding to 39 MOTUs, after the filtration of MOTUs below the threshold of 50 reads. In the second batch it was obtained 1 833 715 reads, corresponding to 114 MOTUs. Data merge of the two runs resulted in 633 978 reads, corresponding to 153 MOTUs. Among the 153 MOTUs, 35 were excluded corresponding to human sequences, sequences with no taxonomic correspondence in NCBI database or to species not present in the study area. Among the remaining MOTUs, 35 were identified as brown hyena sequences. The remaining 83 MOTUs, totalling 571 868 reads were used for the assessment of taxonomic identification of prey items. It was possible to identify to the species level 66 MOTUS, and 17 MOTUS to the genus level. This allowed the identification of 29 different taxa of prey items.

The filtering steps reduced the sample size from 83 initial brown hyena samples down to 64 brown hyena samples (77,1%) with informative metabarcoding data, which were included in the following analyses.

3.5 Cheetah diet

Among the 19 cheetah samples with identified prey items, we found a total of seven different prey taxa: three ungulates - the springbok (*Antidorcas marsupialis*), the gemsbok (*Oryx gazella*) and the steenbok (*Raphicerus campestris*), one lagomorph – the cape hare (*Lepus capensis*), two birds - the common ostrich (*Struthio camelus*) and the buzzard (*Buteo sp*), and one carnivore - the aardwolf (*Proteles cristata*; Table 3).

Table 3: Cheetah prey – Group and scientific name of the species identified in cheetah scat samples, the number of reads in the final filtering process, the number samples containing each prey and the frequency of occurrence (%).

Group and Scientific Name	Number of reads	Counts of Occurrence	FOO (%)
Ungulates	25878	9	47.4
<i>Antidorcas marsupialis</i>	24729	7	36.8
<i>Oryx gazella</i>	192	1	5.3
<i>Raphicerus campestris</i>	957	1	5.3
Lagomorphs	103853	7	36.8
<i>Lepus capensis</i>	103853	7	36.8
Birds	67095	3	15.8
<i>Struthio camelus</i>	11315	2	10.5
<i>Buteo sp</i>	55780	1	5.3
Carnivores	15236	2	10.5
<i>Proteles cristata</i>	15236	2	10.5

The number of prey per scat ranged between one and two, with one prey being the most common. Springbok and cape hare stood out as dominant prey in cheetah's diet, both with a frequency of occurrence (FOO) of 36,8%, occurring in seven samples each. Aardwolf and ostrich followed, each appearing twice in the among all samples, with a FOO of 10,5%. The remaining species had only one occurrence each, with a FOO of 5,3% (Figure 16). In general, ungulates were the most common group in the cheetah diet, with a FOO of 47,4%, then lagomorphs with 36,8% of occurrence, birds with 15,8%, and carnivores with 10,5% (Figure 17). Notably, there were no records of any domestic prey in the cheetah's diet among our samplings.

In terms of dietary diversity for the overall cheetah sampling in the INP calculated using the Shannon's index, it was estimated as $H = 1.62 \pm 0.15$.

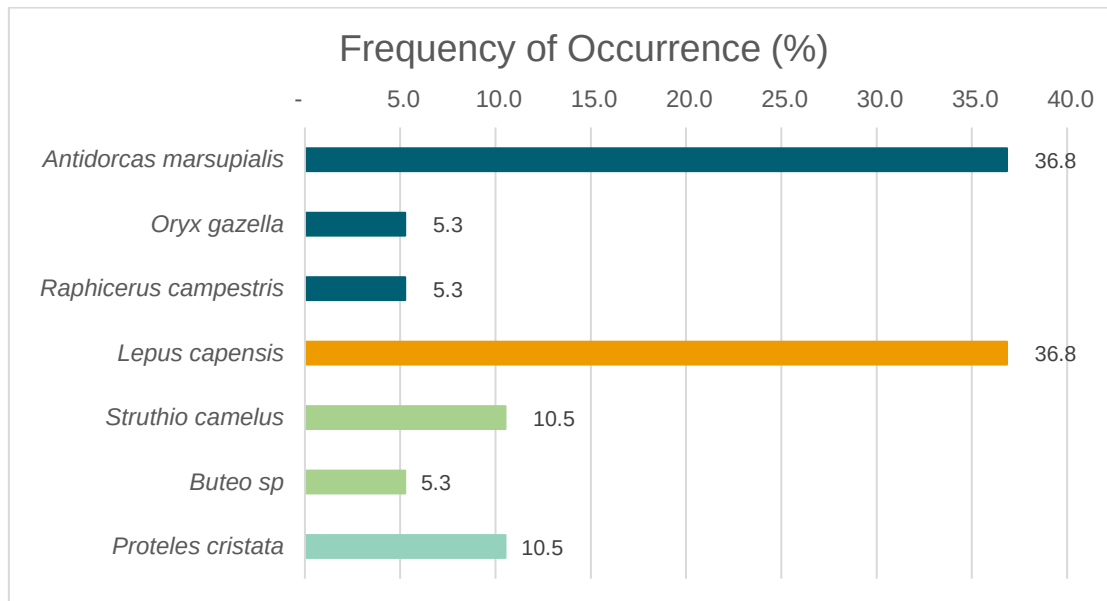


Figure 16: Frequency of Occurrence (%) in the diet of cheetah in INP - Frequency of occurrence (%) of the seven different species detected among the 19 scat cheetah samples. Dark blue bars represent ungulates, orange represent lagomorphs, green represent birds and light blue represent carnivores.

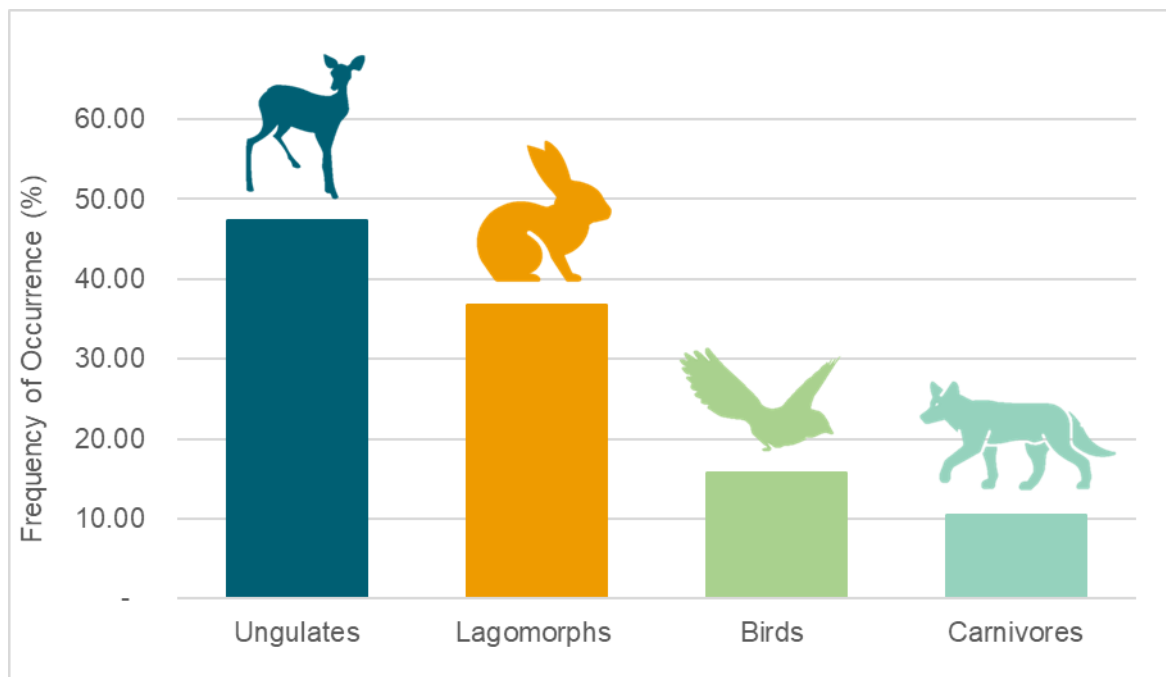


Figure 17: Frequency of occurrence (%) of groups of prey for cheetah in the Iona NP – Frequency of occurrence (%) of the 4 different groups of cheetah prey. Dark blue bar represents ungulates, orange represents lagomorphs, green represents birds and light blue represents carnivores.

3.6 Brown hyena diet

Twenty-nine different taxa were identified as diet items from the 63 scats of brown hyena (Table 4). Among those, birds were the more represented group with 14 species– the common ostrich, the gull (*Larus sp.*), the greater flamingo (*Phoenicopterus roseus*), the common tern (*Sterna hirundo*), a cormorant (*Phalacrocorax sp.*), the grey plover (*Pluvialis squatarola*), the white pelican (*Pelecanus onocrotalus*), a lark (*Eremophila sp.*), the double-banded courser (*Rhinoptilus africanus*), the namaqua sandgrouse (*Pterocles namaqua*), a gyrfalcon (*Falco sp.*), the helmeted guineafowl (*Numida meleagris*), the house sparrow (*Passer domesticus*), and the swift (*Apus sp.*), followed by ungulates with seven species- the springbok (*Antidorcas marsupialis*), the gemsbok (*Oryx gazella*), the steenbok (*Raphicerus campestris*), the mountain zebra (*Equus zebra*), the domestic goat (*Capra hircus*) and two species of domestic cattle (*Bos taurus* and *Bos indicus*), two species of marine mammals – the cape fur seal (*Arctocephalus pusillus*), and the common bottlenose dolphin (*Tursiops truncatus*), two species of mesocarnivores – the black-backed jackal (*Canis mesomelas*), and the wildcat (*Felis silvestris*), two species of lagomorphs - the cape hare (*Lepus capensis*) and the Jameson’s red rock hare (*Pronolagus randensis*), and two other small mammals, the rock hyrax (*Procavia capensis*) and the cape short-eared Gerbil (*Desmodillus auricularis*).

