Carnivore ecology and diet assessment using DNA-based approaches: the elusive black-footed cat (*Felis nigripes*) as a case study

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ABSTRACT

Information obtained from the assessment of diet has been used to reconstruct food webs of elusive and shy predators that are difficult to observe in their natural habitats. The information from these studies has been crucial in developing conservation strategies. Predator-prey dynamics in particular, can also be used as a proxy of ecosystem health. Traditional approaches for diet analysis often involve direct observation or morphological identification of prey remains from scat. These approaches can be challenging for smaller predators that are difficult to track, and are generalist, opportunistic or scavenging carnivores. This is because traditional methods are not well adapted to analyze and identify such a wide variety of prey species, making them liable to bias and inaccuracy.

DNA-based methods for the identification of diet from scats have been shown to provide better resolution, accuracy and consistency when compared to all other methods. Species identification of prey consumed can be achieved using DNA barcoding; identification of species through the amplification of specific genetic markers linked to taxonomically identified species. DNA metabarcoding is a recent technological advancement which allows for identification of multiple species from a single sample using high-throughput sequencing (HTS).

Using the shy and elusive black-footed cat (*Felis nigripes*, Burchell 1824) as a case study, in this dissertation I aimed to determine if DNA-based methods could be used to provide important population-level information (number of individuals in population, relatedness of individuals, dietary components, and parasite load).

The first data chapter (Chapter 2) aimed to provide an in-depth review of the state of DNA reference libraries for small South African mammals. Small mammals constitute a large portion of small and medium carnivore diet. As such, the abundance of DNA reference libraries for small mammals gives a good indication if DNA metabarcoding studies are feasible. Analysis of DNA records revealed that the majority of small mammals are represented by at least one of the five mitochondrial genes. This study supports the use of multiple gene regions when performing scat

metabarcoding particularly when wanting to determine the small mammal component of the diet.

Chapter 3 tested *in silico* if published metabarcoding primers for 12S rRNA, 16S rRNA, COI and cyt *b* could reliably delimit common prey items eaten by black-footed cats. Successful species delimitation using metabarcoding rests on the presence of a "DNA barcode gap". This gap is the difference between intraspecific and interspecific genetic distances within a group of organisms. Eight metabarcoding primer pairs were chosen from the literature. Using an alignment of DNA sequences from South African small mammals, each amplicon produced by the primer pairs was tested to determine if species level identification could be made. An optimal set of primers were developed for use in black-footed cat (and other small and medium carnivores) diet analyses.

Chapter 4 assessed if microsatellites previously designed for use in domestic cat (*Felis silvestris catus*) could be successfully amplified and used to identify individual black-footed cats from scats. All nine microsatellite loci used in this study were amplified successfully and were polymorphic. The loci were found to have sufficient discriminatory power to distinguish individuals and identify clones and could therefore be used in parentage assignments.

Chapter 5 aimed at identification of prey items of black-footed cats in the Benfontein Nature Reserve, Kimberley, South Africa using DNA metabarcoding. Of the four DNA regions selected for high-throughput sequencing, the cyt *b* region did not sequence well and was therefore excluded from the study. The result from this study were compared to a previous study conducted which was based on direct observations of black-footed cat feeding. Unsurprisingly, our DNA data supported previous studies which showed that the diet of black-footed cat consists mainly of rodents. Importantly, the DNA metabarcoding results identify some prey items that have not been recorded in black-footed cat diet previously highlighting the sensitivity of the DNA-based method. For example larger prey items such as antelope were also identified suggesting that the cats may also opportunistically scavenge.

Chapter 6 assessed the presence of parasites found in black-footed cat wild populations. Five pathogens known to be of veterinary importance in domestic cats were found in the black-footed

cat scats. These pathogens had previously not been documented in wild African felids, but are well documented in other taxa. These results add to the growing knowledge of diseases that could possibly contribute to the declining populations of black-footed cats in South Africa.

In conclusion, DNA based methods have been shown to improved species resolution when compared to traditional methods of diet assessments. More so when coupled with high-throughput sequencing technologies. The results of this study show that DNA metabarcoding can be applied successfully to study the diet of South Africa carnivores. The results also indicate that DNA metabarcoding can be used in identification of species that are endemic to southern Africa.

PREFACE

The data described in this thesis were collected in the Conservation Genetics Lab (Willows-Munro Lab) at UKZN from 01 March 2020 to 30 October 2022. Experimental work was carried out while registered at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Sandi Willows-Munro.

This thesis, submitted for the degree of Doctor of Philosophy in the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, School of Life Sciences, Pietermaritzburg campus, represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

Vimbai Isabel Siziba

February 2024

I certify that the above statement is correct and as the candidate's supervisor I have approved this thesis for submission.

Professor Sandi Willows-Munro

Supervisor

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DECLARATION 1 - PLAGIARISM

I, **Vimbai Isabel Siziba**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis.

Publication 1

V. I. Siziba and S. Willows-Munro

Published - African Journal of Ecology

An assessment of South African small mammal barcode sequence libraries: implications for future carnivore diet analyses by DNA.

Author contributions:

VIS and S.W-M designed the research. VIS performed research and analyzed the data. VIS and S.W-M wrote the paper.

Publication 2

V. I. Siziba, Michelle M. Scroeder, B. Wilson, A. Sliwa and S.Willows-Munro

Published - Ecology and Evolution

A method for non-invasive individual genotyping of black-footed cat (*Felis nigripes*)

Author contributions:

VIS and SW-M designed the research. MS, BW, and AS collected samples. VIS performed research. VIS analyzed data. VIS, SW-M, MS, BW, and AS wrote the paper

Signed:

Vimbai Isabel Siziba

February 2024

This dissertation is dedicated to my babies. You boys make the work worth it. I hope one day my achievements will be proof that the sky was never the limit, shoot for the stars and write yourself into history.

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To my best friend, I have always said thank you, I will continue to do so and still feel like it is never enough

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CHAPTER 1

Literature review: Use of DNA metabarcoding in ecological studies of mammalian carnivores

1.1 Background and problem statement

Diet assessments are fundamental to ecologists because they provide essential information on trophic interactions and functioning of ecosystems (Bradshaw *et al.* 2003; Kartzinel *et al.* 2015). Predator-prey interactions in particular, are central to ecology and conservation studies because they play a vital role in control of animal populations and maintaining biodiversity within ecosystems (Mills 1992; Ritchie and Johnson 2009). Carnivores occupy a significant ecological niche at the top of food webs and, as such, are identified as keystone species within any ecosystem (Mills *et al.* 1993). This position allows carnivores to maintain ecosystem function and stability of other species populations in lower trophic levels through trophic cascades (Ripple *et al.* 2014).

The information obtained from diet assessment has been used to reconstruct food webs of elusive predators or predators that are otherwise difficult to observe in their natural habitats (Juen and Traugott 2007). The information from these studies has been crucial in developing conservation strategies and policies, particularly in cases where relocation is required to preserve endangered species (Sergio *et al.* 2008). Diet assessments have also been used in taxonomy and quantifying biodiversity within an ecosystem (Hajibabaei *et al.* 2007).

Determining the dietary profile of a carnivore is necessary to fully understand the role of the carnivore in an ecosystem (de Sousa *et al.* 2019). Various methods have been used for diet assessments, including observation, scat analysis, and more recently, molecular techniques (Greenstone 1999; Symondson 2002a). Traditional methods of diet analysis have been shown to be more accurate for large carnivores such as lions that consume a single large prey once every couple of days. However, traditional approaches can be challenging for smaller predators that are generalist, opportunistic, or scavenging carnivores and feed on a wide variety of prey (Symondson 2002a). This is because traditional methods are not well adapted

to analyze and identify a variety of prey species, making them liable to bias and inaccuracy (Pompanon *et al.* 2012; Symondson 2002a).

Most of the information available on the food habitats of terrestrial animals is based on direct observations and has been integral in understanding the ecology of all wildlife (Allison and Destefano 2006; Rosenberg and Cooper 1990). Direct observations, however, become limited when analyzing carnivores that are either elusive or those who occupy remote habitats. In such cases, the feeding ecology of those carnivores can be assessed using GPS-collaring or radio telemetry (Anderson and Lindzey 2003). Other alternatives for diet assessments include indirect methods such as analyzing scats and stomach contents (Balestrieri *et al.* 2011).

To date, a variety of methods have been used to determine the diet of various taxa (Birkhofer *et al.* 2017). Each of these methods has limitations, either due to inherent bias, low resolution, or the feasibility and practicality of the technique in terms of time and resources (Corse *et al.* 2010; Tambling *et al.* 2012). The methods, application and limitations of each will be discussed in further detail below. Overall, these methods are limited in their capacity to analyze large sample sets (Kaneko and Lawler 2006). This is a requirement when analyzing generalist predators such as small to medium-sized carnivores that still remain largely understudied in South Africa.

DNA-based methods have been shown to have better resolution, accuracy, and consistency compared to all other alternative methods (Casper *et al.* 2007; Damm *et al.* 2010). Species identification of prey consumed is achieved through barcoding; identification of species through gene-specific regions (Hebert *et al.* 2003a). DNA barcoding can be used to correctly identify unknown taxa at the species level with no prior knowledge of possible dietary preferences (Darling and Blum 2007). As such, DNA barcoding is a useful universal tool that is applicable to a variety of studies such as conservation, ecology, and taxonomy (Hollingsworth 2007).

DNA metabarcoding is a recent technological advancement that allows for the identification of multiple species from a single sample through the use of high-throughput sequencing (HTS). DNA metabarcoding makes it possible to analyze diet by identifying multiple unknown species simultaneously (Pompanon *et al.* 2012; Raye *et al.* 2011). This makes DNA

metabarcoding an ideal method for determining the prey preference of small to medium-sized carnivores that feed on a wide variety of species across different taxa.

This review focuses on the feeding ecology of mammalian carnivores. The initial section of this review considers the importance of diet studies and how they apply to studies that include biodiversity, ecology, and taxonomy. The second section of the review describes the different methods used to study the dietary preferences of carnivores and the advantages and limitations of each method. The third section focuses on DNA barcoding, particularly regarding DNA metabarcoding and the various HTS platforms applicable to diet assessments. The last part of the review evaluates the available information on the dietary preferences of carnivores in South Africa. As well as explaining how this study will aid in bridging the knowledge gaps concerning the feeding ecology of small to medium-sized mammalian carnivores.

1.2 Role of diet studies in ecology and biodiversity

The diet of carnivore species is central to the ecological niche they occupy (Mills 1992). This is because a predator's food habits explain its role in an ecosystem and impact on the population densities of the species they feed on and species in lower trophic levels (Klare *et al.* 2011a). Information obtained from the study of carnivore diets gives a good indication of the stability of an ecosystem in regards to resources utilized (Mills 1992). This, in turn, can be used to assess the ability of an area's prey base to sustain or be affected by predation relative to prey selection and prey availability. Consequently, the availability of prey also gives a good indication of the relative abundance of predators and possible distribution (Kerley *et al.* 2015). In the case of predators that rarely feed at random, feeding is determined by availability of prey and competition with other predators (Ritchie and Johnson 2009).

Information pertaining to prey selection and availability can guide us in determining prey species that are either vulnerable or endangered. Studies assessing predator-prey interactions can aid in conservation by identifying predators that have a direct impact on threatened or endangered species (Hopken *et al.* 2016). For example, a study on the feeding ecology of otters (*Lutra lutra*) found that the most prevalent species in the diet was eel (*Anguilla anguilla*), which was rapidly declining across Europe (Britton *et al.* 2006). Differences

observed between prey recovered from different localities can be an indication of prey availability or abundance in that particular area (Deagle *et al.* 2009). This, therefore, means that regions where the feeding ecology differs may require unique conservation strategies (Lyngdoh *et al.* 2014). Ultimately, diet studies, especially those of carnivores, are an important consideration when implementing balanced conservation and species management strategies (Sergio *et al.* 2005).

Diet studies have been used to reconstruct species interactions of rare, shy, or elusive species whose feeding ecology is challenging to visualize directly (Garcia-Robledo *et al.* 2013; King *et al.* 2010). The study by Garcia-Robledo *et al.* (2013), managed to outline plant-herbivore interactions by studying the diet of rolled-leaf beetles in the genera *Cephaloleia* and *Chelobasis* that fed on plants of the order Zingiberales. King *et al.* (2010) used diet assessment to reconstruct the predator-prey interactions between carabid beetles (*Pterostichus melanarius*) and various earthworm species.

Carnivores have been identified as keystone species because their dietary profile has a direct effect on the abundance of other species in an ecosystem (Mills *et al.* 1993). Predators feed on other animals; this places them at the top of the food web (Ripple *et al.* 2014). This trophic position has been shown to have a ripple effect on lower trophic levels. As such, the feeding ecology of carnivores controls the diversity and population densities of other species within the ecosystem (Sergio *et al.* 2008; Sergio *et al.* 2005). Therefore, adding or removing carnivores has subsequent effects on lower trophic levels, such as herbivore-plant interactions (Sergio *et al.* 2008). A classic example is the arrival of whales (*Orcinus orca*) in Alaska, which greatly reduced the sea otter (*Enhydra lutris*) populations as a result of predation (Estes *et al.* 1998). The decline in sea otter populations caused a direct increase in sea urchin densities, which in turn drastically reduced the biomass of kelp.

Results obtained from diet studies of predators have been used in implementation of conservation strategies, some of which use carnivores for bio-control (Symondson *et al.* 2002b). For example, re-introduction of gray wolf (*Canis lupus*) in Yellowstone National Park was shown to significantly restore carbon levels through feeding on herbivores such as moose (*Alces alces*), which would, in turn, directly increase the populations of balsam fir (*Abies*

balsamea) (Wilmers *et al.* 2003). The re-introduction of grey wolf enhanced carbon storage by limiting the number of herbivores that feed on plants which absorb and store CO_2.