Table 4 - Brown hyena prey –Group and scientific name of the species identified in brown hyena scat samples, the number of reads in the final filtering process, the number samples containing each prey and the frequency of occurrence (%).

Group and Scientific Name	Number of reads	Counts of occurrence	FOO
Birds	92235	23	35.9
<i>Struthio camelus</i>	28899	3	4.7
<i>Larus sp.</i>	629	2	3.1
<i>Phoenicopterus roseus</i>	185	1	1.6
<i>Sterna hirundo</i>	280	1	1.6
<i>Phalacrocorax sp.</i>	50325	7	10.9
<i>Pluvialis squatarola</i>	2936	1	1.6
<i>Pelecanus onocrotalus</i>	1392	1	1.6
<i>Eremophila sp.</i>	218	1	1.6
<i>Rhinoptilus africanus</i>	159	1	1.6
<i>Pterocles namaqua</i>	2779	1	1.6
<i>Falco sp.</i>	2174	1	1.6
<i>Numida meleagris</i>	205	1	1.6
<i>Passer domesticus</i>	1630	1	1.6
<i>Apus sp</i>	424	1	1.6

Ungulates	272597	49	75.0
<i>Antidorcas marsupialis</i>	127854	23	35.9
<i>Oryx gazella</i>	106978	18	28.1
<i>Raphicerus campestris</i>	31430	1	1.6
<i>Equus zebra</i>	3618	2	3.1
<i>Capra hircus</i>	870	2	3.1
<i>Bos taurus</i>	704	1	1.6
<i>Bos indicus</i>	877	1	1.6
Marine mammals	130165	21	32.6
<i>Arctocephalus pusillus</i>	87856	17	26.6
<i>Tursiops truncatus</i>	42309	4	6.1
Mesocarnivores	4414	4	6.3
<i>Canis mesomelas</i>	4111	3	4.7
<i>Felis silvestris</i>	303	1	1.6
Lagomorphs	71938	8	12.5
<i>Lepus capensis</i>	71622	7	10.9
<i>Pronolagus randensis</i>	316	1	1.6
Other small mammals	519	2	3.1
<i>Procavia capensis</i>	277	1	1.6
<i>Desmodillus auricularis</i>	242	1	1.6

In brown hyenas, the number of prey per sample ranged from one to five, with one prey per sample being the most common occurrence. The dominant group of prey in the diet of this carnivore were ungulates with a FOO of 75.0, followed by birds (FOO=35.9%) and marine mammals (FOO=32.6; Figure 19). Among the ungulates, two prey are dominant, the *Antidorcas marsupialis*, with a FOO of 35,9%, occurring in 23 scats, followed by *Oryx gazella* with a FOO of 28,1% and occurring in 18 scats. Among birds, cormorants (FOO=10.9%) and the ostrich (FOO=4.7%) were the most consumed, while among the two marine mammals observed, the cape fur seal was the one most consumed, with a FOO of 26,6% and occurring in 17 scats. For other prey, *Lepus capensis* is the one also exhibiting a considerable consumption, with a FOO of 10,9% and occurring in seven samples. The other wild species appear to be less important in the diet of brown hyena (Figure 18). Notably, three different species of domestic mammals were observed among diet items of brown hyena, including domestic goats and cattle. Together, domestic species were found in four samples, presenting a FOO of 6,3%.

The dietary diversity of brown hyena in the INP calculated based on the Shannon's index, was $H=2.66 \pm 0.24$.

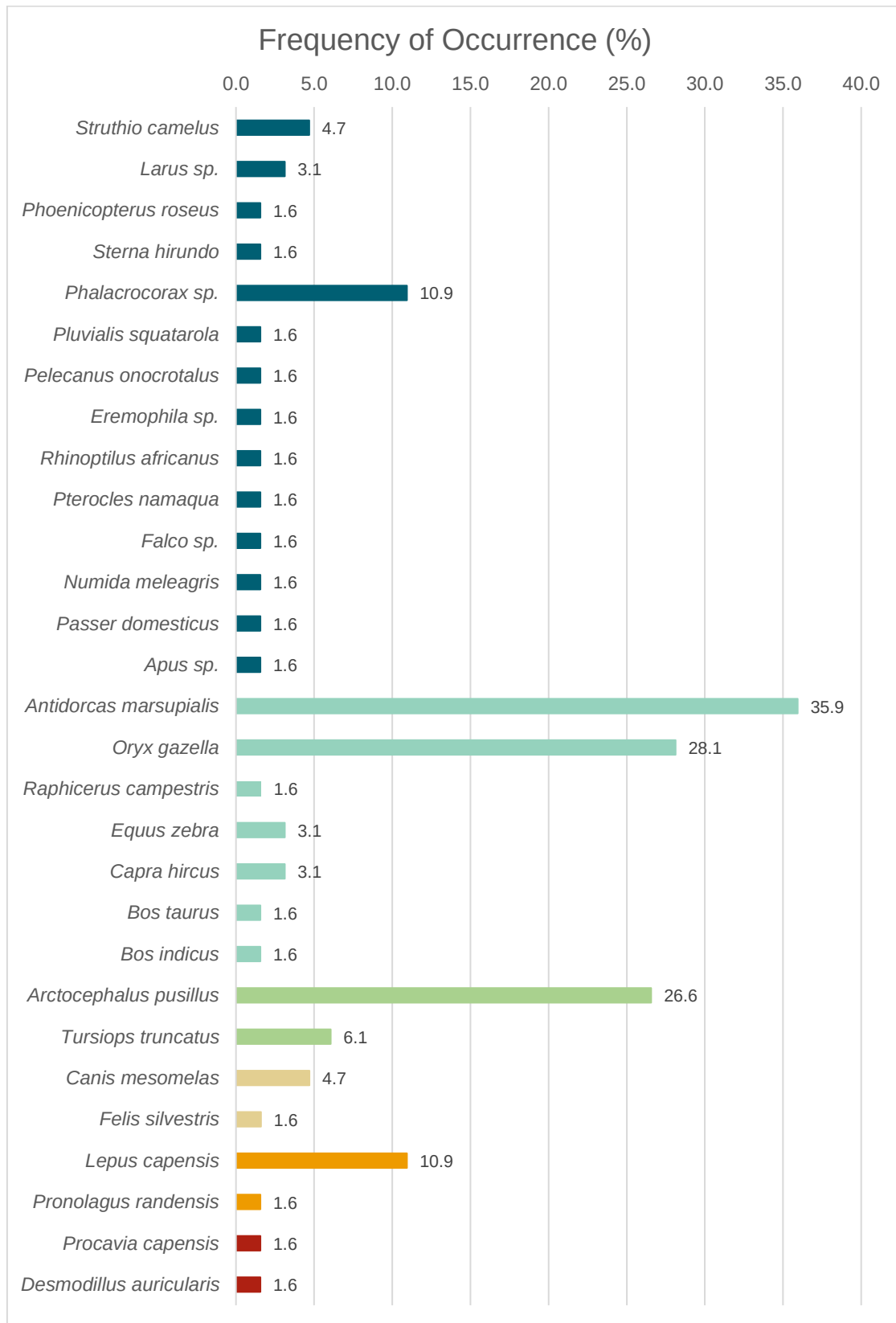


Figure 18: Frequency of Occurrence (%) in the diet of brown hyena in INP - Frequency of occurrence (%) of the 29 different species detected among the 64 scat brown hyena sample. Dark blue bars represent birds, light blue represent

ungulates, green represent marine mammals, yellow represent mesocarnivores, orange represent lagomorphs and red represent the other small mammals.

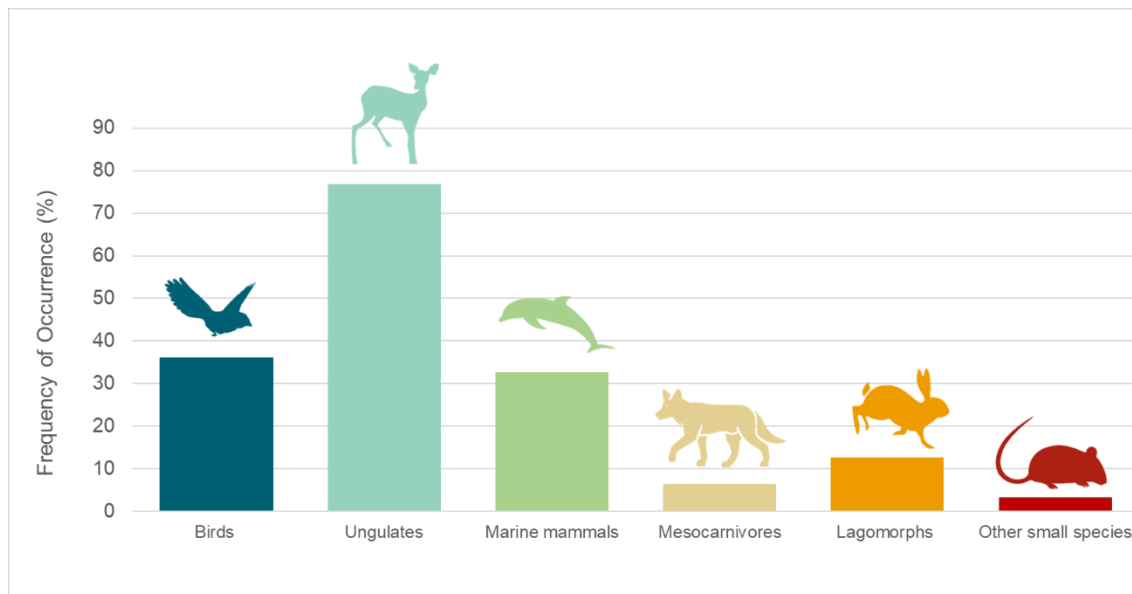


Figure 19: Frequency of occurrence (%) of groups of prey for brown hyena in the INP – Frequency of occurrence (%) of the 6 different groups of brown hyena prey. Dark blue bars represent birds, light blue represent ungulates, green represent marine mammals, yellow represent mesocarnivores, orange represent lagomorphs and red represent the other small mammals.