Analyzing the diet of predators has shown that they control populations of prey which they feed on, and also populations of other competitive predators (Mills 1992). For example, in Southern California, the decline of coyote (*Canis latrans*) populations caused a sharp increase in the populations of mesopredators such as foxes (*Vulpes vulpes*), opossums (*Didelphis virginiana*), and raccoons (*Procyon lotor*). The increase of other competitive predators led to a significantly reduced biodiversity of bird species in the area (Crooks and Soulé 1999). The presence of carnivores has also been linked to an increase in scavenger diversity, which aids in nutrient cycling of an ecosystem (Ripple *et al.* 2014).

Diet studies have been used to provide species level identification and provide complementary data for studies that focus on studies to taxonomy and biodiversity (Hajibabaei *et al.* 2007). The DNA barcode sequences of species identified through diet studies can be deposited into the Barcode of Life Database (Gerloff *et al*. 1995). This would be highly beneficial to regions such as South Africa, where the DNA barcodes of most species are yet to be documented (da Silva and Willows-Munro 2016). Identifying species in various ecosystems would aid in determining the level of bio-diversity across different geographical locations.

Diet assessments have been applied to address the issues surrounding human-wildlife conflicts in an effort to protect wildlife species (Bagchi and Mishra 2006; Namgail *et al.* 2007). In areas where humans and predators co-habit, there have been massive declines in carnivore populations. This was a result of hunting and killing by humans who believed the carnivores feed on their livestock (Bauer and Kari 2001). Some studies analyzing the prey choice of these carnivores have shown that livestock is not the primary prey (Marker *et al.* 2003).

1.3 Sampling methods

Various methods are used as a means of obtaining samples for diet assessments. These sampling methods are categorized as invasive or non-invasive (Taberlet *et al.* 1999). Invasive methods require the predator to be captured or immobilized to obtain samples. Invasive methods are used when collaring animals and involve analyzing stomach and gut contents

(Giller 1986). Analysis of stomach contents requires that the entire contents of the gut be examined, thereby giving a complete dietary profile of ingested prey (Hyslop 1980). The difficulty associated with invasive sampling is mainly due to ethical considerations and the inability to obtain permits for invasive sampling of endangered or threatened species (Kohn and Wayne 1997; Symondson 2002a).

Non-invasive sampling methods are preferred for sampling mammalian carnivores because samples are collected without capturing or disturbing the animal (Sheppard and Harwood 2005; Waits and Paetkau 2005). Scat samples are the most accessible non-invasive samples to gather and can be collected in significant quantities (Fernandes *et al.* 2008).

1.4 Traditional methods for diet analyses

1.4.1 Direct and indirect monitoring of predation

Visual observation is still used to study the diet of carnivores in the field. This approach is simple and gives accurate and direct information about the prey preferences of animals (Mills 1992). Visual observations can directly account for how prey was consumed; either through primary predation, scavenging, or secondary predation (Merfield *et al.* 2004). Correct identification of the mode in which prey is consumed reduces the chances of incorrect trophic links. Although direct observation is considered one of the most reliable methods, it is not without limitations.

Studies have shown that direct observation can have a biased estimate of prey consumed (Gordon 1994). Bias can be introduced when animals are not observed consistently, and only a limited number of feeding events are observed (Mills 1992). Feeds that occur when there is no observation would be missed. Such is the case with smaller carnivores that feed at night or under the canopy of vegetation (Allison and Destefano 2006; Symondson 2002a). For generalist predators, the prey observed would only account for a fraction of the dietary range of the predator (Sheppard and Harwood 2005). In some instances, the presence of an observer can interfere with the predator's access to prey (Mills 1992). Observation of a predator attacking prey does not confirm if the event was common or rare. Technically, direct

monitoring of predation is labor intensive and can be impractical in remote terrains or for highly elusive predators (Ale and Brown 2009).

Remote video monitoring has been used to observe predator and prey interactions without disturbing the animals in field and controlled environments (Merfield *et al.* 2004). In the case of nocturnal animals, specialized night vision cameras are used (Allison and Destefano 2006). The accuracy of this method is highly dependent on animals entering the video camera's field of view. For both day and night video monitoring, even the best equipment will have problems with contrasts and weather. High light pollution can also present background difficulties that constrict viewing angles (Allison and Destefano 2006).

Radio telemetry has been used as an alternative to study predators that are challenging to observe directly. Either because they are highly elusive or occur in remote terrains. Predators are radio-tagged and followed across their natural environment (Anderson and Lindzey 2003). Their prey of choice is determined by remains after a feed. The major disadvantage of this method is that the remains of killed prey may be evident if the predator consumes the entire kill. If predation occurs in remote areas or under extreme weather conditions, following the predator becomes extremely difficult. Radio telemetry has also been shown to be difficult for predators that move erratically, such as the hyena (Mills 1992). In addition, such research cannot be replicated (Birkhofer *et al.* 2017).

Global Positioning System (GPS) collaring and Geographic Information system (GIS) has been used as an alternative to telemetry due to its higher data capturing capabilities and lower labor (Anderson and Lindzey 2003; Sand *et al.* 2005). The major drawback of GPS collaring is that it can take extended periods of time to conduct experiments and produce large amounts of data. A study by Sand *et al.* (2005) to estimate the kill rates of wolves (*Canis lupus*) was conducted over 287 days and produced 6140 hourly GPS coordinates. Radio telemetry and GPS collaring are biased towards identification of larger prey. Smaller prey is easily excluded because it is consumed quickly, and no traces are left (Mills 1992; Sand *et al.* 2005). This would mean the diet profile inferred from telemetry data may not be an accurate representation of the predator's actual diet.

Captive simulations have been used as a means to evaluate the diet of animals in a controlled environment. Laboratory estimates tend to give a reliable indication of dietary preferences. However, simulations can fail to recreate vegetation structure and climate as is in the field. Altering the environment alters the ability of the predator to locate its prey, which can ultimately lead to biased estimates of prey species (Symondson 2002a).

1.4.2 Scat analysis

Fecal (scat) analysis is the examination of undigested parts within scat samples to identify a predator's possible prey (Bagchi and Mishra 2006). Scat analysis provides an alternative method for assessing the diet of elusive animals because scats are readily often available and easy to collect (Putman 1984). Scat analysis is the most commonly used method of diet assessment and has been used to identify prey that has been missed through direct observations (Kamier *et al.* 2012; Klare *et al.* 2011b; Ogara *et al.* 2010). For example, chimpanzees were identified as prey for lions from scat analysis despite the predation of chimpanzees never having been observed in the field (Tsukahara 1993).

The scats of carnivores contain undigested diagnostic parts from prey, such as hair, teeth, bones, and feathers (Tollit *et al.* 2003; Tsukahara 1993). The various scats are compared to a reference guide to identify the prey consumed. Undigested intact bone fragments such as jaws, teeth, and scapulae make identification of prey easier. However, bone fragments that are either digested or fragmented are more challenging to assign to particular prey (Oli 1993). The presence of bone in scat samples does not always accurately determine a particular prey species. Instead, taxonomic resolution of the prey consumed is limited to the order or family level. For example, prey is most likely to be identified as a rodent or shrew or a larger prey such as antelope, but species identification is very limited (Klare *et al.* 2011b).

Hair samples collected from scats are used for microscopic histological examination (Oli 1993). The pattern of the hair imprint is examined and compared to that of known hair imprints to identify possible prey items. The disadvantage associated with analyzing hair is that some samples may be degraded by stomach acids, causing the hair to be unrecognizable (Ogara *et al.* 2010). Microscopic examination of hair samples is a laborious process that

requires an advanced skill set. Some prey items can be missed because hair samples are unidentified due to lack of a complete reference guide (Mills 1992).

Morphological identification of undigested remains in fecal samples can lead to biased results due to differences in digestion rates between hard and soft-bodied prey (Braley *et al.* 2010; Putman 1984). Soft-bodied or smaller prey is usually completely digested and less likely to be identified (Mills 1992). Moreover, fecal samples represent the short-term diet profile of a predator and do not always give the full prey range unless sampled over an extended period of time (Deagle *et al.* 2006). Furthermore, studies based on morphological identification require thorough prior knowledge of prey morphological diversity to correctly identify prey remains (Corse *et al.* 2010).

Interpretation of scat analysis is either through biomass calculation, frequency of occurrence, or estimated mass of food items (Klare *et al.* 2011b). Most researchers use the frequency of occurrence, which is the least reliable method. Scat analysis is more reliable through biomass calculations done after feeding trials (Balestrieri *et al.* 2011). The predator is placed on a fast for about 40-90 hours until no fresh scat is produced. Thereafter, the predator is fed a specific diet, and scat is re-collected and compared to that of free-ranging predators. This method was used to determine the diet of free-ranging cheetahs in Namibia (Marker *et al.* 2003). The study showed that cheetahs were consuming birds, hares, and antelope and not domestic cattle as assumed by the locals.

A comparison of various traditional methods showed that there is no ideal method of diet assessment. Comparison of results from different methods will most likely produce more accurate dietary profiles (Mills 1992). However, when studying carnivores with an inconsistent diet, there is a higher chance of disagreement between various methods (Klare *et al.* 2011b). Molecular-based approaches have been used to limit bias and overcome the disadvantages posed by traditional methods of diet assessment (Pompanon *et al.* 2012; Symondson 2002a).

1.5 Molecular identification methods for diet analyses

In some cases, observation of predators as a means of identifying their prey is not possible (Sopp *et al.* 1992), particularly with evasive or small predators (Sheppard and Harwood 2005). Analysis of fecal remains may be misleading if the predator ingests soft-bodied prey, leaving no indigestible remains to analyze. Therefore, observation and scat analysis would overlook many trophic interactions (Symondson 2002a). The best assessment of diet in such situations would be using molecular identification methods (Sheppard and Harwood 2005; Symondson 2002a). The techniques used are enzyme electrophoresis, polyclonal and monoclonal immunoassays, and DNA-based methods (Symondson 2002a). Most of the molecular methods were initially developed to study the dietary profile of marine animals because of the difficulty associated with underwater observations (Kaneko and Lawler 2006). Studies on the feeding ecology of terrestrial carnivores relied heavily on observations. As such, the majority of literature on molecular-based methods is skewed toward marine animals.

1.5.1 Protein electrophoresis

Protein electrophoresis is the analysis of homogenized extracts of stomach contents on a polyacrylamide gel (Greenstone 1999; Symondson 2002a). This method is usually done in combination with stains for enzyme activity such as esterases and isomerases (Traugott 2003; Walrant and Loreau 1995). The separation of protein bands characterizes the different prey types present in the gut of a predator (Giller 1986). Reference samples are used to identify each prey and differentiate it from the predator. The reference samples consist of gels of the starved predator and that of potential prey (Traugott 2003). The banding pattern on the polyacrylamide gel of a predator being analyzed is compared to that of reference prey, and digested prey species are identified (Walrant and Loreau 1995).

The electrophoresis approach is ideal in situations where prey is entirely consumed, leaving no recognizable remains (Giller 1986), and has mainly been used in studies of arthropod predation (Murray *et al.* 1989; Solomon *et al.* 1996). Another advantage of protein electrophoresis is that it is extremely sensitive, such that even prey consumed in low quantities can be detected (Walrant and Loreau 1995).

The effectiveness of protein electrophoresis is limited to predators with a narrow prey range. In predators that consume a variety of prey, the results are a series of complex banding patterns (Walrant and Loreau 1995). Identifying species-specific bands becomes difficult due to superimposed protein bands that are extremely difficult to interpret (Pompanon *et al.* 2012). The resolution of protein electrophoresis can be limited by the choice of enzyme stain used (Greenstone 1999). Estearases are polymorphic; therefore, a single species can have multiple banding patterns. This can make it challenging to identify the banding pattern of prey if it is in the zone of polymorphism of the predator. Secondly, it can also complicate differentiation of prey species with similar isoenzyme activity (Walrant and Loreau 1995).

1.5.2 Immunoassays

Various immunoassays such as protein precipitation (Berth and Delanghe 2004; Nickerson and Doucette 2020), western blotting (Hoyt *et al.* 2000; Mahmood and Yang 2012) and enzyme-linked immunosorbent assays (ELISA) (Sopp *et al.* 1992) have been used to determine the diet of predators from their stomach contents. Each of these assays depends on specific antigen-antibody coupling interactions (Grisley and Boyle 1985). Antibodies are created with the required properties targeting specified epitopes (Symondson *et al.* 1999). The prey protein acts as an antigen. Specific amino acids on the prey protein are used as an epitope to which the antibody binds (Grisley and Boyle 1985). Antibodies can be designed to detect prey of predators that feed on broad taxonomic groups (polyclonal antibodies), a particular genus or species (monoclonal antibodies), and even different sexes and stages of growth (Symondson 2002a).

Serological studies can be modified to quantify the proportional estimates of a particular prey species relative to the total prey consumed (Hoyt *et al.* 2000; Naranjo and Hagler 2001; Sopp *et al.* 1992). ELISA was used to study the predators of the glass-winged sharpshooters (*Homalodisca vitripennis*) (Fournier *et al.* 2008). The study accurately identified the glass-winged sharpshooter in various predator gut contents. Similar to protein electrophoresis, immunoassays can detect prey in minute quantities (Hoyt *et al.* 2000). Additionally, immunoassays can screen large numbers of predators for prey (Birkhofer *et al.* 2017).

During protein precipitation, monoclonal immunoglobins can induce interference, leading to false positive or negative results (Berth and Delanghe 2004). Blocking agents can be used, but they are not as effective at blocking the effects of monoclonal immunoglobins (Ismail *et al.* 2002). Serological studies have been largely used to study predation of invertebrates and marine mammals but rarely for terrestrial mammals because of their invasive nature (Sheppard and Harwood 2005; Symondson 2002a).