3.7 Niche overlap between cheetahs and brown hyena

The diet of cheetahs and brown hyenas revealed five species in common, three ungulates (*Antidorcas marsupialis*, *Oryx gazella*, and *Raphicerus campestris*), a bird (*Struthio camelus*) and a lagomorph (*Lepus capensis*). Notably, the springbok was a significant component in the diet of both species, presenting in both cases FOO values above 35%. The calculation of the Jaccard index indicates 15% of the total items are shared between the two species, while the assessment of the niche overlap using the Pianka's Index resulted in a value of 0.59, indicating an important overlap of resources between the two carnivores.

4. Discussion

4.1 Non-invasive samples and DNA Metabarcoding

In this study, non-invasive samples of cheetahs and hyenas were collected in Iona National Park, Angola, during the years 2021 and 2022. These samples were used to study several aspects of these species through a molecular ecology approach, including their social organization and how it influences space use. Additionally, DNA metabarcoding was employed to study the diet of these two carnivores in INP.

Cheetahs display diurnal activity (IUCN, 2022), while brown hyenas predominantly exhibit nocturnal activity (IUCN, 2015). Both species generally live in low population densities, challenging ecological studies (Haag et al., 2009). Therefore, techniques using non-invasive sampling, which do not require capturing, handling, or direct observation of individuals, have gained importance in the field of carnivore research (Kelly et al., 2012). They minimize stress and disturbance to the animals (Haag et al., 2009), provide easy access and sample collection, allowing for studies across regions, seasons, and years (Trites & Joy, 2005) and yielding valuable insights into population dynamics, social interactions, movement patterns through fragmented habitats, territory occupancy patterns, dietary preferences, and genetic diversity (Haag et al., 2009; Kelly et al., 2012). Additionally, non-invasive techniques enable the collection of many samples efficiently and cost-effectively, saving both time and resources (Kelly et al., 2012).

Nonetheless, there are some limitations associated with scat sampling. Both the quantity and quality of DNA in these samples can be low, eventually hampering its use (Rutledge et al., 2009). In this study the genotyping was conducted with a 2 steps PCR, pre and post, to overcome the challenges of low DNA quantity and quality. The timing of sample collection is also important for the success of the molecular work, and in the absence of freezing temperatures in Angola (Huntley et al., 2019), proper preservation techniques after collect the samples are needed (Rutledge et al., 2009). In this study, samples were preserved in silica beads immediately after collection to dehydrate, as water is a highly damaging factor for DNA. Additionally, it is essential to consider how sampling carnivoran species from a particular location may affect their behaviour, especially when the scats are a way to mark territory (Kelly et al., 2012).

In this study's context, non-invasive samples were used both for species and individual identification to study the social organization of brown hyenas and cheetahs in INP, and in a DNA metabarcoding approach to investigate these predators' diet. Using this dietary

approach, it is possible to determine the frequency of occurrence of each prey item in scats (Wachter et al., 2012). However, in general, studies of diet assume that each diet item is independent among the samples analyses, when the same item may be found in multiple samples. This can lead to distorted results where the presence of the same individual or prey species is counted multiple times, giving the impression of a higher frequency (Morin et al., 2019).

Another limitation of DNA metabarcoding and molecular scat-based methods is the higher prevalence of predator DNA compared to prey DNA (Egeter et al., 2019), which in the present study resulted in the exclusion of several samples due to the inability to detect prey DNA beyond predator DNA. Furthermore, distinguishing between predator's prey and prey of the prey, i.e. secondary predation, is another limitation. In this study, it is unlikely that such has happened in cheetahs because mostly of the samples had only one detected prey, and when two prey items were detected in the same sample, they corresponded to herbivores or insectivore species. For brown hyena that could have happened as it was detected two fish species and one reptile species, respectively in samples in which marine mammals and birds were found.

Overall, it is still a challenge to rely only on one approach for diet studies involving non-invasive samples.

4.2 Individual and sex identification

Individual identification for both species was conducted using microsatellites previously designed for similar species. In the case of cheetahs, it was possible to individual identify 27 (64.3%) of the 42 total samples, which is lower than what was achieved in Khalatbari et al., (2022), reporting a success of 72,5% for individual identification.

Regarding brown hyenas, it was possible to identify 51 (61,4%) of the 83 total samples.

In several samples, it was not possible to establish a molecular identification of the individual due to challenges encountered during allele identification. This could have occurred as a result of potential sample contamination or the presence of DNA in low quantity and quality, which is often the case in non-invasive samples (Rutledge et al., 2009).

Another issue arose in distinguishing individuals based on the genetic profiles created. In some instances, it was challenging to differentiate individuals, due to the low level of polymorphism, or even the presence of monomorphic loci in the case of brown hyenas, which decreased the informativeness of the complete genotype (Vladimir et al., 2020).

Also, if individuals within a population are closely related, it is more difficult to distinguish them (Vladimir et al., 2020).

4.3 Social organization and space use

4.3.1 Social organization and space use for cheetah

In cheetahs, only males were detected, and no clear patterns were found between their familial relationship and their use of the space based on sample collection. In two pairs of related individuals, we found samples of both individuals at the same latrine, which may not be directly related to their genetic kinship, as the other individuals appeared to mark seemingly randomly in the area in terms of their familial relationships. However, cheetahs are known to be solitary animals, spending most of their time alone, hunting and feeding on their own (Wachter et al., 2018). Therefore, even individuals of the same coalition do not necessarily live together. The absence of females and thus cubs, further reinforces the idea that the individuals detected in this study likely live solitarily and apart from each other (Wachter et al., 2018).

Male cheetahs have smaller home ranges compared to females and occupy areas where resources such as prey, water, or shelter are abundant (Kusler et al., 2019). Several individuals deposited their faeces in the same location or nearby (within 1 km), indicating possible resource abundance in those areas, which would attract multiple cheetahs. Male cheetahs are also known to be territorial animals and mark their territory through marking trees, including defecation, to signal their territory to other males, thus avoiding confrontations. This behaviour is even more driven by territorial overlap between females and males, with males marking to establish their presence and potentially initiate mating (Kusler et al., 2019). Territorial marking is a highly common behaviour among carnivores (Murphy et al., 2018). The lack of female samples in the study can be explained by their rare visits to these marking sites, while males visit them multiple times establishing dominance (Boast et al., 2016; Cornhill & Kerley, 2020a). Furthermore, regarding the proximity of some deposition sites, it is possible that cheetahs use locations close to each other as an adaptive behaviour to other predators visiting, as observed by Cornhill & Kerley (2020b). This behaviour is not unique to cheetahs, as it has been documented in other carnivores such as cougars, lynx, and white rhinos (Allen et al., 2014; Marneweck et al., 2018; Vogt et al., 2014). Females and males may also have different habitat and prey preferences, leading them to occupy different areas. According to the resource dispersion hypothesis, the size of the territory used is determined by the pattern of food distribution (Broomhall et al., 2003).

4.3.2 Social organization and space use for brown hyena

Overall, the analysis of scat deposition patterns did not reveal a distinct association with the familial relationships identified among individuals. Only two pairs of full siblings had their samples collected from the same latrine, while in both cases, their maternal progenitor deposit faeces at a considerable distance. Additionally, the siblings were also using different latrines apparently not shared among them. Male individuals of brown hyenas are known to may adopt a nomadic behaviour, often leaving their natal clans (Mills, 1990; Westbury et al., 2018), which may provide an explanation for the use of different latrines between them and also between them and their respective progenitors.

Although, brown hyenas can form clans, their social structure can often exhibit a dynamic nature, and this flexibility may result in familial individuals not necessarily sharing the same latrine (Vitale et al., 2020). Additionally, we noted that various latrines were used by individuals not related. Communal latrines used by individuals of the same species have been seen in several mammal species, from primates (Dröscher & Kappeler, 2014), to felids (Rodgers et al., 2015) and mustelids (Laurentino et al., 2023), and serve as gathering point deposits (Vitale et al., 2020). These communal latrines serve multiple functions, including marking territory, facilitating mate defence, or enabling inter-species communication (Vitale et al., 2020). Regarding to the presence of single scats in certain latrines, it is also known that latrines are only used by one individual (Skinner, 1981), as we also observed for brown hyena in INP. Furthermore, brown hyenas, despite belonging to clans, tend to engage in solitary hunting and feeding activities (D Owens & Owens, 1996).