Serology can lead to false positives if there are any shared antigens between prey species (Hoyt *et al.* 2000). This can be problematic because phylogenetically related taxa can share numerous antigens (Feller and Gallaher 1982). Furthermore, generating clones requires specialized tissue culture facilities, which most laboratories do not have. Buying antibodies from labs that develop them is expensive. Serological methods are not widely used because they are labor-intensive and time-consuming (Chen *et al.* 2000; Sheppard and Harwood 2005).

1.5.3 Fatty acid analysis

Fatty acid analysis estimate the diet of predators based on the fatty acid signatures of its prey (Iverson *et al.* 2004). Particular fatty acids present in prey are known to be distinctive of specific species and are assimilated into predator adipose tissue and digestive glands untransformed (Arnould *et al.* 2005; Phillips *et al.* 2002; Stowasser *et al.* 2006). These biomarker fatty acids cannot be synthesized by the predator and can only be taken up through the diet. Over time, the fatty acid profile of a predator will resemble that of its prey (Stowasser *et al.* 2006). Fatty acid analysis has been used extensively to study the diet of marine animals because they store fat for buoyancy (Bradshaw *et al.* 2003; Piche *et al.* 2010; Remili *et al.* 2023). The same method may not be as efficient for terrestrial carnivores (Iverson *et al.* 2004). For example, the actual diet of Canadian Inuit sled dogs (*Canis familiaris borealis*) could not be reliably constructed based on fatty acid signatures. This was as a result of the similarity of fatty acid signatures of predator and prey (ungulates) (Berkley *et al.* 2014). Nonetheless, fatty acid analysis is reliable for determining the diet of terrestrial carnivores that consume fish and invertebrates that have specific fatty acid signatures remarkably different from those of the predator (Iverson *et al.* 2004).

Unlike the above-described molecular methods, fatty acid analysis can differentiate different prey species even if multiple prey are consumed (Stowasser *et al.* 2006). Lipids stored in digestive glands can be used for diet assessment even after extended periods of starvation (Phillips *et al.* 2002). Fatty acid analysis provides information on the long-term diet without the need for multiple sampling. Prey can be detected up to 14 days after consumption (Birkhofer *et al.* 2017). Prey identification can be achieved even if the prey has been completely digested, leaving no diagnostic hard remains (Casper *et al.* 2007).

Fatty acid analysis presents a number of challenges when used for diet assessments. Firstly, determining diet is difficult without prior knowledge of the fatty acid signatures of prey (Phillips *et al.* 2002). This can be further complicated if the fatty acid signatures of the same prey species differ depending on geographical locations (Phillips *et al.* 2003). Data obtained for fatty acid analysis can be skewed if fatty acids are sampled from different tissues (Arnould *et al.* 2005). This is because deposition and mobilization of fatty acids is not uniform across the body (Arnould *et al.* 2005). Fatty acid biomarkers are unique to certain species, however, they can vary within species due to changes associated with age (Iverson *et al.* 2004). Fatty acid analysis gives little indication of the relative proportions of consumed prey (Phillips *et al.* 2003). Due to its many limitations, the resolution of fatty acid analysis is low (Herman *et al.* 2005). Resolution is improved when used in conjunction with another technique (Phillips *et al.* 2003) and in most studies fatty acid analysis has been used together with stable isotope analysis (Herman *et al.* 2005; Nyssen *et al.* 2005; Stowasser *et al.* 2006).

1.5.4 Stable isotope analysis

Isotopic enrichment is a method to assess the diet of animals through characterization of naturally occurring isotopes. The isotopic composition of stable isotopes such as carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) present in an animal is a reflection of the diet of the animal (DeNiro and Epstein 1978; DeNiro and Epstein 1981; Hobson *et al.* 1997). Stable carbon ratios can be used to differentiate between different food groups and are used to indicate the distance between a predator and its food base in a trophic system (Hobson *et al.* 1997; Stowasser *et al.* 2006). Stable nitrogen ratios, on the other hand, similar to fatty acid analysis, have been used largely to determine the diet of marine mammals and rarely for avian diets and terrestrial carnivores (Cherel and Hobson 2005; Hobson and Clark 1992; Stowasser *et al.* 2006). For terrestrial

carnivores, stable isotope analysis is used to show the seasonal shift in diet. A study by Darimont and Reimchen (2002) used stable isotope analysis to show that grey wolf (*Canis lupus*) fed largely on salmon (*Onchorynchus* spp.) during the fall.

The major advantage of this method is that it gives an indication of the long-term diet and can be used to detect food consumed 3-4 weeks prior (Tieszen *et al.* 1983). If fossil remains are used, the diet of a species over a lifetime can be determined (Garcia *et al.* 2009; Pérez-Claros and Palmqvist 2008). Isotopic enrichment is the only method that shows temporal changes in diet without the need for re-sampling (Darimont and Reimchen 2002).

The major disadvantage of isotopic enrichment is that it does not identify species consumed. Instead, isotopic enrichment can only distinguish broad prey categories, such as marine prey from terrestrial prey (Darimont and Reimchen 2002; Klare *et al.* 2011b). Secondly, the method of sampling can introduce bias and inaccuracy. This is because no single tissue is ideal for analyzing stable isotopic signatures due to differences in their metabolic rates and turn-over ratios (DeNiro and Epstein 1978). For example, tissues with a high lipid content will have decreased carbon irrespective of the diet (Tieszen *et al.* 1983). Therefore, in order to obtain more accurate results, various tissues should be sampled. Contradictory to this, there are studies that show that for omnivores, it is preferable to only sample from a single tissue (Sweeting *et al.* 2005). Additionally, stable isotope analyses are usually laboratory-based or controlled studies and are sometimes not applicable in the wild (Sweeting *et al.* 2005). Therefore, the results obtained from stable isotope analysis can be misleading for terrestrial carnivores that feed in the wild.

1.6 DNA-based identification of diet

DNA-based techniques have proved to be the most informative, versatile, and accurate method for analysis of the feeding ecology across various taxa and sampling sources (Damm *et al.* 2010; de Sousa *et al.* 2019; Pompanon *et al.* 2012; Symondson 2002a). Their application was initially applied to study the feeding ecology of marine mammals (Deagle *et al.* 2005b; Dunshea *et al.* 2008) and marine invertebrates (Braley *et al.* 2010). However, the use of DNA-based techniques has since extended to studying the diet of birds (Deagle *et al.* 2010; Deagle *et al.* 2007a; Oehm *et al.* 2011; Valentini *et al.* 2009), larvae (Garros *et al.* 2008), rodents

(Soininen *et al.* 2009), bovids (Pegard *et al.* 2009; Valentini *et al.* 2009), carnivores (Kim *et al.* 2017; Shehzad *et al.* 2012b), reptiles (Swinehart *et al.* 2023), and chiroptera (Bohmann *et al.* 2011; Zeale *et al.* 2011).

DNA techniques have been used successfully across a variety of samples from stomach contents (Deagle *et al.* 2005a; Marshall *et al.* 2010; Troedsson *et al.* 2009), regurgitates (Taberlet and Fumagalli 1996) and scats (Corse *et al.* 2010; Deagle and Tollit 2007b; Pegard *et al.* 2009; Valentini *et al.* 2009). The wide application of DNA-based methods across various taxa and sampling sources is due to improved accuracy and fewer shortcomings compared to other methods of diet assessment (Sheppard and Harwood 2005).

Unlike traditional methods, DNA identification does not depend on morphological identification that can be altered during capture, feeding, and digestion of prey (Jarman *et al.* 2002; King *et al.* 2008). A comparative study assessed the effectiveness of DNA-based methods to determine the diet of the generalist predator arrow squid (*Nototodarus gouldi*) (Braley *et al.* 2010). DNA-based techniques identified 84% of the prey species consumed compared to morphological techniques that identified 50%. Another study compared a DNA-based methodology to microscopy to assess the diet of deer. The total number of taxa, as well as the taxonomic precision to species level identified, was higher with DNA techniques compared to microscopy (Nichols *et al.* 2016). These studies show clear evidence that DNA-based techniques provide a much higher resolution of prey identification when compared to morphological analysis.

1.7 DNA barcoding

A DNA barcode is a gene sequence from a standardized part of the genome (Arnot *et al.* 1993; Hebert *et al.* 2003a). The primary function of DNA barcoding is to identify unknown organisms to the species level using molecular data (Hajibabaei *et al.* 2007). The query sequence of the unknown sample is compared to a DNA barcode library based on the sequences of known species (Hajibabaei *et al.* 2007). The patterns of sequence variation are used to identify the unknown species and potential new or cryptic species (Hebert and Gregory 2005). DNA barcoding is more accurate and consistent when compared to morphology-based identification, which does not always support species level resolution (Hajibabaei *et al.* 2011).

DNA barcoding is considered one of the most important measures of comparing diversity and has been used to establish protected areas (Hebert and Gregory 2005; Kress *et al.* 2014). DNA barcoding has been applied in creating barcode reference libraries that have been used to study species diversity (Blagoev *et al.* 2013) and species adaptation (Dinca *et al.* 2011).

A variety of mitochondrial gene regions are used for barcoding, but the global standard for animals is the 658bp fragment of the 5' end of the mitochondrial gene cytochrome *c* oxidase I (COI, *cox1*) (Hebert *et al.* 2003b). Secondary barcodes such as the 16S rRNA have been used as an alternative to the COI barcode, which amplifies poorly in invertebrates, amphibians, and reptiles (Kress *et al.* 2014). The advantage of the COI gene is that it is located within a protein-coding region. Therefore, errors can be identified by checking if the obtained sequence is translatable (Jinbo *et al.* 2011). Previously, the application of the COI gene was limited by the absence of universal primers for the amplification of unknown species (Creer *et al.* 2010). However, to date, DNA barcodes can be evaluated *in silico* using software that can identify barcodes and their associated primers (Ficetola *et al.* 2010; Govendor *et al.* 2019; Riaz *et al.* 2011).

In the case of degraded DNA, as that obtained in scats after digestion (Kohn and Wayne 1997) or other environmental samples (Taberlet *et al.* 2012), a smaller section of the COI gene region (130bp) can be used (Meusnier *et al.* 2008). Mini-barcodes (between 100bp-250bp) have also been successfully used for species identification (Govendor *et al.* 2019; Hajibabaei *et al.* 2006). Mini-barcodes were also used to assess the dietary profile of the pyrean desman (*Galemys pyrenaicus*). This approach identified more species than previous dietary studies (Hawlitschek *et al.* 2018). These studies confirm the application of mini-barcodes where longer length barcode sequences cannot be obtained to identify various taxa correctly, even at the species level.

When working with degraded DNA, there is no alternative to very short mini-barcodes (Bienert *et al.* 2012) because fecal samples have fragmented and highly digested state of samples (Rosenberg and Cooper 1990; Taberlet *et al.* 1999). The mini-barcode marker drawback can be overcome by using more than one barcode to increase species resolution and identification of closely related species (Hoffmann *et al.* 2016; Zhang *et al.* 2018). DNA barcoding can also lead to the identification of new species. However, this should be

confirmed by using barcodes from different sequence markers combined with morphological, ecological, and biogeographical datasets (Damm *et al.* 2010).

Multiple genetic markers, both mitochondrial and nuclear, should be used in barcoding studies (da Silva and Willows-Munro 2016; Hopken *et al.* 2016). Studies have shown that a combination of both mitochondrial and genetic markers improves species resolution (Chan *et al*. 2021). However, mitochondrial markers are the preferred choice in most studies because they evolve more rapidly than nuclear markers (Allio *et al*. 2017). Rapid evolution of mitochondrial markers mean that they will show a higher degree of sequence variation as compared to nuclear markers (Shaw 2002).

1.7.1 Amplification using universal markers

The diet of any species can be accurately determined with no prior knowledge of possible prey items through the use of primers that amplify a broad taxonomic range of potential prey taxa (Blankenship and Yayanos 2005; Corse *et al.* 2010; Taberlet *et al.* 2007). For example, the diet assessment of leopard cats (*Prionailurus bengalensis*) using universal primers of the cytochrome *b* (cyt *b*) gene amplified a range of animal prey from amphibians, birds, fish mammals, to reptiles (Lee *et al.* 2013).

The use of broadly applicable primers can, however, be a challenge when studying the diet of carnivores because cells from the intestinal lining are shed in the scats (Kohn and Wayne 1997) and can be amplified simultaneously with prey DNA (Deagle *et al.* 2007a; Jarman *et al.* 2006). Minute quantities of prey DNA can be easily missed because their amplification is overshadowed by the presence of dominant predator DNA (Jarman *et al.* 2004; Jarman *et al.* 2006). The COI gene is a protein-coding gene and can therefore complicate design of universal markers because every third base of each codon is variable (Taberlet *et al.* 2012). The use of primers not designed specifically for the detection of certain taxa can introduce errors. This is more so when using environmental DNA (eDNA) samples that contain a variety of taxa as is the case with generalist predators. The errors are introduced in the form of chimeric fragments during the PCR. Incompletely amplified PCR products act as primers in subsequent PCR cycles (Acinas *et al.* 2005). Some studies have shown that universal primers are in fact not universal across all taxa. In such cases, alternative forward and reverse primers should be

designed for amplification of the remaining taxa (Sharma and Kobayashi 2014). To avoid the limitations posed by universal primers, some studies have opted to use multiple universal primers across various gene regions to improve species detection (Rosli *et al.* 2011). The study by Rosli *et al*. 2011 used the 12S rRNA, COI, and cytochrome *b* (cyt *b*) region to identify the diet of Malayan gaur (*Bos gauras hubbacki*). This was further supported by other studies that show that species detection is increased when multiple barcode markers are used (Zhang *et al.* 2018).

To overcome the limitations imposed by predator DNA, restriction enzymes that digest predator DNA can be used (Dunshea 2009; Leray *et al.* 2013). Restriction enzymes pose a number of technical constraints. Firstly, for restriction digestion to be successful, predator and prey DNA sequences have to be sufficiently different such that the restriction enzymes do not cleave prey DNA (Green and Minz 2005). Secondly, only double-stranded predator DNA can be digested by restriction enzymes. Any single-stranded fragments will be missed and amplified during HTS. Thirdly, this method is only applicable to DNA extracts prior to barcode marker amplification. After amplification, the DNA of some prey species will already have been missed. Lastly, prior knowledge of the DNA sequences of both predator and prey species is essential to be certain that the selected restriction enzyme will not digest prey DNA (Leray *et al.* 2013). To test the effectiveness of restriction enzymes, it is necessary to do a test before and after amplification to check for predator amplicons (Dunshea 2009; Leray *et al.* 2013).

1.7.2 Amplification using species-specific primers

Species-specific primers have been designed to avoid amplification of predator DNA associated with universal markers (Juen and Traugott 2007; Vestheim *et al.* 2005; Walter *et al.* 2000). However, this is only possible with prior knowledge of the genome of the prey species. When multiple species make up the diet profile of a predator and some of the species are unknown, species-specific primers may introduce bias (Jarman *et al.* 2002). In such situations, group-specific markers that target a broad range of prey can be used (Jarman *et al.* 2004; Jarman *et al.* 2006). Designing group-specific primers can be difficult if the conserved primer binding regions of the prey are similar to the homologous sequence of the predator.

Some studies have combined group-specific and species-specific primers to elucidate the dietary profile of generalist predators (Jarman *et al.* 2004; Kuusk and Agusti 2008). Group-specific primers that amplify a barcode with low taxonomic resolution can be used together with species-specific primers that target a barcode region with high resolution (Pompanon *et al.* 2012). This approach was used to study the diet profile of carabid beetles (*Pterostichus melanarius*) that feed on earthworms that have high genetic diversity within morphological species (King *et al.* 2010).

Shotgun barcoding has been used as a means of species identification for feeding ecology studies. Shotgun barcoding is the amplification of a barcode region from an environmental sample containing unknown taxa. The individual PCR products are cloned into bacterial vectors and selected for pyrosequencing. The environmental sample's biodiversity would be quantified by analyzing the various clones (Darling and Blum 2007). Sanger sequencing (Sanger *et al.* 1977) has been used to provide robust DNA sequences.

Standard DNA barcoding (using Sanger sequencing technology), despite its many advantages, does not adequately fulfill the needs of ecological studies because it is limited in the number of species it can identify. Use thereof, for diet studies that use environmental samples which can contain numerous species in a single sample would be tedious, time consuming, and can be inefficient (Pegard *et al.* 2009; Shokralla *et al.* 2012).

1.8 DNA metabarcoding

DNA metabarcoding is the combination of DNA barcoding and high-throughput sequencing to identify multiple species simultaneously (Taberlet *et al.* 2012). DNA metabarcoding has been used to determine multiple species that make up the diet profile of animals across various taxa (Raye *et al.* 2011). The ability of metabarcoding to identify multiple species simultaneously makes it the ideal method for determining the diet of generalist predators (Pompanon *et al.* 2012).

A study by Hajibabaei *et al*. (2011) compared species identification of Ephemeroptera and Trichoptera species present in the river Benthos using morphological analysis, Sanger sequencing, and high-throughput sequencing (HTS). HTS provided evidence of nine species

that were undetected using the Sanger sequencing method. However, HTS missed six low-abundance species. Overall, the study showed that HTS has the potential to provide more accurate information from mixed-source samples when compared to morphological examination and cloning/Sanger sequencing combined. Metabarcoding has also been shown to be more effective compared to shot-gun barcoding; some prey items that were not recovered by cloning were detected through HTS (Shokralla *et al.* 2014). The versatility of DNA metabarcoding has also been used to study both animal and plant species that make up the diet of free-ranging wild pigs (*Sus* scrofa) (Robson et al. 2017).

1.9 High-throughput sequencing

High-throughput sequencing (HTS) (Kircher and Kelso 2010) is a relatively new technological advancement that has enormous potential (Schuster 2008). HTS technologies enable a much higher degree of parallel sequencing (Pegard *et al.* 2009; Schadt *et al.* 2010). Up to a million reads can be sequenced simultaneously in a single experiment as compared to Sanger sequencing, which is limited to a 96-sequencing template (Hert *et al.* 2008; Schuster 2008) or 384-sequencing template (Shibata *et al.* 2000). Compared to Sanger sequencing HTS technologies have lower cost per Mb. This means they are cheaper than Sanger sequencing when analyzing large volumes of data (Glenn 2011).

The main disadvantage for some HTS platforms such as Illumina, is that the read length is shorter and less accurate than Sanger sequencing (Shendure and Ji 2008). This in turn makes *De-novo* assembly challenging owing to difficulties in assembling very short reads (Hert *et al.* 2008). PCR amplification prior to sequencing performed by some HTS platforms may introduce errors in the bases of the DNA strand (Glenn 2011). In some cases, bias can be introduced if some sequences are amplified more than others. This changes the frequency and abundance of DNA fragments that were present before amplification and means that the proportion of reads from different taxa cannot be used to determine the relative abundance of each species that makes up the dietary profile (Hert *et al.* 2008).

High-throughput sequencing comprises various sequencing platforms that are either PCR or Single Molecule Sequencing (SMS) based (Shokralla *et al.* 2012). The PCR-based technologies also known as second generation platforms (Glenn 2011) are Roche 454 Genome Sequencer

(Roche Diagnostics Corp., Branford, CT, USA), Hiseq 2000 and MiSeq (Illumina Inc., San Diego, CA, USA), AB SOLiD™ System (Life technologies Corp., Carlsbad, CA, USA), and Ion Personal Genome Machine (Life Technologies South San Francisco, CA, USA) (Shendure and Ji 2008; Shokralla *et al.* 2012). SMS technologies, also known as third generation platforms (Glenn 2011) are non-PCR based and do not include an amplification step prior to sequencing such as HeliScope (Helicos BioSciences Corp., Cambridge, MA, USA) and PacBio RSSMRT system (Pacific Biosciences, Menlo Park, CA, USA). Choosing a suitable sequencing platform requires detailed understanding of the capabilities of each platform and if it can satisfy the research objectives. Important issues to consider are sources of error, error rates, speed, and cost of sequencing and downstream analysis (Glenn 2011; Kircher and Kelso 2010).

1.9.1 PCR-based next generation DNA-sequencing technologies

The biochemistry of the various sequencing platforms are different, but the generation of the array and workflow is similar. The library is prepared by fragmenting DNA and ligation of common adapter sequences for an emulsion of bridge PCR reaction.

The 454 GenomeSequencer FLX

The 454 platform's sequencing method is based on DNA synthesis, whereby nucleotides are added individually in the order specified by the DNA template. After emulsion PCR, DNA is fragmented, and each fragment is attached to a bead and deposited on a plate containing more than a million wells. A solution containing one type of deoxynucleoside triphosphate (DNTPs) is passed along the wells in a specified order. DNTPs are incorporated into the individual wells based on the template DNA. In this way, up to a million DNA fragments can sequenced simultaneously (Ansorge 2009; Liu *et al.* 2012).

The 454 platform has been used to determine the diet of little penguins (*Euduptula minor*) (Deagle *et al.* 2010). The 454 sequencing platform provides the longest sequence reads compared to other first and second generation sequencing platforms. Other advantages of the 454 platform include a relatively short run time and high range of applications (Shokralla *et al.* 2012). However, 454 has the highest cost per Mb compared to other HTS platforms (Glenn 2011; Metzker 2010).

Illumina Solexa Genome Analyzer

The Illumina platform is based on sequencing by synthesis chemistry. Adapters are ligated on one end of DNA fragments and to the other end complementary adapters (Metzker 2010). The adapters are used as primers for PCR amplification, and the complementary adapters are used to immobilize each single-stranded DNA to a solid support. After several PCR cycles, the DNA fragments that are clustered on the solid support are supplied with a sequencing reaction mixture. The sequencing reaction mixture contains primers, reversible terminator nucleotides, and DNA polymerase. As each different nucleotide is incorporated into the DNA strand, it is detected and identified via its fluorescent dye. About 40 million DNA clusters can be simultaneously sequenced, resulting in very high sequence throughput (Ansorge 2009; Liu *et al.* 2012).

The Illumina platform was used to determine the dietary profile of the leopard cat (*Prionailurus bengalensis*) in Pakistan and snow leopard (*Panthera uncial)* in South Gobi (Shehzad *et al.* 2012b; Shehzad *et al.* 2012a). Illumina is the most widely used platform, and this is because it has the widest range of applications and lowest cost per Mb (Glenn 2011; Metzker 2010). Except for the Miseq Platform, Illumina platforms have the longest run times (Liu *et al.* 2012).

Applied Biosystems ABI SOLiD system

The ABI SOLiD platform uses two-base ligation chemistry (Liu *et al.* 2012). DNA is ligated to adapters and bound to beads that contain emulsion PCR reagents. After the PCR cycle, the DNA fragments are denatured and hybridized to oligonucleotides, and sequencing primers are hybridized to the adapters. The doublet of the fourth and fifth bases corresponds to one of four fluorescent labels at the end of the octamer. After the fourth and fifth bases are detected, the octamer is cleaved off. The hybridization and ligation cycles are repeated to detect bases nine and ten. The following hybridization and ligation cycle will detect base 14 and 15 and so forth. To determine bases three and four, eight and nine, 13 and 14, the sequencing process is continued with a primer shorter than the one previously used. This is done up to the maximum sequence read of about 35 bases (Hashmi *et al.* 2015). Two-base encoding provides inherent error correction (Metzker 2010). DNA can be sequenced

simultaneously for up to 50 million bead clusters, resulting in very high throughput (Ansorge 2009). The main advantage of SOLiD is that it has the lowest cost per Gb compared to all the other platforms (Glenn 2011). This makes it ideal for sequencing of high sample volumes. The SOLiD platform has the shortest read length of 35-75bp and, consequently, the longest run time of up to eight days. The main advantage of the SOLiD platform is that it has the highest number of reads per run, highest sequence output, and lowest error rates (Liu *et al.* 2012).

Ion torrent

The Ion torrent platform relies on real-time measures of pH concentration as a result of hydrogen ions released when a nucleotide is incorporated into a newly synthesized strand of DNA. The sequencing process is performed on micro wells. Each micro well contains a single DNA template from the library. An ion sensor underneath each well detects the change in pH due to the nucleotides incorporated (Liu *et al.* 2012).

The ion torrent platform uses one of three ion chips; 314, 316, and 318, which generate up to 10Mb, 100Mb, and 1Gb, respectively, according to the required sequence coverage (Shokralla *et al.* 2012). Ion torrent has the shortest run time, which is adaptable to the amount of data being sequenced (Shokralla *et al.* 2012). Ion torrent has a lost cost per run. On the other hand, however, it has a high cost per Mb (Glenn 2011). A study compared Ion Torrent to Illumina sequencing for diet analysis. The results from the Illumina sequencing showed a higher sequence quality and detected more species variation compared to Ion Torrent (Forin-Wiart *et al.* 2018).

1.9.2 Single-molecule DNA sequencing technologies

The Helicos single molecule sequencing device, Heliscope

The systems previously discussed require amplification of DNA fragments prior to sequencing. The Helicos platform sequences directly from nucleic fragments and primers anchored in a flow cell on a glass cover slip. Labeled nucleotides, primers, and the polymerase enzyme are added to the glass. The labelled nucleotide omit a light as they are incorporated into the newly synthesized DNA strand. The emitted light determines which base was incorporated through sequencing by synthesis technique.

The Helicos platform sequences millions of DNA fragments, resulting in throughput in the Gigabase range. Compared to other platforms, the Helicos platform is newer but has been tested and validated in several applications (Ansorge 2009). The Helicos platform has a non-biased representation of templates due to the exclusion of the PCR step (Metzker 2010). Unfortunately, the Helicos platform has very short read lengths, and sequencing is only through a service center because the company no longer sells instruments (Glenn 2011; Shokralla *et al.* 2012).

Pacific BioSciences SMRT DNA sequencer

The BioSciences platform is a real-time, fluorescent-based SMS sequencer with a single molecule sequencing-by-synthesis approach. A waveguide is planted together with DNA polymerase that detects real-time DNA polymerazation as the complementary DBNA strand is synthesized. Different fluorescent labels are used for each of the four nucleotides. The fluorescent label is attached to the terminal phosphate group, unlike other technologies where the fluorescent label is attached to the nucleotide base. Therefore, the fluorescent label is released as the nucleotide is incorporated (Liu *et al.* 2012).

The PacBio platform does not need a washing step between each nucleotide flow, thus reducing the overall sequencing time. Additionally, the natural capacity of DNA polymerase to incorporate ten or more nucleotides per second is utilized in several parallel reactions (Shokralla *et al.* 2012). The PacBio sequencing platform is still in development and, therefore, has the highest error rate compared to all other sequencing platforms (Metzker 2010). However, it has the highest read length, shortest sequencing output, and shortest run time (Liu *et al.* 2012).