The frequent use of latrines closer to the coast by some individuals may indicate an abundance of food resources in that area. It is possible that during the season when the samples were collected, these individuals fed mostly on coastal resources. It is known that higher concentrations of seals lead to more frequent visits by brown hyenas to the coast (Wiesel, 2010). On the other hand, individuals that did not use the coastal area and instead defecate in latrines located inland apparently exhibited a more dispersive deposition pattern. When multiple scats were collected from the same individual, they were found more dispersed, suggesting a larger foraging range compared to those hyenas near the coast. This dispersion may indicate that brown hyenas inhabiting inland Iona may have to forage more extensively. Additionally, their diet was more diverse compared to those individuals primarily near the coast whose diet relied more on marine mammals and some birds.

It was also observed that some individuals had samples collected in the same latrine in both years of the study. This pattern may suggest that these latrines are “long term latrines”, a concept described by Bearder & Randall (1978), to indicate that latrines are visited over a long time period (Vitale et al., 2020).

Further research is needed to understand the pattern of scat deposition. To achieve this understanding, it is important to gather some information, including the spatial distribution of latrines, the temporal use of latrines across seasons, individual-specific patterns, the contribution of each individual through scat deposition, and the signals encoded within the scats (Buesching & Jordan, 2022).

4.4 Cheetah diet

In the diet of cheetahs, seven different prey items were found, with the most common being one prey per scat, along with some samples containing two prey items, consistent with findings in other studies (Thuo et al., 2020).

Two prey items, the springbok and the cape hare were the most important prey in terms of frequency of occurrence. Cheetah diets are known to primarily consist of small to medium-sized ungulates, like the Thomson's gazelle, Grant's gazelle, and impala (Hilborn et al., 2012; Thuo et al., 2020).

Both species have been reported to be consumed by cheetah. However, springbok is often one of the most consumed species when its distribution overlap with cheetah, while the cape hare is a less common prey item (Marker et al., 2003; Mills, 1984; Wilson et al., 2013). Springbok is highly abundant in the grasslands of Iona (Nunes, 2020), a habitat that particularly favours cheetahs as they are recognized as eximious runners and can achieve high speeds in these open grasslands (Hayward et al., 2006; Hilborn et al., 2012).

With a weight range varying from 26.5 kg to 47.6 kg, the springbok is classified as a medium-sized ungulate. This provides cheetahs with an optimal balance between hunting effort and nutritional content. Additionally, springboks have diurnal activity, like cheetahs, which can increase the frequency of encounters between them. The fact that springboks often move in groups further enhances the hunting opportunities for cheetahs, as there are multiple potential prey targets (Reid, 2005).

The cape hare is also common in the INP and like the springbok, can be found in open habitats like grasslands (Laia et al., 2021). Species of hare are a significant prey item in cheetah diet (Marker et al., 2003; Mills, 2015; Mutoro et al., 2022). The availability together

with the vulnerability of springbok and cape hare seem important factors contributing to their high consumption by cheetahs, as it was previously observed (HILBORN et al., 2012).

Species like gemsbok and steenbok are also common in cheetah diet (Mills, 2015). Although gemsbok is a large ungulate, much bigger than cheetahs, it can be prey by cheetahs in coalition, as cheetahs have hunting techniques to overcome the large size of the prey (Mills, 1984). Nevertheless, DNA metabarcoding does not allow to differentiate the size of the prey, and it may be more likely that our observation of gemsbok in the diet of cheetah came from preying on gemsbok calves rather than adults, as this is more common for large prey species (Mills, 1984, 2015).

Species of birds are typical prey items for cheetahs, which can diversify their diets with small species or seek alternative prey when more common species are unavailable (Khalatbari et al., 2022). The ostrich was observed twice in the diet, a species previously found in cheetah diets (Marker et al., 2003; Mills, 2015) and common in the INP (African Parks, 2023b). Cheetahs can hunt either adults when in coalitions or chicks and it is pointed as the principal predator of ostriches (Cooper et al., 2010; Mills, 2015). It is also possible that they eat ostriches' eggs (Cooper et al., 2010).

Carnivores are not frequent prey for cheetah, but on our sampling, we found aardwolf twice in scats from different years. Although this is not common, aardwolf could have been preyed as an opportunistic catch, or may have been preyed upon by other predators, as occasional scavenging behaviour has been reported in cheetahs (Broekhuis & Irungu, 2017; Thuo et al., 2020). The presence of aardwolf in the cheetah diet could also be possible by opportunistic feeding on naturally dead animals.

No domestic animals were found in the cheetah diet in our sampling. This conforms to results from other studies, where the presence of domestic prey in cheetah diet has very low frequency or is non-existent, suggesting clearly that livestock is not important in the diet of cheetahs, which prey mostly on wild prey (Marker et al., 2003).

It is important to note that the relatively small sample size could not fully represent what cheetahs might be feeding in INP, which could lead to potential bias in the results, and therefore the results of this study must be interpreted with caution (Broekhuis et al., 2018).

4.5 Brown hyena diet

As far as I could understand, this is the first study of the brown hyena's diet using DNA metabarcoding. The analysis of 64 faecal samples revealed a notable diversity of food

items, with a total of 29 different items identified. Brown hyenas are capable of exploit a wide variety of food sources and this richness diet is concordant with their opportunistic and adaptable behaviour, as has been also observed in previous studies from several locations on the coast of Namibia (Kuhn et al., 2008), in South Africa (Faure et al., 2019) and in the Kalahari Desert (Mills, 2019).

The presence of a single prey item per sample was the most common pattern observed, as in previous studies (Comley et al., 2018; Faure et al., 2019). However, we found samples that contained up to five different prey items, which was also reported in South Africa (Slater & Muller, 2014). This variation can be attributed to the size or quantity of the food item consumed, leading brown hyenas to consume multiple prey when needed to satisfy their nutritional needs (Mills, 2019). Additionally, when variable sources of food are available, brown hyenas typically move from one small prey to the next (Mills, 2019; Slater & Muller, 2014). This behaviour demonstrates the flexibility of brown hyena diets and their ability to adapt to different available food resources.

Overall, ungulates were the most frequent group found in brown hyenas' diet at the INP, as seen in other regions (Comley et al., 2018), with springbok being the most common prey, possibly due to its abundance in INP (Nunes, 2020) and its status as a primary food source for brown hyenas, corroborating previous observations of the frequency of this species in brown hyenas' diet (Fourvel et al., 2015). These observations may likely be indicative of scavenging events, given the high frequency of springbok in cheetah's diet. Additionally, brown hyenas are known scavengers that often feed on carcasses resulting from cheetah kills (Comley, 2016).

The gemsbok was the second most common prey in brown hyena diet, which also conforms to previous studies founding this prey species (Comley, 2016; Fourvel et al., 2015). Together with springbok, gemsbok was the most frequently spotted ungulate inside INP (Nunes, 2020), making them an available prey. Gemsbok is one of the largest antelopes in Africa (Hoffman et al., 2020), and therefore brown hyena are unlikely to hunt them. However, cheetahs and leopard can prey on gemsbok making the carcass available to brown hyena (Bothma et al., 1997; Mills, 1984, 2015). Another explanation for the high occurrence of gemsbok in the diet of brown hyena is that the same gemsbok individual can serve as food source more than once and can be shared by multiple brown hyenas, which can result in a high number of scats based on a few gemsbok individuals (Morin et al., 2019).

The cape fur seal was highly frequent in the diet of brown hyenas, especially in samples collected near the coast. This marine mammal is abundant in the coastal region of the Namib Desert, and the possible absence of competitors besides black-backed jackal, in this area may explain hyenas' preference for this prey (Wiesel, 2010). Several previous studies have also documented the frequent presence of the seal in brown hyenas' diet, indicating its importance as a food source (Skinner et al., 1995; Wiesel, 2006, 2010). Hyenas exhibit a versatile feeding strategy, involving both scavenging behaviour and predation, although this last is rare among brown hyenas. The predation behaviour often occurs upon the seal pups, due to their vulnerability and often lack of protection from their female parent that during nursing spend five days in the ocean and only two days near their pups to nursing them (Curtis et al., 2021). Direct predation of newborns may have occurred, in samples collected in November, as the Cape Fur Seal are born between the months of November and January (Curtis et al., 2021), with the pups becoming accessible prey in the following months. In the case of the second batch of samples, collected in March, direct predation of juveniles is likely. Also, pups can die from different causes besides predation, such as starvation and heat stress in hot days, resulting in resources easily available to brown hyenas (Wiesel, 2006). The second marine mammal consumed, although in much lower frequency, was the dolphin, also reported previously (Skinner et al., 1995), which certainly shows scavenging behaviour on stranded animals.

Birds were a considerable component of brown hyena diet, as previously observed (Kuhn et al., 2008; Mills & Mills, 1978; Slater & Muller, 2014). Notably, sea birds were the most frequent in their diet, namely cormorants. These birds, along with seagulls, terns and pelicans often nest on offshore islands, with the consequent washing up of bird carcasses along the coastline providing a source of carrion for brown hyenas (Kuhn et al., 2008; Simmons et al., 2006). However, the consume of birds can either be from predation or scavenging (Owens & Owens, 1978) and the aggregation behaviour exhibited by certain bird species, such as flamingos, can facilitate the hunting efforts of brown hyenas (Kuhn et al., 2008). Additionally, brown hyenas have been observed consuming bird eggs, and it is common for them to feed on ostrich eggs, sometimes carrying them over considerable distances (Cooper et al., 2010; Kandel, 2004; Mills, 1978; Skinner & Van Aarde, 1991).

Two carnivores were observed as prey resources for brown hyenas, the black-backed jackal and the wildcat. Other studies have also reported that brown hyena incorporates canids into their diet, though it is likely through scavenging rather than active hunting (Kuhn et al., 2008; Skinner et al., 1995).

Furthermore, the consumption of livestock was identified with a frequency of occurrence of 6.3%. This event may result from the exploitation of carcasses left by other predators that have indeed killed domestic animals or from carcasses of animals that died due to diseases, starvation, or old age (Maude & Mills, 2005). It is not common for brown hyenas to hunt upon domestic prey, and the choice of these animals may be a response to the availability of this food source or the scarcity of other food sources (Maude & Mills, 2005).

These findings highlight the adaptable and opportunistic feeding habits of brown hyenas. They also provide valuable insights into the abundance and availability of prey species within their habitats (Kuhn et al., 2008).

4.6. Niche overlap between cheetahs and brown hyena

Five prey species were found in both diets with springbok as the dominant species in both cheetah and hyena diets. In this habitat where cheetahs and brown hyenas coexist, it is possible that they share the same resources and that brown hyena take advantage of cheetah's prey, showing the reliance of brown hyenas on scavenging and kleptoparasitism opportunities present by other carnivores (Faure et al., 2019; Yarnell et al., 2013).

The Jaccard similarity index and Pianka's index provided distinct views of dietary analysis, suggesting that while the number of shared species might be low, the proportions of these species in the diets of hyenas and cheetahs are significant and similar. This index demonstrates the importance of considering not just presence but also abundance when assessing diet overlap.

4.7 Conservation of brown hyena and cheetah in the INP

Large carnivores play a crucial role not only in ecosystem functioning by regulating populations of smaller carnivores and herbivores, thus influencing vegetation composition and structure, but they also hold significant economic value through tourism (Vissia et al., 2021). Additionally, scavengers contribute significantly to nutrient recycling and the removal of carcasses from the environment. Understanding their distribution, population dynamics, and dietary preferences becomes imperative for effective conservation efforts (Monterroso et al., 2019; Vissia et al., 2021). Cheetahs have received more research attention, including previous uses of DNA approaches to study their diet, while brown hyena is understudied, including this being the first study using DNA metabarcoding to study its diet.

The proximity of these carnivores to human populations has a profound impact on their population densities, which can decline and even lead to local extinctions. This conflict

arises more from the perceived threat these carnivore species pose to livestock rather than actual livestock losses to predation (Boast et al., 2016; Dickman, 2008; Marker et al., 2003).

Over the past decade, conservation measures have been rising, and many farmers are beginning to recognize the necessity of protection and coexistence. Nevertheless, trapping practices persist, although the outcomes have evolved. Whereas in the past, these animals were killed, today, efforts are made to release or relocate them to safer areas (Marker et al., 2008). Although some advances have been made, the continuous growth of the human population has consequences for the habitat of species inhabiting Iona National Park. INP is home to several endangered species and has experienced significant extinctions over the past decades (Braga-Pereira et al., 2020). Carnivores such as cheetahs, leopards, and brown hyenas are present but at low population densities (African Parks, 2023a).

Species inventories also help determine the diversity of species in a particular area, allowing for comparisons with other regions, investigations into the individual space use, and assessments of the impact of human activities on species (Tobler et al., 2008). Additionally, understanding the distribution and abundance of species aids in the more efficient planning and evaluation of conservation strategies, thereby increasing knowledge of regional biodiversity. To assess the current population of animals in Iona National Park, a camera trapping program is currently being implemented. This initiative aims to provide valuable information about the occupancy, abundance, and trends of some of the most elusive wildlife species (African Parks, 2023a). Additional molecular monitoring using non-invasive samples can help in population estimates, and furthermore identify population dynamics and familial relationships that are more difficult assessed using other ecological approaches.

There is also a great need to increase efforts to combat illegal hunting, which has contributed to the decline of carnivore species in Iona National Park.

5. Conclusion

This study represents a significant contribution to the understanding of cheetahs and brown hyenas' ecology. Unlike cheetahs, this research is the first study of brown hyenas' diet using DNA metabarcoding, proving that it is appropriate for the species. This study contributed to a better knowledge of space use patterns, social structures, interactions between prey and predator and prey use by cheetahs and brown hyenas in Iona National

Park. Furthermore, it provides valuable insights into the presence of other species within INP.

As previously observed, species such as springbok are highly abundant in INP and represent a significant part of both cheetahs' and brown hyenas' diet. As expected, cheetahs primarily feed on ungulates, while brown hyenas had a more varied diet, exhibiting variations in their diet based on their space use. Hyenas closer to the coast consumed mainly cape fur seal while the remaining hyenas living in inland consume mostly ungulates, and completed their diet with smaller preys like birds, lagomorphs, rodents, and mammals. Two carnivores were also found in brown hyenas' diet.

Comparisons between the diet of brown hyenas and cheetahs showed that they share some prey species, highlighting the dependence of brown hyenas on prey hunted by other predators in the park interior.

Overall, the non-consumption of livestock by cheetahs is a positive aspect that may contribute to their conservation. However, more extensive studies with a larger number of samples are needed. In the case of brown hyenas, livestock consumption may indicate scavenging and opportunistic behaviour.

Several limitations may have affected the results of this study, including the small number of samples, particularly for cheetahs, resulting in a limited number of different individuals in both species, and the absence of female individuals in the cheetah samples. With a larger sample size and thus more individuals, it would be possible to infer dietary specialization within a population and between males and females. Although there is no sexual dimorphism in brown hyenas, there is in cheetahs, and males and females may have different prey choices. A larger number of individuals could also provide better insights into familial relationships, social organization, and a more detailed understanding of space use and home ranges.

In addition, the prevalence of predator DNA in many samples, sometimes as the only presence, limited the sample size for dietary analysis. The use of multiple barcodes could be an improvement for more strong dietary results.

Looking forward, future research could focus on variations of the same parameters here studied across seasons and years, so it would be possible to understand differences in space use patterns, social behaviours, and prey preferences. This would allow to understand better the global ecological relationships in INP and benefit the development of more effective conservation strategies.

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Supplementary Information

Table SI 1: Information about each cheetah scat sample- Information about laboratory ID, year, latitude and longitude of collection, the individual identification, sex, and prey species found in the scats.

Lab Sample-ID	Year	Latitude	Longitude	Individual	Diet
SANI627	2021	-16.615469	12.101078		<i>A. marsupialis</i>
SANI629	2021	-16.615637	12.101509	♂ AJU02	x
SANI630	2021	-16.615488	12.101485		<i>L. capensis</i>
SANI637	2021	-16.867856	12.364522		<i>P. cristata</i>
SANI638	2021	-16.879801	12.410955	♂ AJU03	<i>R. campestris</i>
SANI639	2021	-16.879801	12.410955		x
SANI640	2021	-16.879801	12.410955		x
SANI642	2021	-16.879785	12.410931		<i>A. marsupialis</i>
SANI643	2021	-16.879825	12.410848	♂ AJU03	<i>L. capensis</i>
SANI646	2021	-16.612329	12.087039	♂ AJU04	x
SANI647	2021	-16.612478	12.086776		<i>L. capensis</i>
SANI648	2021	-16.61244	12.086825	♂ AJU05	x
SNI23-319	2022	-16.60120597	12.08141699	♂ AJU09	x
SNI23-320	2022	-16.60122198	12.08141103	♂ AJU08	x
SNI23-321	2022	-16.601226	12.08143601	♂ AJU08	<i>S. camelus</i>
SNI23-322	2022	-16.60121997	12.081419	♂ AJU06	x
SNI23-326	2022	-16.60991301	12.08454	♂ AJU05	x
SNI23-327	2022	-16.60991703	12.08453304	♂ AJU10	<i>A. marsupialis</i>
SNI23-328	2022	-16.60990203	12.08454201	♂ AJU08	<i>L. capensis, P. cristata</i>
SNI23-329	2022	-16.61546501	12.10146197	♂ AJU08	<i>S. camelus</i>
SNI23-330	2022	-16.61547599	12.10147798		x
SNI23-332	2022	-16.61551103	12.10144596	♂ AJU07	x
SNI23-333	2022	-16.61550298	12.10149399	♂ AJU08	x
SNI23-385	2022	-16.81389903	12.35449201	♂ AJU04	<i>A. marsupialis</i>
SNI23-387	2022	-16.81390397	12.35451004	♂ AJU04	x
SNI23-388	2022	-16.81388604	12.35451699	♂ AJU04	<i>A. marsupialis</i>
SNI23-390	2022	-16.81391302	12.35450802	♂ AJU05	x
SNI23-399	2022	-16.881616	12.32702997		x
SNI23-400	2022	-16.88161802	12.32703399		<i>A. marsupialis</i>
SNI23-401	2022	-16.88162598	12.32701597	♂ AJU05	x
SNI23-402	2022	-16.88163503	12.327036		x
SNI23-406	2022	-16.99726003	12.37814398		x
SNI23-407	2022	-16.997268	12.378147	♂ AJU04	<i>L. capensis</i>
SNI23-408	2022	-16.99725199	12.378132		<i>L. capensis</i>

SNI23-409	2022	-16.99725299	12.378118	♂ AJU04	x
SNI23-410	2022	-16.99735902	12.37822101		<i>Buteo sp.</i>
SNI23-412	2022	-16.879789	12.41090298	♂ AJU04	x
SNI23-413	2022	-16.87977601	12.41088697	♂ AJU04	<i>L. capensis</i>
SNI23-414	2022	-16.87976402	12.41088596	♂ AJU04	x
SNI23-420	2022	-16.68530999	12.31639802	♂ AJU04	<i>A. marsupialis</i>
SNI23-421	2022	-16.68534603	12.316379		x
SNI23-426	2022	-16.685284	12.31635997	♂ AJU09	x

Table_S1 2: Information about each brown hyena scat sample- Information about laboratory ID, year, latitude and longitude of collection, the individual identification, sex, and prey species found in the scats.