1.10 Application of DNA metabarcoding to determine the diet of South African carnivores

The feeding ecology of predators in South Africa has been extensively studied with regard to large carnivores such as leopard (Braczkowski *et al.* 2012a; Ott *et al.* 2007), lion(Tambling *et al.* 2012), jackal (Kamier *et al.* 2012; Van de Ven *et al.* 2013) and brown hyena (Burgener and M. 2003; Slater and Muller 2014). There is extensive duplication of research on the diet assessments of large carnivores (Balme *et al.* 2013; Hartstone-Rose *et al.* 2016). This is

because carnivores have been shown to have different feeding patterns in different environments (Lyngdoh *et al.* 2014). For example, the feeding ecology of black-backed jackals has been studied across different provinces, such as Free State (Kamier *et al.* 2012), Northern Cape (Klare *et al.* 2010), Eastern Cape (Van de Ven *et al.* 2013) and in the North West (van der Merwe *et al.* 2009). The same applies to leopard studies conducted in different parts of the Western Cape (Braczkowski *et al.* 2012a; Martins *et al.* 2010) and the Eastern Cape (Ott *et al.* 2007). The dietary analyses of jackals have been extensively researched in South Africa such that it includes how the presence of other predators, as seasonal changes, have an effect on diet selection (Brassine and Parker 2012; Do Linh San *et al.* 2009). Additional information is essential as the diet of carnivores can evolve based on the environment or the presence of other predators that provide possible competition for prey (Corse *et al.* 2010).

This information is, however, distinctly lacking for small to medium-sized carnivores, which make up the majority of the carnivore species in South Africa (Stuart 1981). The most recent study of the diet of a small carnivore was that of caracal (Braczkowski *et al.* 2012b), cape fox (Klare *et al.* 2014), bat-eared fox (Klare *et al.* 2011a), serval (Ramesh and Downs 2015) and cape genet (Roberts *et al.* 2007). The majority of dietary studies of small carnivores are older (MacDonald and Nel 1986; Stuart 1981). Those that are recent are largely obtained from unpublished Honors and Master's theses (Amiard 2014; Bizani 2014; Matolengwe 2010; Mbatyoti 2010). Such as the diet profile of the African civet, yellow mongoose (Bizani 2014), small spotted genet (Matolengwe 2010), and cape grey mongoose (Mbatyoti 2010). None of the studies, both published and unpublished have used a DNA-based method to adequately quantify the dietary profiles of small to medium-sized carnivores. The majority of information available does not specify the species name of the animals consumed by the carnivores. Instead, the majority of the prey items are classified in terms of families.

Current studies of dietary composition and prey preference in South African carnivores have been assessed using traditional methods such as scat morphology (Braczkowski *et al.* 2012a). Despite the limitations associated with using scat morphology to determine diet profile, very few of these studies have used scat analysis in combination with an additional method in order to validate the results. The studies that have used a combination of methods used scat analysis in combination with GPs positioning for predators that were either elusive or if the geographical terrain was not easily accessible (Martins *et al.* 2010; Tambling *et al.* 2012).

Although scat analysis has been shown to be the least ideal method, it has been used in almost every dietary assessment study, and in most cases, it is the only method used. This is despite recommendations by different authors on using a combination of methods to avoid bias and the pitfalls associated with scat morphological analysis (Klare *et al.* 2011b; Mills 1992; Pompanon *et al.* 2012). Despite all the recent advancements in molecular work, none of these studies used any molecular data to determine dietary components. In some cases where scat morphology was used, identification of prey items was not possible because some hairs could not be identified (Davidson *et al.* 2013). In other cases, prey was omitted because the prey remains occurred in trace amounts (Klare *et al.* 2010).

Current studies on the diet of carnivores in South Africa do not fully exploit the range of potential prey species of a predator as would be expected if DNA barcoding was used (Corse *et al.* 2010). Due to methods used to determine the diet of South African carnivores, uncertainties remain considering the limitations associated with analyzing undigested remains (Deagle *et al.* 2009).

The limited focus on large carnivores may have been due to a need to focus on human-wildlife conflicts (Kamier *et al.* 2012). Very few of these studies have addressed dietary overlap between carnivores occupying the same ecological niche (van der Merwe *et al.* 2009). In addition, information relating to the diet of small to medium-sized carnivores is distinctly lacking. This can imply that current trophic levels of South African ecosystems may have crucial links missing. This has major limitations for our current understanding of the structure and function of ecosystems (Braley *et al.* 2010).

1.11 Black-footed cat (*Felis nigripes*) as a case study

The black-footed cat (*Felis nigripes*) (Burchell 1824) is one of three endemic felids (African wild cat (*Felis lybica*) and caracal (*Felis caracal*)) and is the smallest and rarest wild cat species in Africa (Macdonald *et al.* 2010; Sliwa 2013). The species has the most restricted distribution of any African felid and is endemic to the arid regions of southern Africa (Sliwa 2004; Sliwa *et al.* 2016). Black-footed cat populations are found in South Africa, which has the largest population, Botswana, Namibia, Angola and Zimbabwe (Wilson *et al.* 2016). Currently, black-footed cats are listed as Vulnerable by the International Union for Conservation of Nature

(IUCN) due to limited range and small population sizes (Sliwa *et al.* 2016). The threats facing black-footed cats and other small felids are considered anthropogenic (Loveridge *et al.* 2010). The ecology, behavior, and population dynamics of black-footed cats remain largely unknown (Molteno *et al.* 1998; Sliwa 1994; Sliwa 2004; Sliwa 2006).

Traditional methods of studying carnivores are challenging to implement for black-footed cats because of low trapping success (Sliwa *et al.* 2010; Sliwa *et al.* 2018). Most studies of black-footed cats have been based on direct observations (Sliwa 1994; Sliwa 2004; Sliwa 2006) and camera traps (Sliwa *et al.* 2018). Direct observations are incredibly time-consuming and laborious as the species is highly mobile and travels long distances (Sliwa 2004). Camera traps have been shown to have low efficacy for the species because individuals do not travel predictable routes marked by scent (Molteno *et al.* 1998; Sliwa 2006). In rare cases where the cats have been recorded, they often move too fast for behavior to be captured by camera (Sliwa *et al.* 2018). Despite these challenges, direct observations have aided in answering questions about the relationships of black-footed cats with other canids (Kamler *et al.* 2015), estimating home ranges and habitats (Sliwa 2004), social organization and seasonal prey preferences (Sliwa 1994; Sliwa 2006). However, significant knowledge gaps still exist in the black-footed cats' taxonomy, behavior, population dynamics, and ecology. For example, the taxonomic status of the two proposed subspecies, Felis *nigripes nigripes* and *Felis nigripes thomasi* (Meester *et al.* 1986), remains to be tested. To this end, DNA data could contribute towards the conservation of the species by identifying population structure and resolving the taxonomy. Recent approaches such as metabarcoding have been used in diet studies in carnivores (Forin-Wiart *et al.* 2018) and could also provide additional crucial ecological information on the feeding behavior of black-footed cats.

A non-invasive approach to monitor populations of scarce species such as the black-footed cat, could be more sustainable and reliable (Banks and Piggott 2022; Piggott *et al.* 2008). DNA extracted from scats reduces the need to handle the animal (Ramon-Laca *et al.* 2015) and could provide information on individual occupancy (Fernando *et al.* 2003), paternity and kinship (Constable *et al.* 2001; Wang *et al.* 2015), genetic diversity (Mengulluoglu *et al.* 2019) species identification and sex (Dalén *et al.* 2004; Kurose *et al.* 2005), identifying hybrids (Adams *et al.* 2003), and estimating population sizes (Banks *et al.* 2002; Eggert *et al.* 2003; Piggott *et al.* 2006). DNA amplification from scat material can be challenging as DNA may be

degraded, leading to low amplification success (Ramon-Laca *et al.* 2015; Taberlet *et al.* 1996) or may contain genotyping errors due to allelic dropout (Fernando *et al.* 2003; Taberlet and Fumagalli 1996).

1.12 Research rationale

The aim of this Ph.D is to develop an innovative non-invasive method for assessing the feeding ecology of black-footed cats in South Africa. The first objective is to provide a detailed assessment of species coverage of the current South African DNA reference libraries for small mammals. This assessment will aid in identifying possible species whose DNA references are unavailable and, therefore, will not be identifiable using DNA metabarcoding in further downstream analyses. The second objective is to use DNA collected from scat samples collected in the field to identify individual black-footed cats. Microsatellite primers will be used for the individual analysis. The accuracy and efficacy of these markers will be tested on non-invasive collected samples. The efficacy of the microsatellites will be tested by comparing the scat genotypes to blood genotypes of the corresponding cats. The third objective is to design and test a versatile suite of mini DNA barcode primers that amplify very short variable DNA barcode regions. These primers will be tested *in silico* to assess their suitability in amplifying potential prey items. The fourth objective is a detailed assessment of the diet of black-footed cats using HTS techniques and comparing the metabarcoding results to those obtained from previous studies that used physical observations. Scat samples will be used as evidence to show that good quality DNA can be obtained for studies (Stech *et al.* 2011). The results of this study will also be used to add to the South African barcode reference library of small to medium-sized carnivores which have been shown to currently have knowledge gaps (da Silva and Willows-Munro 2016). The results of the study will be used to identify and document the possible parasites infecting wild populations of black-footed cats. Most importantly, this study will provide an evaluation of DNA metabarcoding for diet assessments, as these studies are distinctly lacking in Africa.

Chapter two to six of this dissertation has been written as manuscripts for publication. Some information is repeated but every attempt has been made to reduce repetition.

1.13 References

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CHAPTER 2

An assessment of South African small mammal barcode sequence libraries: implications for future carnivore diet analyses by DNA

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2.1 Abstract

DNA metabarcoding requires reference libraries that link sequences to species. Mitochondrial gene regions cytochrome c oxidase I (COI), 12S ribosomal RNA (12S rRNA), 16S ribosomal RNA (16S rRNA), cytochrome *b* (cyt *b*), and the hypervariable control region (D-loop) are routinely used to delimit animal species. This study aimed to review the state of DNA reference libraries for small South African mammals as small mammals constitute a large portion of small and medium carnivore diet. Analyses of DNA records revealed that of 193 small mammal species in South Africa, only 141 have DNA sequences available for one or more of the mitochondrial genes examined. Cyt *b* had the highest species coverage, with 59.1% of South African species represented in the reference libraries. COI only has 33.7%, 12S rRNA has 23.8%, D-loop has 17.6%, and 16S rRNA has the lowest species coverage of 15%. This study supports the use of multiple gene regions when performing scat metabarcoding, particularly when wanting to determine the small mammal component of the diet. Additionally, it emphasizes the need to build comprehensive DNA reference libraries linking sequences to taxonomically identified species.

Keywords: barcoding**,** COI, 12S rRNA, 16S rRNA, cyt *b*, Control region D-loop

2.2 Introduction

High-throughput sequencing technology has led to the emergence of metabarcoding and environmental DNA (eDNA) fields, allowing rapid species inventories to be produced from complex bulk samples such as scats (Ruppert *et al.* 2019). Scat DNA metabarcoding is increasingly used to track the feeding ecology of wildlife species and provides the opportunity for non-invasive sampling and monitoring of elusive species (Berry *et al.* 2017; Shehzad *et al.* 2012). The use of metabarcoding in predator-prey studies has proved far more successful in determining prey items in scats than morphological methods (Klare *et al.* 2011; Long *et al.* 2007; Pompanon *et al.* 2012). Feeding patterns of carnivores are considered essential for ecology and conservation studies (Xiong *et al.* 2017). Carnivores are keystone species, and their feeding patterns have a cascading effect on species further down the food chain (Ripple *et al.* 2014). Small mammals represent an essential component of small to medium-sized predator diet (Spencer *et al.* 2017), and their abundance and diversity can be an important measure of ecosystem health (Avenant 2011). Additionally, small mammals feed on and pollinate many plant species and are crucial to many ecosystems (Francis *et al.* 2010).

The standard animal barcoding region is the mitochondrial cytochrome *c* oxidase 1 (COI) (Hebert *et al.* 2003). However, the 658bp region is not informative in all taxa (Lv *et al.* 2014) and is too large for metabarcoding and eDNA methodologies (Deagle *et al.* 2014; Hajibabaei *et al.* 2019), which usually rely on fragments of less than 200bp (Bylemans *et al.* 2018). Other regions of the mitochondrial genome, namely 12S rRNA (Kocher *et al.* 1989), 16S rRNA (Palumbi *et al.* 1991), and cytochrome *b* (cyt *b*) (Irwin *et al.* 1991), have all been used to delimit mammal species. The hypervariable control region (D-loop) is also commonly used to delimit closely related taxa (Hoelzel *et al.* 1991; Stoffberg *et al.* 2012). Most carnivore scat DNA metabarcoding and eDNA studies have used either the 12S rRNA region for species identification (Riaz *et al.* 2011; Shehzad *et al.* 2012; Shu *et al.* 2021) or the 16S rRNA region (Hardy *et al.* 2017; Shu *et al.* 2020; Taylor 1996). Unfortunately, the potential of scat metabarcoding has not been fully realized in Africa (Elsaied *et al.* 2021). Lack of standardization regarding which gene region to amplify reduces comparability among different studies. This paper aims to review the current South African sequence-based reference libraries for small mammals. Other factors, such as misidentification and

pseudogenes, may also affect classification accuracy in metabarcoding and eDNA studies (Porter and Hajibabaei 2021). However, this study only examined the size and composition of the DNA barcode reference libraries. This work compares the available data for COI, 12S rRNA, 16S rRNA, cyt *b,* and D-loop and suggests which markers should be prioritized for future carnivore scat DNA metabarcoding studies in Africa.

2.3 Materials and methods

The small mammal list used in this study was compiled from volumes III and IV of Mammals of Africa (Kingdon *et al.* 2013) and was cross-referenced with the Red List of Mammals of South Africa, Lesotho, and Swaziland (www.ewt.org.za). Small mammals from the Orders Afrosoricida, Chiroptera, Eulipotyphla, Lagomorpha, Macroscelidae, and Rodentia were included in this study (Appendix 1).

Using the binomial names, publicly available DNA records and locations for five mitochondrial regions COI, 12S rRNA, 16S rRNA, cyt *b,* and D-loop for South African small mammals were mined from BOLD (Barcode of Life Database) and GenBank in August 2022. Scat metabarcoding often uses short-read, high-throughput sequencing technologies. For this reason, we also noted as present in the database sequences < 300bp.