Lab Sample-ID	Year	Latitude	Longitude	Individual	Prey species
SANI583	2021	-16.61547	12.10143		x
SANI584	2021	-16.61547	12.10143		<i>S. camelus; A. marsupialis; O. gazella; L. capensis</i>
SANI585	2021	-16.61547	12.10143	♂ BH03	<i>S. camelus; A. marsupialis</i>
SANI586	2021	-16.61547	12.10143		x
SANI587	2021	-16.68522	12.31636		<i>A. marsupialis</i>
SANI588	2021	-16.68522	12.31636		<i>A. marsupialis</i>
SANI590	2021	-16.6724	12.29772		x
SANI601	2021	-16.864203	11.933385	♀ BH02	<i>Phalacrocorax sp.; T. truncatus; Larus sp.</i>
SANI603	2021	-16.864203	11.933385	♀ BH02	<i>T. truncatus</i>
SANI604	2021	-16.864203	11.933385	♂ BH03	<i>L. capensis; A. marsupialis</i>
SANI608	2021	-16.864198	11.933762	♀ BH02	<i>Phalacrocorax sp.; Larus sp.</i>
SANI609	2021	-16.864203	11.933754	♂ BH03	<i>A. pusillus</i>
SANI610	2021	-16.864286	11.933738	♀ BH02	<i>A. pusillus</i>
SANI611	2021	-16.864314	11.93385	♂ BH03	<i>A. pusillus</i>
SANI612	2021	-16.864314	11.933747	♀ BH02	x
SANI613	2021	-16.864314	11.933747		<i>O. gazella</i>
SANI618	2021	-16.836959	12.447514		<i>A. marsupialis; B. taurus; P. randensis</i>
SANI619	2021	-16.787355	12.359289		<i>A. marsupialis; L. capensis</i>
SANI621	2021	-16.672344	12.29773		<i>O. gazella</i>

SANI622	2021	-16.615466	12.10145	♂ BH15	<i>A. marsupialis</i>
SANI623	2021	-16.615469	12.101078	♂ BH15	<i>O. gazella</i>
SANI624	2021	-16.615469	12.101078	♂ BH15	<i>O. gazella</i>
SANI628	2021	-16.615637	12.101509	♀ BH09	<i>A. marsupialis</i>
SANI631	2021	-16.61542	12.101476		x
SANI636	2021	-16.896896	12.355065		x
SANI641	2021	-16.879801	12.410955		x
SANI649	2021	-16.600771	12.088343		<i>A. marsupialis</i>
SANI650	2021	-16.600676	12.08839	♂ BH03	<i>A. marsupialis</i>
SNI23-292	2022	-16.86435398	11.93400001	♂ BH01	<i>A. pusillus</i>
SNI23-293	2022	-16.86435699	11.93400001	♀ BH02	<i>A. pusillus</i>
SNI23-294	2022	-16.86435498	11.93400999	♀ BH02	<i>A. pusillus</i> ; <i>P. roseus</i> ; <i>T. truncatus</i>
SNI23-295	2022	-16.86435096	11.93400403	♂ BH03	<i>Phalacrocorax sp</i> ; <i>S. hirundo</i> ; <i>A. Pusillus</i>
SNI23-296	2022	-16.86429002	11.93338302	♂ BH01	<i>A. pusillus</i>
SNI23-297	2022	-16.86428499	11.93339501	♂ BH01	<i>A. pusillus</i>
SNI23-298	2022	-16.86428801	11.93340104	♀ BH02	<i>T. truncatus</i>
SNI23-299	2022	-16.86427703	11.93340104	♂ BH03	<i>C. mesomelas</i>
SNI23-300	2022	-16.86427401	11.93340003	♂ BH01	<i>A. Pusillus</i> ; <i>E. zebra</i>
SNI23-302	2022	-16.864401	11.933307	♂ BH03	<i>Phalacrocorax sp.</i>
SNI23-303	2022	-16.86440301	11.933293	♂ BH01	<i>A. pusillus</i>
SNI23-304	2022	-16.86440402	11.93328696	♂ BH03	<i>A. pusillus</i>
SNI23-305	2022	-16.864387	11.93329903	♀ BH02	<i>A. pusillus</i>
SNI23-306	2022	-16.86439103	11.93331496	♂ BH03	<i>A. pusillus</i>
SNI23-307	2022	-16.86431902	11.93372902	♀ BH02	<i>Phalacrocorax sp.</i>
SNI23-308	2022	-16.86431098	11.93373196	♂ BH04	<i>A. pusillus</i>
SNI23-309	2022	-16.86431701	11.93373104	♂ BH05	<i>Phalacrocorax sp.</i> ; <i>A. pusillus</i> ; <i>P. squatarola</i> ; <i>C. mesomelas</i>
SNI23-310	2022	-16.86431198	11.93371796		<i>L. capensis</i> ; <i>Phalacrocorax sp.</i> ; <i>P. onocrotalus</i> ; <i>C. mesomelas</i>
SNI23-311	2022	-16.86432397	11.93373196		x
SNI23-312	2022	-16.60074203	12.088395		<i>A. marsupialis</i> ; <i>F. silvestris</i>

SNI23-313	2022	-16.60072996	12.08840699	♂ BH03	<i>A. marsupialis</i> ; <i>Eromophila</i> sp.; <i>R. africanus</i>
SNI23-314	2022	-16.60071203	12.08839199	♂ BH06	<i>A. marsupialis</i> ; <i>O. gazella</i>
SNI23-315	2022	-16.60071404	12.08840196	♂ BH06	<i>A. marsupialis</i> ; <i>Fulvetta</i> sp.
SNI23-316	2022	-16.60072602	12.088423		<i>A. marsupialis</i> ; <i>O. gazella</i>
SNI23-317	2022	-16.60123799	12.08144699	♂ BH06	<i>E. zebra</i> ; <i>A. marsupialis</i> ; <i>O. gazella</i>
SNI23-318	2022	-16.60124101	12.081448	♂ BH06	<i>P. namaqua</i> ; <i>F. peregrinus</i>
SNI23-323	2022	-16.609926	12.08454402	♂ BH08	x
SNI23-324	2022	-16.60991703	12.08455299	♀ BH09	x
SNI23-325	2022	-16.60990303	12.08458099		<i>A. marsupialis</i>
SNI23-380	2022	-16.77889201	12.20082097	♂ BH10	x
SNI23-381	2022	-16.77889201	12.20079599	♀ BH11	<i>O. gazella</i>
SNI23-382	2022	-16.77887801	12.20077101		x
SNI23-383	2022	-16.77886804	12.20081703	♀ BH11	<i>O. gazella</i>
SNI23-384	2022	-16.77888304	12.20083798	♂ BH10	<i>O. gazella</i>
SNI23-389	2022	-16.81388503	12.35452797		<i>O. gazella</i>
SNI23-391	2022	-16.82014899	12.35417199	♂ BH12	<i>A. Marsupialis</i> ; <i>C. hircus</i> ; <i>P. capensis</i> ; <i>N. meleagris</i>
SNI23-395	2022	-16.91363202	12.34933698	♀ BH13	<i>S. camelus</i> ; <i>A. marsupialis</i>
SNI23-397	2022	-16.913642	12.34933798		<i>L. capensis</i> ; <i>O. gazella</i> ; <i>B. indicus</i> ; <i>C. hircus</i>
SNI23-398	2022	-16.913643	12.34933396		<i>A. marsupialis</i>
SNI23-404	2022	-16.99871496	12.37860398		x
SNI23-418	2022	-16.68530303	12.31637003		<i>O. gazella</i>
SNI23-419	2022	-16.68529398	12.31638797		x
SNI23-422	2022	-16.68536799	12.31640599	♂ BH10	x
SNI23-423	2022	-16.68537604	12.31641999		<i>L. capensis</i>
SNI23-424	2022	-16.68535802	12.316409		<i>A. marsupialis</i>
SNI23-425	2022	-16.68531401	12.31637103		<i>O. gazella</i>
SNI23-427	2022	-16.67245399	12.29774301		<i>A. marsupialis</i> ; <i>P. domesticus</i>
SNI23-428	2022	-16.672413	12.297756	♂ BH10	x
SNI23-429	2022	-16.67240697	12.297741		<i>O. gazella</i>
SNI23-430	2022	-16.672413	12.297741		x

SNI23-432	2022	-16.64736897	12.27965101	♂ BH10	<i>L. capensis; O. gazella; Apus sp.; D. auricularis</i>
SNI23-433	2022	-16.64737802	12.27971002	♀ BH09	<i>O. gazella</i>
SNI23-434	2022	-16.787044	12.35828097	♂ BH16	x
SNI23-435	2022	-16.78711499	12.35822498	♂ BH14	x
SNI23-443	2022	-16.68523	12.31633	♂ BH04	<i>R. campestris</i>

Table_SI 3: PCR Mix used for ATP6- The mix was prepared by adding 5 µL of Master Mix, 0.4 µL of both primers and 2.2 µL of H₂O per sample (1x).

ATP 6 Mix

Reagents	Quantity per sample (1x)
Master Mix	5 µL
ATP6-DF3	0.4 µL
ATP6-DR3	0.4 µL
H ₂ O	2.2 µL

Table_SI 4: Thermocycling conditions for the PCR for species identification using ATP6 primer- The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95 °C for 15 minutes; then 10 cycles with a decrease of 1 °C per cycle of denaturation, annealing and elongation at 95 °C, 60 °C and 72 °C respectively, during 30, 45 and 30 seconds, respectively; 35 cycles of a second denaturation, annealing and elongation at 95 °C, 50 °C and 72 °C respectively, during 30, 45 and 30 seconds, respectively; and a final elongation at 60 °C for 3 minutes.

Thermocycling conditions ATP 6 PCR

Temperature (°C)	Duration	Number of cycles
95 °C	15 min	1x
95 °C	30 sec	10x (-1 °C)
60 °C	45 sec	
72 °C	30 sec	
95 °C	30 sec	35x
50 °C	45 sec	
72 °C	30 sec	
60 °C	3 min	1x
10 °C	∞	

Table_SI 5: Sanger Sequencing Mix used for ATP6 fragment and species identification- The mix was prepared by adding 0.5 µL of TRR Mix, 1 µL of TRR Buffer, 0.5 µL of one of the primers and 7 µL of H₂O.

Sanger Sequencing Mix	
Reagents	Quantity per sample (1x)
TRR Mix	0.5 µL
TRR Buffer	1 µL
Primer Forward or Reverse (1:10)	0.5 µL
H ₂ O	7 µL

Table_SI 6: Multiplex Mix used for pre and post PCR of genotyping- The mix was prepared for both cheetah and brown hyenas' samples by adding 5 µL of Master Mix, 1 µL of mix primers and 2 µL of H₂O in pre-PCR and 3 µL in post-PCR.

Multiplex Mix		
Reagents	Quantity per sample (1x) pre-PCR	Quantity per sample (1x) post-PCR
Master Mix	5 µL	5 µL
Mix primer	1 µL	1 µL
H ₂ O	2 µL	3 µL

Table_SI 7: Thermocycling conditions for the pre-PCR for Multiplex 1, 2, 3 and 4 of cheetah- The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95 °C for 15 minutes; then 9 cycles with a decrease of 0.5 °C per cycle of denaturation, annealing and elongation at 95 °C, 62 °C and 72 °C respectively, during 30, 90 and 30 seconds, respectively; 11 cycles of a second denaturation, annealing and elongation at 95 °C, 58 °C and 72 °C respectively, during 30, 90 and 30 seconds, respectively; and a final elongation at 60 °C for 15 minutes.

Thermocycling conditions for cheetah multiplex pre- PCR		
Temperature (°C)	Duration	N ^a of cycles
95°C	15 min	1x
95°C	30 secs	9x (-0.5°C)
62°C	90 secs	
72°C	30 secs	
95°C	30 secs	11x
58°C	90 secs	
72°C	30 secs	
60°C	15 min	1x
10°C	∞	

Table_SI 8: Thermocycling conditions for the post-PCR for Multiplex 1, 2, 3 and 4 of cheetah- The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95 °C for 15 minutes; then 9 cycles with a decrease of 0.5 °C per cycle of denaturation, annealing and elongation at 95 °C, 62 °C and 72 °C respectively, during 30, 60 and 30 seconds, respectively; 36 cycles of a second denaturation, annealing and elongation at 95 °C, 58 °C and 72 °C respectively, during 30, 45 and 30 seconds, respectively; and a final elongation at 60 °C for 30 minutes.

Thermocycling conditions for cheetah multiplex post- PCR

Temperature (°C)	Duration	N ^a of cycles
95°C	15 min	1x
95°C	30 secs	9x (-0.5°C)
62°C	60 secs	
72°C	30 secs	
95°C	30 secs	36x
58°C	45 secs	
72°C	30 secs	
60°C	30 min	1x
10°C	∞	

Table_SI 9: Thermocycling conditions for the pre-PCR for Multiplex 1, 2, 3 and 4 of brown hyena- The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95 °C for 15 minutes; then 5 cycles with a decrease of 0.5 °C per cycle of denaturation, annealing and elongation at 95 °C, 58 °C and 72 °C respectively, during 30, 90 and 30 seconds, respectively; 15 cycles of a second denaturation, annealing and elongation at 95 °C, 56 °C and 72 °C respectively, during 30, 90 and 30 seconds, respectively; and a final elongation at 60 °C for 15 minutes.

Thermocycling conditions for brown hyena multiplex pre- PCR

Temperature (°C)	Duration	N ^a of cycles
95°C	15 min	1x
95°C	30 secs	5x (-0.5°C)
58°C	90 secs	
72°C	30 secs	
95°C	30 secs	15x
56°C	90 secs	
72°C	30 secs	
60°C	15 min	1x
10°C	∞	

Table_SI 10: Thermocycling conditions for the post-PCR for Multiplex 1, 2, 3 and 4 of brown hyena- The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95 °C for 15 minutes; then 5

cycles with a decrease of 0.5 °C per cycle of denaturation, annealing and elongation at 95 °C, 58 °C and 72 °C respectively, during 30, 60 and 30 seconds, respectively; 40 cycles of a second denaturation, annealing and elongation at 95 °C, 56 °C and 72 °C respectively, during 30, 45 and 30 seconds, respectively; and a final elongation at 60 °C for 30 minutes.

Thermocycling conditions for brown hyena multiplex post- PCR

Temperature (°C)	Duration	N ^a of cycles
95°C	15 min	1x
95°C	30 secs	5x (-0.5°C)
58°C	60 secs	
72°C	30 secs	
95°C	30 secs	40x
56°C	45 secs	
72°C	30 secs	
60°C	30 min	1x
10°C	∞	

Table_SI 11: Information of each locus used for individual identification in cheetah- Locus divided in multiplex, sequence of the primer forward and reverse, allele size range observed and number of alleles per locus.

Multiplex	Locus	Primer sequence (5'-3')	Obs. allele size range (bp)	Nº of alleles
M1	FCA075	(F) ATGCTAATCAGTGGCATTG (R) GAACAAAAATTCCAGACGTGC	147-155	3
	FCA171	(F) CGGCACCTACAGACGGAC (R) AGTAGCTTAGCAGCCAAGCG	128-136	4
	FCA310	(F) TTAATTGTATCCAAGTGGTCA (R) TAATGCTGCAATGTAGGGCA	123-133	3
	FCA97	(F) TAATGTTCAACTTGAATTGCTTCC (R) GAACAGTAGTTTGCCCATACAGG	163-167	2
	Fca008	(F) ACTGTAAATTTCTGAGCTGGCC (R) TGACAGACTGTTCTGGGTATGG	169-181	5
M2	FCA005	(F) CCTAAGGAAACAGTAATCCTGGCC (R) TGGCAGGCATACCAGGAT	166-172	3
	FCA126	(F) GCCCCTGATACCTGAATG (R) CTATCCTTGCTGGCTGAAGG	143-153	3
	FCA205	(F) CCTGCTCTCAAGGAGCTCC (R) CCCATTTCTCCTACCAGTTCC	128-132	2
	FCA224	(F) CTGGGTGCTGACAGCATAGA (R) TGCCAGAGTTGTATGAAAGGG	181-193	5

	FCA230	(F) AAGAATGGACTTGGGAAATGG (R) AAACCACAACAGGCCAAAAGG	119-133	3
M3	FCA096	(F) CACGCCAACTCTATGCTGA (R) CAATGTGCCGTCCAAGAAC	223-235	5
	FCA133	(F) ATGAGGACCGATGACTCTTTT (R) GGAAATGTTGACAGAGGGACA	153-165	5
	FCA14	(F) AGCCAAGAGGGGAAACAAC (R) TGTCACAAATGGCAGGTTGT	174-184	4
	FCA212	(F) TTTGATTCACGTAAAGGTGCC (R) GACATTGTATGCTTCTGTGGTG	137-149	3
	FCA247	(F) GGAAATTAGGAGCTCTGCCA (R) AAGATTTACCCAGTTGCCCC	182-190	3
	FCA85	(F) CTGTACATTTCTTCCCATTCG (R) CCCCTACTGGGTGCACTG	149-171	5
M4	FCA026	(F) GGAGCCCTTAGAGTCATGCA (R) TGTACACGCACCAAAAACAA	159-173	5
	FCA045	(F) TGAAGAAAAGAATCAGGCTGTG (R) GTATGAGCATCTCTGTGTTTCGTG	162-174	3
	FCA105	(F) TTGACCCTCATACTTCTTTGG (R) TGGGAGAATAAATTTGCAAAGC	206-220	5
	FCA132	(F) ATCAAGGCCAACTGTCCG (R) GATGCCTCATTAGAAAAATGGC	133-137	3
	FCA214	(F) AGAATCCAACCTTGCCAGG (R) GGGAAGCAAAGAGCACAGTC	190-204	4
	FCA69	(F) AATCACTCATGCACGAATGC (R) AATTTAACGTTAGGCTTTTTGCC	121-131	5

Table_SI 12: Information of each locus used for individual identification in brown hyena- Locus divided in multiplex, sequence of the primer forward and reverse, allele size range observed and number of alleles per locus.