2.4 Results

As of August 2022, small mammal records in South Africa totalled 4 774. Of the 193 small mammal species in South Africa, at least one or more of the DNA markers (COI, 12S rRNA, 16S rRNA, cyt b, and D-loop) are available for 136 species (71%). Out of 22 families analyzed, 20 had DNA sequence records for one or more regions examined. The Cistugonidae and Sciuridae families had no DNA sequences. Six families, Pepetidae, Hystricidae, Petromuridae, Thyronomidae, Hipposideridae, and Miniopteridae had 100 % of species represented by at least one gene region. The rest of the families had > 70% representation except for Nesomyidae (31%), Gliridae (40%), Emballonuridae (50%) and Nycteridae (67%). South African specimens had low records. Seven families (Gliridae, Hystricidae, Pepetidae, Petromuridae, Thyronomidae, Nycteridae, and Emballonuridae) had less than five records.

Table 2.1. Summary of sequence records (COI, 12S rRNA, 16S rRNA, cyt *b*, and D-loop) available for South African (SA) small mammal species. The percentage of species with records is calculated as the number of species with at least one or more of the DNA markers examined.

Order	Family	Species in SA	Species with records	% species with records	Total available records	Total SA records
Afrosoricida	Chrysochloridae	17	16	94	99	77
Eulipotyphla	Soricidae	16	14	88	15716	397
Lagomorpha	Leporidae	8	7	88	4664	144
Macroscelidae	Macroscelididae	8	7	88	723	321
	Muridae	41	30	73	44552	2079
	Bathyergidae	8	6	75	1322	953
	Nesomyidae	13	4	31	1665	52
	Gliridae	5	2	40	549	4
Rodentia	Hystricidae	1	1	100	328	4
	Sciuridae	6	0	0	8298	0
	Pepetidae	1	1	100	120	4
	Petromuridae	1	1	100	3	1
	Thyronimidae	1	1	100	161	3
Chiroptera	Molossidae	9	8	89	1598	17
	Cistugonidae	2	0	0	2	0

Hipposideridae	3	3	100	1698	159
Pteropodidae	4	3	75	3316	0
Vespertilionidae	25	17	68	11675	131
Miniopteridae	3	3	100	211	125
Nycteridae	3	2	67	61	5
Emballonuridae	2	1	50	731	3
Rhinolophidae	12	10	83	3457	293

Marker cyt *b* was available for 59% of small mammals, followed by the COI marker with 34% and the 12S rRNA with 24% coverage. The D-loop and 16S rRNA had the lowest species coverage, with only 18% and 15% of species having data for these gene regions, respectively. Of the 22 families, 19 had at least one species with COI sequence, while 18 had at least one species with cyt *b*. The 12S rRNA and 16S rRNA markers were less represented. Eight families had at least one species with 12S rRNA, while only five had species with 16S rRNA records (Table 2.2). Chrysochloridae, Macroscelididae, Muridae, and Thyronomidae were the only three families with DNA sequences for all five markers (Figure 2.1). Three of the families were represented by a single marker. Gliridae only had 12S rRNA data, Petromuridae only had cyt *b* data, and Pepetidae only had COI data.

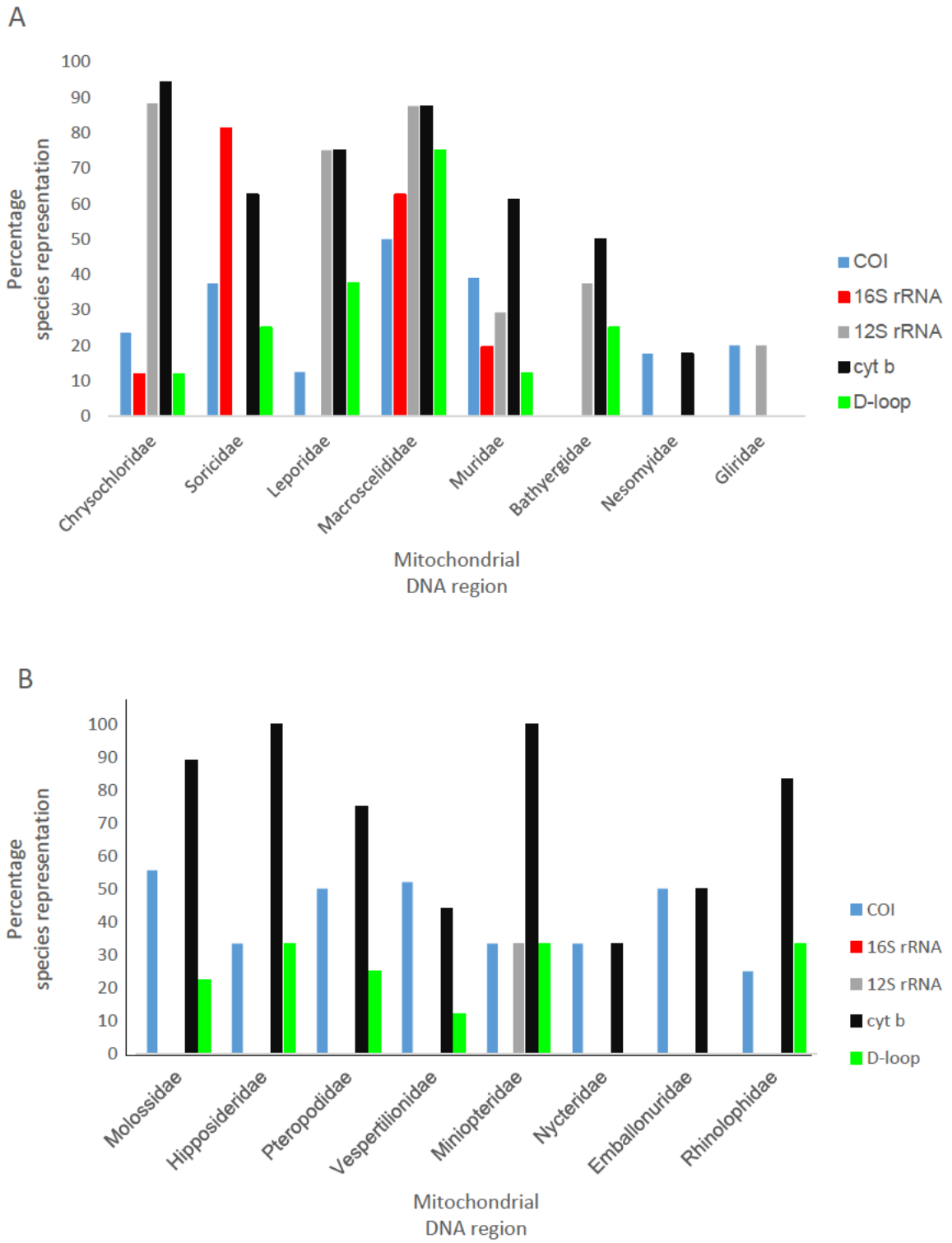

Figure 2.1. Percentage species for each small mammal family for which data is available for the five genes (COI, 12S rRNA, 16S rRNA, cyt *b*, and D-loop). Families have been ranked from highest species representation (A) to lowest species representation (B).

Table 2.2. Records per marker for each small mammal family found in South Africa.

Family	No. of species	16S rRNA		COI		D-loop		12S rRNA		Cyt *b*	
		Available records	Species with records	Available records	Species with records	Available records	Species with records	Available records	Species with records	Available records	Species with records
Chrysochloridae	17	2	2	4	4	2	2	16	15	32	16
Soricidae	16	37	13	15	6	40	4	0	0	15	10
Leporidae	8	0	0	1	1	155	3	16	6	14	6
Macroscelididae	8	10	5	7	4	306	6	9	7	285	7
Muridae	41	33	8	546	16	7	5	27	12	694	25
Bathyergidae	8	0	0	1	1	445	2	22	3	472	4
Nesomyidae	13	0	0	3	3	0	0	0	0	25	3
Gliridae	5	0	0	0	0	0	0	2	1	0	0
Hystricidae	1	0	0	1	1	0	0	0	0	2	1
Sciuridae	6	0	0	0	0	0	0	0	0	0	0
Pepetidae	1	0	0	1	1	0	0	0	0	0	0
Petromuridae	1	0	0	0	0	0	0	0	0	1	1
Thyronimidae	1	1	1	1	1	1	1	1	1	1	1
Molossidae	9	0	0	17	5	356	0	0	0	273	8
Cistugonidae	2	0	0	0	0	0	0	0	0	0	0
Hipposideridae	3	0	0	2	1	8	1	0	0	0	0
Pteropodidae	4	0	0	17	2	0	0	0	0	11	3
Vespertilionidae	25	0	0	89	13	45	3	0	0	207	11
Miniopteridae	3	0	0	11	1	31	1	3	1	140	3
Nycteridae	3	0	0	5	1	0	0	0	0	1	1
Emballonuridae	2	0	0	3	1	0	0	0	0	2	1
Rhinolophidae	12	0	0	8	3	229	4	0	0	204	10

2.5 Discussion

This study provides the first assessment of the completeness of South Africa's sequence-based reference libraries for small mammals. Previous studies have either assessed flora contribution (Bezeng *et al.* 2017), insect contribution (Myburgh *et al.* 2021), or fauna contribution as a whole (da Silva and Willows-Munro 2016). Our assessment shows that 73.1% of small mammal species in South Africa have sequence data for the primary genetic markers used in DNA barcoding, metabarcoding, and eDNA studies. This is an improvement to the barcode resources for South African mammals previously assessed in 2016 (da Silva and Willows-Munro 2016).

Our study, like others, shows that BOLD tends to grossly underestimate the level of biodiversity by the available DNA barcodes (Kim 2012). This is because COI sequences dominate BOLD, whereas GenBank extends to the other available genes and includes the option of whole genomes. This is the case with species such as *Elephantulus branhyrhynchus*, *Eremitalpa granti*, *Thyrynomys swinderus*, and *Chrysochloris asiatica* where individual sequences are unavailable. Instead, whole mitochondrial sequences are available on GenBank. So, in such cases where a mitochondrial gene is not available, it can be mined from the whole genome sequence. This can be particularly useful for the Chrysochloridae family, where the COI DNA sequences are unavailable for 13 known species. An important distinction between GenBank and BOLD is that GenBank is just a sequence repository, while BOLD is a curation tool that stores sequences. BOLD sequences include important information such as photographs, sampling locality, details of who collected and identified the specimen, and the primers used to generate the sequence data. Although many sequences are shared between the two databases, misidentifications and errors are more common in GenBank (Leray *et al.* 2019; Pentinsaari *et al.* 2020).

Availability of manpower and funds is thought to be the greatest cause of low records in countries with very high biodiversity, such as Brazil, Indonesia, and South Africa. Compared to Canada, China, Russia, and the USA, where many more records are available (Yang *et al.* 2020). It should, however, be noted that some specimens that were collected in developing countries were sent to the Centre for Biodiversity Genomics (CBG) in Canada for sequencing

because up until 2015, the CBG offered this service free of charge (da Silva and Willows-Munro 2016). This could be why Canada has contributed the most barcode records across almost all taxa. Despite this considerable support from the CBG for COI sequencing, our study has shown that cyt *b* has the most complete reference library for South African small mammals (59% of species). This is driven primarily by the popularity of this gene for phylogenetic and phylogeographic studies (Engelbrecht *et al.* 2011; Willows-Munro and Matthee 2011). Considerable effort is needed to increase the number of South African small mammal records. In particular, species belonging to *Elephantulus, Paraxerus, Petromus, and Xerus* as they are endemic to the southern African region (Kingdon *et al.* 2013). For some families, sequence data from a single specimen is available. This is insufficient to establish any genetic differences in species across different geographical locations. As well as possibly missing any cryptic species documented in many small mammal groups (Engelbrecht *et al.* 2011; Phukuntsi *et al.* 2016; Taylor *et al.* 2013). Initiatives such as the Foundational Biodiversity Information Programme (FBIP, https://fbip.co.za) – a joint initiative of the Department of Science and Innovation (DSI South Africa), the National Research Foundation (NRF South Africa) and the South African National Biodiversity Institute (SANBI) have been established to promote DNA barcoding efforts in South Africa.

This study emphasizes the utility of a multi-marker approach in future diet metabarcoding studies in South Africa. Moreover, using other genes, such as 12S rRNA and 16S rRNA, in addition to COI will allow for more accurate species assignment and will allow for the elucidation of phylogenetic relationships at higher taxonomic levels.

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2.7 Supplementary data

Table S2.1 List of all small mammals used in this study.