Multiplex	Locus	Primer sequence (5'-3')	Obs. allele size range (bp)	N° of alleles
M1	Ccr12	(F) TAATGCTCTACTGTTGCCTTCT (R) CTCACAATCAGGACTGGCTATA	171-173	2
	Ccroc04	(F) TGGGCTAAGTCTTCCCTGA (R) ATGGGGGCTCCTTCACTC	339-343	2
	Ccroc08	(F) TTTTTTAAAGTTCTTAGGCAGGAC (R) GGAGCCTGTTTCAGTCTGTG	186-187	2
M2	Ccr16	(F) ATGATCTGTCTATTTTAAATGCAC (R) GCCTCCATTTTCTTGCTCT	200-202	2
	Ccr17	(F) TTCACAGCCAAAGGAATATTAT (R) ACATGAGGATGATAGCACAGAT	169-171	2

	Ccroc03	(F)TGAAACAAGGATAAATTAATGAAAA GA (R)CAGTAGAAAAATAATCCAAGTATAAA CC	135-137	2
	Ccr07	(F) CTCGGAGACGGGAAACTG (R) CTAAGATGCCCTGCTGC	190-196	3
	Ccroc09	(F) CAGAATTAATCCATATCACAAACTGC (R) TAGGAACCTCTTGCCGTCAG	239-243	3
	Ccr11	(F) AAAATATGCAGAACTTTTCTAAGTCA (R) GATCCTGCTTCAGATTCATGC	162	1
	Ccr13	(F) TCTCCGAAGAACAGCAAGA (R) CTACCCCTCTCACCTCTTCA	129-145	4
M3	Ccr15	(F) TCATTGGTTACAGAAAGTTGAATT (R) AAAGATTACATGGGGAGACGA	148	1
	Ccroc01	(F) CCTCAATTAGGAACATAAAAAAGTG (R) GAAGGAAGGAAGCAATATGC	226-234	2
	Ccroc02	(F) GCATGCAGATAATTTGAAGATG (R) CAAAAAGGAGAAATTTAGCAGA	137-141	2
	Ccr05	(F) GAGG ACCCACAACCT CAAACC (R) GAGAAT	171-175	3
M4	Ccroc07	(F) TCCCTCAAGTCACTCGGTGT (R) TGCTAATGTTTCATTGCAGGG	288-336	6
	Ccr14	(F) GTGATGTTAAGAAGAACTGAATTAGA (R) ACGCCCCAAAATGAATGTA	213-217	2

Table_SI 13: PCR Mix used for 12S fragment- The mix was prepared for both cheetah and brown hyenas' samples by adding 5 µL of Master Mix, 0.3 µL of both primers and 2.4 µL of H₂O per sample (1x)

12S Mix

Reagents	Quantity per sample (1x)
Master Mix	5 µL
Mix Primer forward 12S (1:10)	0.3 µL
Mix Primer reverse 12S (1:10)	0.3 µL
H ₂ O	2.4 µL

Table_SI 14: Thermocycling conditions for the PCR for the 12S fragment- The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95 °C for 15 minutes; then 40 cycles of denaturation, annealing and elongation at 95 °C, 47 °C and 72 °C respectively, during 30, 30 and 20 seconds, respectively; and a final elongation at 60°C for 10 minutes.

Thermocycling conditions 12S PCR

Temperature (°C)	Duration	N ^a of cycles
95°C	15 min	1x
95°C	30 secs	40x
47°C	30 secs	
72°C	20 secs	
60°C	10 min	1x
10°C	∞	

Table_SI 15: INDEX PCR Mix- The mix was prepared for both cheetah and brown hyenas' samples by adding 7 µL of, Kappa HiFi Hot Start enzyme, 1.4 µL of index and 2.8 µL of H₂O and DNA per sample (1x)

INDEX PCR Mix

Reagents	Quantity per sample (1x)
Kappa HiFi Hot Start enzyme	7 µL
Index	1.4 µL
H ₂ O	2.8 µL
DNA	2.8 µL

Table_SI 16: NGS Library Prep Index PCR- The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95 °C for 3 minutes; then 8 or 10 cycles of denaturation, annealing and elongation at 95 °C, 55°C and 72 °C respectively, during 30 seconds each; and a final elongation at 72 °C for 5 minutes.

Thermocycling conditions INDEX PCR

Temperature (°C)	Duration	N ^a of cycles
95°C	3 min	1x
95°C	30 secs	8x(10x)
55°C	30 secs	
72°C	30 secs	
72°C	5 min	1x
10°C	∞	

Table_SI 17: Relatedness between the nine males of cheetahs- Combinations among individuals of cheetah and respective relatedness.

Individual 1	Individual 2	Relatedness	Individual 1	Individual 2	Relatedness
AJU01	AJU02	0.022	AJU03	AJU10	0.020
AJU01	AJU03	0	AJU04	AJU05	0
AJU01	AJU04	0	AJU04	AJU06	0
AJU01	AJU05	0	AJU04	AJU07	0
AJU01	AJU06	0	AJU04	AJU08	0
AJU01	AJU07	0	AJU04	AJU09	0
AJU01	AJU08	0	AJU04	AJU10	0.724
AJU01	AJU09	0	AJU05	AJU06	0.624
AJU01	AJU10	0	AJU05	AJU07	0
AJU02	AJU03	0	AJU05	AJU08	0.139
AJU02	AJU04	0.536	AJU05	AJU09	0.224
AJU02	AJU05	0	AJU05	AJU10	0
AJU02	AJU06	0	AJU06	AJU07	0.255
AJU02	AJU07	0	AJU06	AJU08	0.138
AJU02	AJU08	0	AJU06	AJU09	0.784
AJU02	AJU09	0	AJU06	AJU10	0
AJU02	AJU10	0.324	AJU07	AJU08	0.334
AJU03	AJU04	0.032	AJU07	AJU09	0.260
AJU03	AJU05	0	AJU07	AJU10	0
AJU03	AJU06	0	AJU08	AJU09	0.031
AJU03	AJU07	0	AJU08	AJU10	0
AJU03	AJU08	0	AJU09	AJU10	0
AJU03	AJU09	0			

Table_SI 18: Relatedness between the 15 individuals of brown hyena- Combinations among individuals of brown hyena and respective relatedness.

Individual 1	Individual 2	Relatedness	Individual 1	Individual 2	Relatedness
BH01	BH02	0.005	BH05	BH10	0.3019
BH01	BH03	0.634	BH05	BH11	0
BH01	BH04	0	BH05	BH12	0
BH01	BH05	0	BH05	BH13	0.234
BH01	BH06	0.490	BH05	BH14	0.068
BH01	BH08	0	BH05	BH15	0.023
BH01	BH09	0.356	BH05	BH16	0.030
BH01	BH10	0	BH06	BH08	0

BH01	BH11	0.308	BH06	BH09	0
BH01	BH12	0	BH06	BH10	0.008
BH01	BH13	0	BH06	BH11	0.5
BH01	BH14	0	BH06	BH12	0
BH01	BH15	0.5	BH06	BH13	0
BH01	BH16	0	BH06	BH14	0
BH02	BH03	0	BH06	BH15	0.416
BH02	BH04	0	BH06	BH16	0
BH02	BH05	0.646	BH08	BH09	0.138
BH02	BH06	0	BH08	BH10	0
BH02	BH08	0	BH08	BH11	0
BH02	BH09	0	BH08	BH12	0.506
BH02	BH10	0.332	BH08	BH13	0
BH02	BH11	0	BH08	BH14	0.044
BH02	BH12	0	BH08	BH15	0
BH02	BH13	0.543	BH08	BH16	0.05
BH02	BH14	0	BH09	BH10	0.092
BH02	BH15	0	BH09	BH11	0
BH02	BH16	0.037	BH09	BH12	0
BH03	BH04	0.129	BH09	BH13	0
BH03	BH05	0	BH09	BH14	0
BH03	BH06	0.832	BH09	BH15	0
BH03	BH08	0	BH09	BH16	0.203
BH03	BH09	0	BH10	BH11	0
BH03	BH10	0.041	BH10	BH12	0
BH03	BH11	0.5	BH10	BH13	0.774
BH03	BH12	0	BH10	BH14	0.243
BH03	BH13	0	BH10	BH15	0.039
BH03	BH14	0	BH10	BH16	0.283
BH03	BH15	0.526	BH11	BH12	0
BH03	BH16	0	BH11	BH13	0
BH04	BH05	0.243	BH11	BH14	0
BH04	BH06	0.075	BH11	BH15	0.23
BH04	BH08	0	BH11	BH16	0
BH04	BH09	0	BH12	BH13	0
BH04	BH10	0.617	BH12	BH14	0.461
BH04	BH11	0	BH12	BH15	0
BH04	BH12	0	BH12	BH16	0.278

BH04	BH13	0.5	BH13	BH14	0
BH04	BH14	0	BH13	BH15	0
BH04	BH15	0.198	BH13	BH16	0.5
BH04	BH16	0	BH14	BH15	0
BH05	BH06	0	BH14	BH16	0.290
BH05	BH08	0.127	BH15	BH16	0
BH05	BH09	0			