Order	Family	Genus	Species	No. of records	Locality	Voucher number
Rodentia	Nesomyidae	Cricetomys	Cricetomys ansorgei	0		
		Dendromus	Dendromus melanotis	0		
			Dendromus mesomelas	0		
			Dendromus mystacalis	2	KwaZulu Natal	DM_11727
			Dendromus nyikae	0		
		Malaconthrix	Malacothrix typica	0		
		Mystromys	Mystromys albicaudatus	0		
		Petromyscus	Petromyscus barbouri	0		
			Petromyscus collinus	0		
			Petromyscus monticularis	0		
		Saccostomus	Saccostomus campestris	24	KwaZulu Natal	DM_11688
		Steatomys	Steatomys krebsii	1	South Africa	KY754158.1
			Steatomys pratensis	1	KwaZulu Natal	DM_11713
	Muridae	Acomys	Acomys spinosissimus	1	South Africa	Z96068.1
			Acomys subspinosus	2	South Africa	FJ415486.1

Aethomys	Aethomys chrysophilus	13	KwaZulu Natal	VS_M3
		31	South Africa	AY585676.1
	Aethomys ineptus			
Dasymys	Dasymys capensis	0		
		0		
	Dasymys incomtus			
		0		
	Dasymys robertsii			
Desmodillus	Desmodillus auricularis	7	Northern Cape	VS_KM3
		4	South Africa	MW537415.1
Gerbilliscus	Gerbilliscus afra			
		23	South Africa	MW537448.1
	Gerbilliscus brantsii			
		16	Northern Cape	VS_KM2
	Gerbilliscus leucogaster			
		2	South Africa	MW537553.1
	Gerbilliscus paeba			
		0		
	Gerbilliscus vallinus			
		4	South Africa	
Grammomys	Grammomys cometes			
		2	South Africa	EU275253.1
	Grammomys dolichurus			
		14	KwaZulu Natal	Ash_D
Lemniscomys	Lemniscomys rosalia			
			KwaZulu Natal	DM_11914
			Gauteng	KJ466161
		79	North West	KJ466167
Mastomys	Mastomys coucha			
			KwaZulu Natal	DM_11914
			KwaZulu Natal	KJ466183
		88	East London	KJ466191
	Mastomys natalensis			

			Mpumalanga	ThgMas
Micaelamys	Micaelamys granti	5	South Africa	MN226666.1
	Micaelamys namaquensis	304	South Africa	MT424228
			South Africa	HG934978.1
Mus	Mus indutus	25		
	Mus minutoides	96	KwaZulu Natal	VS_11693
	Mus neavei	0		
	Mus orangiae	0		
Myomyscus	Myomyscus verreauxii	12	South Africa	MNHN_VV1999102
Otomys	Otomys auratus	0		
	Otomys irroratus	139	KwaZulu Natal	VS_1001
	Otomys karoensis	0		
	Otomys laminatus	4	South Africa	AF492722.1
	Otomys sloggetti	3	South Africa	AF492750.1
	Otomys unisulcatus	1	South Africa	AF492755.1
Parotomys	Parotomys brantsii	15	South Africa	AF492731.1
	Parotomys littledalei	18	South Africa	AF492732.1
Rhabdomys	Rhabdomys bechuanae	40	South Africa	MT093553.1
	Rhabdomys dilectus	116	South Africa	FR837654.1
	Rhabdomys intermedius	1	South Africa	MT093550

				KwaZulu Natal	VS_1004
		Rhabdomys pumilio	273		MT093529
				South Africa	MT093550
	Thallomys	Thallomys nigricauda	16		
		Thallomys paedulcus	0		
		Thallomys shortridgei	0		
	Zelotomys	Zelotomys woosnami	0		
Bathyergidae	Bathyergus	Bathyergus janetta	3	South Africa	MH186533.1
		Bathyergus suillus	369	South Africa	KJ866865.1
	Cryptomys	Cryptomys hottentotus	28	South Africa	MH186560.1
		Cryptomys mahali	0		
		Cryptomys natalensis	6	South Africa	AY427064.1
		Cryptomys pretoriae	0		
	Fukomys	Fukomys damarensis	1	KwaZulu Natal	VS_M17
	Georuchus	Georychus capensis	533	South Africa	MG496925.1
Gliridae	Graphiurus	Graphiurus microtis	0		
		Graphiurus murinus	0		
		Graphiurus ocularis	1	South Africa	AJ536355.1
		Graphiurus platyops	1	South Africa	AJ536354.1

Lagomorpha	Sciuridae	Paraxerus	Graphiurus rupicola	0		
			Paraxerus cepapi	0		
			Paraxerus palliatus	0		
			Paraxerus palliatus ornatus	0		
			Paraxerus palliatus tongensis	0		
		Xerus	Xerus inauris	0		
			Xerus princeps	0		
	Pedetidae	Pedetes	Pedetes capensis	1	South Africa	HE983623
	Thryonomyidae	Thryonomys	Thryonomys swinderianus	5	South Africa	AJ301644
	Petromuridae	Petromus	Petromus typicus	1	South Africa	MH186591.1
	Hystricidae	Hystrix	Hystrix africaeaustralis	3	South Africa	MH186593.1
	Leporidae	Bunolagus	Bunolagus monticularis	72	South Africa	MZ871439.1
		Lepus	Lepus capensis	32	South Africa	AY292732.1
			Lepus saxatilis	58	South Africa	AY292732.1
			Lepus victoriae	0		
		Pronolagus	Pronolagus crassicaudatus	5	South Africa	U31034.1
			Pronolagus randensis	6	Limpopo	U31038.1
					Northern Cape	U31042.1
					Free State	U31039.1
			Pronolagus rupestris	10	Mpumalanga	U31040.1

Afrosoricida	Chrysochloridae	Amblysomus	Pronolagus saundersiae	2	South Africa	AY292736.1
			Amblysomus corriae	3	South Africa	KM388913.1
			Amblysomus hottentotus	16	KwaZulu Natal	VS_M31
			Amblysomus marleyi	3	South Africa	KM388915.1
			Amblysomus robustus	3	South Africa	KM388916.1
			Amblysomus septentrionalis	3	South Africa	KM388917.1
		Calcochloris	Calcochloris obtusirostris	3	South Africa	KM388926.1
		Chlorotalpa	Chlorotalpa duthieae	2	South Africa	KM388921.1
			Chlorotalpa sclateri	2	South Africa	KM388922.1
		Chrysochloris	Chrysochloris asiatica	5	Cape Town	AJ428944
			Chrysochloris visagiei	0		
		Chrysosplax	Chrysospalax trevelyani	2	South Africa	KM388927.1
			Chrysospalax villosus	2	South Africa	KM388928.1
		Crypochloris	Cryptochloris wintoni	1	South Africa	KM388925.1
			Cryptochloris zyli	2	South Africa	KM388924.1
		Eremitalpa	Eremitalpa granti granti	5	South Africa	AM904729
		Neamblysomus	Neamblysomus gunningi	2	South Africa	KM388919.1
			Neamblysomus julianae	4	Gauteng South AFrica	DNMNH M-046707
Eulipotyphla	Soricidae	Crocidura	Crocidura cyanea	14	KwaZulu Natal	Ash_C

			Crocidura flavescens	5	South Africa	FJ486861.1
			Crocidura fuscomurina	4	South Africa	FJ486865.1
			Crocidura hirta	12	KwaZulu Natal	VS_11689
			Crocidura maquassiensis	1	South Africa	FJ486888.1
			Crocidura mariquensis	5	South Africa	FJ486892.1
			Crocidura silacea	2	South Africa	FJ486921.1
		Mysorex	Myosorex cafer	10	KwaZulu Natal	VS_1002
			Myosorex cf. tenuis	0		
			Myosorex longicaudatus	3	South Africa	FJ486976.1
			Myosorex longicaudatus boosmani	0		
			Myosorex sclateri	8	South Africa	KC505658.1
			Myosorex varius	35	KwaZulu Natal	VS_1006
		Suncus	Suncus infinitesimus	3	South Africa	FJ486946.1
			Suncus lixus	1	KwaZulu Natal	VS_11724
			Suncus varilla	2	South Africa	FJ486955.1
Macroscelidea	Macroscelididae	Elephantulus	Elephantulus brachyrhynchus	12	Mpumalanga	DNMNH M-046474
			Elephantulus edwardii	255	South Africa	JH947130.1
			Elephantulus intufi	13	South Africa	EU136162.1
			Elephantulus myurus	18	Limpopo	NZG ES1021

					Gauteng	DNMNH M-046419
					KwaZulu Natal	VS_M6
Chiroptera	Molossidae	Macroscelides	Elephantulus pilicaudus	0		
			Elephantulus rupestris	169	South Africa	EF141695.1
			Macroscelides proboscideus	142	South Africa	MG949278.1
					South Africa	DQ901190.1
		Petrodromus	Petrodromus tetradactylus	5		
		Chaerephon	Chaerephon ansorgei	1	South Africa	Y377967.1
			Chaerephon pumilus	455	South Africa	MF947529
		Mops	Mops condylurus	8	South Africa	HM802913.1
			Mops midas	4	South Africa	HM802916.1
		Otomops	Otomops martiensseni	169	South Africa	EF216425.1
		Sauromys	Sauromys petrophilus	2	South Africa	HM802931.1
		Tadarida	Tadarida aegyptiaca	5	South Africa	HM802930.1
			Tadarida fulminans	2	South Africa	HQ384486.1
			Tadarida ventralis	0		
	Cistugonidae	Cistugo	Cistugo lesueuri	0		
			Cistugo seabrae	0		
	Hipposideridae	Cloeotis	Cloeotis percivali	1	South Africa	FJ185181.1
		Hipposideros	Hipposideros caffer	11	Eastern Cape	LRR131017HNR_HCA1

		Hipposideros vittatus	18	South Africa	KT371778.1
Pteropodidae	Eidolon	Eidolon helvum	0	South Africa	MT441517.1
	Epomophorus	Epomophorus crypturus	3		
		Epomophorus wahlbergi	17	Eastern Cape	LRR310817MBSF_EW2
	Rousettus	Rousettus aegyptiacus	18	South Africa	MK982175.1
Vespertilionidae	Eptesicus	Eptesicus hottentotus	3	South Africa	KF452622.1
	Glauconycteris	Glauconycteris variegata	0		
	Hypsugo	Hypsugo anchietae	0		
	Kerivoula	Kerivoula argentata	0		
		Kerivoula lanosa	1	Eastern Cape	DM14997
	Laephotis	Laephotis botswanae	20	Eastern Cape	MM011217GSF_LBO1
		Laephotis namibensis	0		
		Laephotis wintoni	3	South Africa	EU797446.1
	Myotis	Myotis bocagii	0		
		Myotis tricolor	63	Eastern Cape	LRR131017HNR_MTR3
		Myotis welwitschii	0	Eastern Cape	LRR041017MzBH_NC1
				South Africa	MF947526
	Neoromicia	Neoromicia capensis	49	Eastern Cape	VS_BB3

			Neoromicia cf. melckorum	0		
				1	South Africa	JX508829.1
			Neoromicia nana	1	South Africa	JX508832.1
			Neoromicia rendalli	16	South Africa	KX375186.1
			Neoromicia zuluensis	7	KwaZulu Natal	VS_15083
		Nycticeinops	Nycticeinops schlieffeni	76	Eastern Cape	DM14988
		Pipistrellus	Pipistrellus hesperidus	1	South Africa	KF452648.1
			Pipistrellus rueppellii	17	South Africa	ON402513.1
			Pipistrellus rusticus	0		
		Scotoecus	Scotoecus albofuscus		Eastern Cape	LRR111017HNR_SDI1
		Scotophilus	Scotophilus dinganii	61	South Africa	MF947528
			Scotophilus leucogaster	6	South Africa	KF452703.1
			Scotophilus nigrita	2	South Africa	KF305857.1
			Scotophilus viridis	14	South Africa	AY754074.1
					Eastern Cape	DM14999
	Miniopteridae	Miniopterus	Miniopterus fraterculus	84		
			Miniopterus inflatus	1	South Africa	AY614737.1
			Miniopterus natalensis	100	Eastern Cape	DM15003
	Nycteridae	Nycteris	Nycteris hispida	1	South Africa	MK837138.1

		Nycteris thebaica	5	South Africa	KF452654.1
		Nycteris woodi	0		
Rhinolophidae	Rhinolophus	Rhinolophus blasii	4	South Africa	FJ185188.1
		Rhinolophus capensis	51	Eastern Cape	DM14998
		Rhinolophus clivosus	183	Eastern Cape	DM14993
		Rhinolophus cohenae	0		
		Rhinolophus damarensis	32	South Africa	KU531293.1
		Rhinolophus darlingi	52	South Africa	KX548029.1
		Rhinolophus denti	15	South Africa	KX548038.1
		Rhinolophus fumigatus	2	South Africa	FJ185197.1
		Rhinolophus landeri	13	South Africa	MG980681.1
		Rhinolophus simulator	9		FJ185212.1
		Rhinolophus smithersi	0		
		Rhinolophus swinnyi	80	Eastern Cape	MM011217GSF_RCL1
Emballonuridae	Taphozous	Taphozous mauritianus	5		KF452718.1
		Taphozous perforatus	0		

CHAPTER 3

In silico validation of metabarcoding primers for species-level identification of South African small mammals

3.1 Abstract

DNA metabarcoding has been increasingly applied across various studies for species identification. Unlike traditional Sanger sequencing, DNA metabarcoding allows for simultaneous rapid species identification of bulk samples. The success of metabarcoding relies on primer choice and the ability of the chosen markers to amplify the target loci. Amplicons should be less that 400 base pairs (bp) in length to be amplifiable in samples that contain degraded DNA (such as scats) and to exploit high-throughput sequencing platforms such as Illumina, but be variable enough to accurately delimit the targeted species. This study aimed to evaluate the utility of previously published metabarcoding primers to identify a taxonomic rage of small mammals that are routine prey for small to medium carnivores. Based on Jeffries-Matusita (J-M) values and barcode gap analysis, the best markers for delineation of small mammals are the 12S rRNA, 16S rRNA, COI, and cyt *b*.

Keywords: primers, environmental DNA, high-throughput sequencing, DNA barcoding, Barcode gap analysis, J-M analysis.

3.2 Introduction

PCR amplification of small regions of primarily mitochondrial genes, combined with high-throughput sequencing technologies, referred to as DNA metabarcoding, has become an increasingly popular method for rapid species identification and delineation (Taberlet *et al.* 2012). These advances enable rapid cataloging of biodiversity (Bohmann *et al.* 2014; Cristescu 2014) and allow for monitoring of species richness at an ecosystem level (Compson. *et al.* 2020). DNA metabarcoding is also the preferred choice when working with bulk samples such as environmental DNA (eDNA) samples, which could contain the DNA of many organisms. The

results of such analyses can be used for a wide range of studies, including phylogenetics (Elbrecht *et al.* 2018b; Turon *et al.* 2020), diet analysis through the use of scats (Monterroso *et al.* 2019; Shehzad *et al.* 2012), wildlife forensics (Staats *et al.* 2016), clinical forensics (Karlsson and Holmlund 2007), and bioassessments of freshwater samples (Elbrecht and Leese 2017).

DNA barcoding in animals relies heavily on a 658bp portion of the cytochrome oxidase I (COI) mitochondrial DNA gene (Hebert *et al.* 2003). Though widely used for animal barcoding, the COI gene is considered by some authors (Deagle *et al.* 2014; Ficetola *et al.* 2010; Vences *et al.* 2005) unsuitable for metabarcoding. This is because the COI a protein-coding gene which lacks sufficient conserved regions for the design of metabarcoding primers across different taxa (Deagle *et al.* 2014; Ficetola *et al.* 2010). The COI gene region is not conserved across certain taxonomic groups (Creer *et al.* 2011; Derycke *et al.* 2010; Hoareau and Boissin 2010; Meyer 2003; Tautz *et al.* 2003). In some studies, the primers used to amplify the COI gene region have been shown to have mismatches (Ficetola *et al.* 2010) which in turn result in unpredictable often lower amplification rates (Deagle *et al.* 2014; Geller *et al.* 2013). In metabarcoding, minimalist barcodes or mini-barcodes are used to improve amplification success (Govendor *et al.* 2019; Hajibabaei *et al.* 2006) and facilitate sequencing using short-read technology. The most common sequencing strategy for metabarcoding is short-read technology, which requires amplicons ideally less than 400 base pairs (bp) in length (Engelbrektson *et al.* 2010; Huber *et al.* 2009). This is particularly useful in cases where DNA may be degraded; as is the case with most eDNA associated with scat samples collected in the field for diet assessments (Deagle *et al.* 2006; Pompanon *et al.* 2012; Shehzad *et al.* 2012).

In order to improve species resolution, there has been a growing call for metabarcoding studies to use a multi-gene approach (Ritter *et al.* 2019; Silva *et al.* 2019; Zhang *et al.* 2020b). Especially in cases where it has been shown that “universal” primers do not work equally across all taxa expected in the sample (Sharma and Kobayashi 2014). Even if primers amplify successfully, different DNA regions have been shown to have varying performances in some taxa (Ficetola *et al.* 2010). Therefore, the use of multiple markers can improve species identification (Hollingsworth *et al.* 2009; Kress *et al.* 2005). For example, COI metabarcoding primers tend to identify 80-90% or even less of the available species due to primer bias (Brandon-Mong *et al.* 2015; Leray *et al.* 2013; Sikder *et al.* 2020). A number of mitochondrial

rRNA genes have been shown to provide taxonomic resolution similar to the COI region while allowing for the design of conserved primers (Ficetola *et al.* 2010; Klindworth *et al.* 2013).

Selection of metabarcoding primers is usually dependent on 1) ability to amplify a short region of DNA because of the degraded state of eDNA (Bylemans *et al.* 2018); 2) amplify a DNA region that is robust in species resolution (Clarke *et al.* 2017); 3) amplify the specific targeted taxonomic group in order to avoid amplification of non-target taxa (Coissac *et al.* 2012); and 4) amplify all or most of the targeted species equally well (Elbrecht and Leese 2017). It is also essential that markers are tested beforehand to confirm suitability for the chosen application (Bylemans *et al.* 2018; Klindworth *et al.* 2013) and selection of the best markers for the taxon to be identified (Carradec *et al.* 2018). Some studies have evaluated available DNA metabarcode markers to ascertain their suitability for a specific study. Ranging from metabarcoding studies for analysis of soil nematodes (Sikder *et al.* 2020), zooplankton (Meredith *et al.* 2021), insects (Brandon-Mong *et al.* 2015) to the most common, analyzing the biodiversity of fresh water fish (Bylemans *et al.* 2018; Elbrecht and Leese 2017; Kumar *et al.* 2022; Zhang *et al.* 2020a).

One way to determine the ability of a DNA marker to accurately delimit species is to determine the presence of a "DNA barcode gap" (Čandek and Kuntner 2015; Jinbo *et al.* 2011; Meyer and Paulay 2005). The barcode gap is defined as the separation between interspecific variation and intraspecific genetic distance (Čandek and Kuntner 2015; Meyer and Paulay 2005; Puillandre *et al.* 2012; Wiemers and Fiedler 2007), such that the interspecific genetic divergence is greater than that of the intraspecific genetic variation (Barrett and Hebert 2005; Chapple and Ritchie 2013; Luo *et al.* 2011; Wiemers and Fiedler 2007). Examples of a barcode gap have been observed when using the 16S rRNA, 18S rRNA and COI markers to delineate phytoplankton species in Mediterranean aquatic ecosystems (Tzafesta *et al*. 2022). As well as in spider species with different morphologies and from different geographical locations (Čandek and Kuntner 2015). When the intraspecific and interspecific genetic distances overlap, the DNA barcode gap is no longer present, and this means the markers used cannot reliably distinguish species (Chapple and Ritchie 2013; Jinbo *et al.* 2011; Meyer and Paulay 2005; Puillandre *et al.* 2012). In some cases the barcode gap is absent. This can be caused by high levels of genetic diversity below the species level (Chapple and Ritchie 2013; Jinbo *et al.*

2011), or closely related species can often cause an absent barcode gap (Chapple and Ritchie 2013; Jinbo *et al.* 2011; Wiemers and Fiedler 2007).

Species biodiversity has been shown to vary between different geographic regions (Abell *et al.* 2008; Olson *et al.* 2001; Spalding *et al.* 2007). Therefore, marker performance may vary across species from different geographic locations (Bylemans *et al.* 2018) or ecosystems (Zeale *et al.* 2011). Some primers are designed to target specific taxonomic groups and may not be intended for use beyond that taxonomic group (Clarke *et al.* 2014; Van Houdt *et al.* 2010). The availability of DNA reference sequences can also affect the performance of an *in silico* approach to assess marker performance (Elbrecht and Leese 2017). Incomplete reference libraries are a liability for eDNA metabarcoding studies, especially in ecoregions that have many endemic species (Bylemans *et al.* 2018) or have incomplete reference libraries such as South Africa (da Silva and Willows-Munro 2016). To date, no studies have assessed whether the currently available metabarcoding markers would be sufficient for species identification and delineation of South African small mammals. This study aimed to analyze available metabarcoding markers for the suitability of species identification and delimitation of South African small mammals by assessing the presence or absence of the DNA barcode gap when each of these markers was tested.

3.3 Materials and methods

3.3.1 Literature review and screening of metabarcoding primers

A literature search was done for available metabarcoding primers. Using the search terms “12S rRNA”, “16S rRNA”, “COI”, “cyt *b*”, “metabarcoding”, “mini-barcodes”, “eDNA” using the Google search engine. The search also included metabarcoding studies of diet assessments of carnivores in order to assess which markers were used for each study. The search was restricted to primers specifically designed for mammals or previously tested for use in mammals. The most common mitochondrial regions used were identified. Genetic databases were constructed by batch downloading 12S rRNA, 16S rRNA, COI, and cyt *b* sequences from the National Centre for Biotechnology Information (NCBI) GenBank portal, and Barcode of Life Database [accessed August 2022]. Sequences were downloaded for each gene region for all the available small mammal families in South Africa. In instances where no barcodes were

found for South African small mammals, international barcodes were used. In order to test the performance of each primer on South African small mammal species, additional de novo sequences were generated to complement the gaps already present in DNA databases.

3.3.2 Creating reference library for South African small mammals

The small mammal list used in this study was compiled from volumes III and IV of Mammals of Africa (Kingdon *et al.* 2013) and was cross- referenced with the Red List of Mammals of South Africa, Lesotho and Swaziland (www.ewt.org.za). Small mammals from the orders Afrosoricida, Chiroptera, Eulipotyphla, Lagomorpha, Macroscelidae, and Rodentia were included in this study (Appendix S1).

Using the binomial names, publicly available DNA records and collection localities for five mitochondrial regions COI, 12S rRNA. 16S rRNA, cyt *b* and D-loop for South African small mammals were mined from BOLD (Barcode of Life Database) and GenBank in August 2022.

Additional sequence data was generated for small mammal taxa of 46 species from 6 orders, and 12 families. Tissues samples were available in the Conservation Genetics Lab Biobank. These samples were collected by past students of the Willows-Munro lab and also included samples contributed by the University of KwaZulu Natal animal house.

Genomic DNA extraction of the tissue sampling and the subsequent PCR amplification of the various barcoding regions namely 16S rRNA using the 16SA and 16SB primers (Palumbi *et al.* 1991), COI region using either the VF1 and VR1 primers (Ivanova *et al*. 2006) and the LCO1490 and HCO2198 primers (Hebert *et al.* 2003), cyt *b* using the H15915 and L14724 primers (Irwin *et al.* 1991) and D-loop using the DLH1 and N777 primers (Aplers *et al*. 2004; Hoelzel *et al*. 1991). DNA from tissue samples was extracted using the E.Z.N.A® Tissue DNA kit (Omega BioTek, Georgia, USA) following the standard protocol. The PCR reactions were made up of 5µl of 5x MyTaq reaction buffer (Bioline, USA), 0.5µl of 200nM of each primer, 0.5µl of MyTaq DNA polymerase (Bioline, USA), 17.5µl distilled autoclaved water and 1µl of 30ng/µlDNA, making up a total volume of 25µl. The PCR cycling conditions were as follows: 95°C for 10 mins, followed by 40 cycles of 94°C for 30 secs, 56°C for 30 secs and 72°C for 45 secs, then finally at 72°C for 10 mins. PCR clean-up and sequencing reactions were performed at the Central Analytical Facility (CAF) at the University of Stellenbosch, South Africa. All sequences

were checked for species specificity using the nucleotide BLAST tool (BLASTn) on NCBI GenBank. Percentage identification between 97 - 100% (Edgar 2010) was used to confirm the exact species match.

3.3.3 Data analyses

DNA sequences were assembled and edited in Mega and aligned using Clustal X2.1 (Larkin *et al.* 2007) with an open gap penalty of 10.0 and an extend gap penalty of 5.0. The selected primers were aligned to the DNA sequences and the alignments were trimmed to the correct amplicon length. Intra- and interspecific genetic distances were calculated for each amplicon using the K2P nucleotide substitution model in MEGA 6.0 and plotted. The K2P model of sequence evolution (Kimura 1980) was used as this is the method implemented in BOLD. The maximum intraspecific distance was subtracted from the minimum interspecific distance to determine the barcoding gap (Meier *et al.* 2006), and the Jeffries-Matusita distance (J-M) statistic was used to test whether the intra- and interspecific distances were statistically separable. The J-M statistic takes into consideration the means of the intra- and interspecific genetic distances and the distribution of values from the mean and measures the separability between the two probability distributions (Dabboor *et al.* 2014). The J-M distance cut off value is 1.414. A value of 1.414 or greater suggests that inter and intra-specific genetic distances are statistically separable (Trigg and Flasse 2001).

3.4 Results

3.4.1 Literature review and screening of metabarcoding primers

A total of 8 metabarcoding primer pairs were obtained from the literature. These included two sets of 12S rRNA primers (Karlsson and Holmlund 2007; Riaz *et al.* 2011), two sets of 16S rRNA primers (Horreo *et al.* 2012; Karlsson and Holmlund 2007; Taylor 1996), COI primer pairs (Geller *et al.* 2013; Leray *et al.* 2013; Meusnier *et al.* 2008), and one cyt *b* primer pair (Galan *et al.* 2012) as shown in Table 3.1.

Table 3.1. Primer pairs used for the *in silico* analyses of metabarcoding primers that can be used for species delineation of small South African mammals.

Locus	Taxonomic group	Primer name	Primer sequence (5' - 3')	Amplicon length (bp)	J-M Analysis	Reference
COI	Universal animal	Uni-MinibarR1 Uni-MinibarF1	GAAAATCATAATGAAGGCATGAGC TCCACTAATCACAARGATATTGGTAC	130	-	Meusnier *et al*. 2008
COI	Universal animal	illmlCOIintF jgHCO2198	GGWACWGGWTGAACWGTWTAYCCYCC TAIACYTCIGGRTGICCRAARAAYCA	330	**1.933603**	Geller *et al.* 2013 Leray *et al.* 2013
16S	Universal animal	16S-HF 16S-HR1	ATAACACGAGAAGACCCT CCCACGGTCGCCCCAAC CCCGCGGTCGCCCCAAC	80-125	**1.334814**	Horreo *et al*. 2012
12S	Universal vertebrate	12SV5 12SV5B2	TTAGATACCCCACTATGC TAGAACAGGCTCCTCTAG	98	**1.68431**	Riaz *et al*. 2011
Cytb	Universal rodent	L15411F H15546R	GAYAAARTYCCVTTYCAYCC AARTAYCAYTCDGGYTTRAT	136	**1.23153**	Galan *et al*. 2012
16S	Mammal and ancient DNA	16Smamm1 16Smamm2	CGGTTGGGGTGACCTCGGA GCTGTTATCCCTAGGGTAACT	140	**1.621692**	Taylor 1996
12S	Mammal	Partial 12S rRNA	CCCCACGGGAAACAGCAGT CGCGGTGGCTGGCACGAAAT	111	**1.342145**	Karlsson and Holmlund 2007
16S	Mammal	Partial 16S rRNA	GACGAGAAGACCCTATGGAGC TCCGAGGTCGCCCCAACC	98	**1.464031**	Karlsson and Holmlund 2007

3.4.2 Creating reference library for South African small mammals

DNA sequences were downloaded from BOLD and GenBank for the 12S rRNA, 16S rRNA, COI, and cyt *b*. Figure 3.1 shows gel pictures of the amplification of some of these DNA sequences. The available number of sequences per gene region are indicated in Figure 3.3.

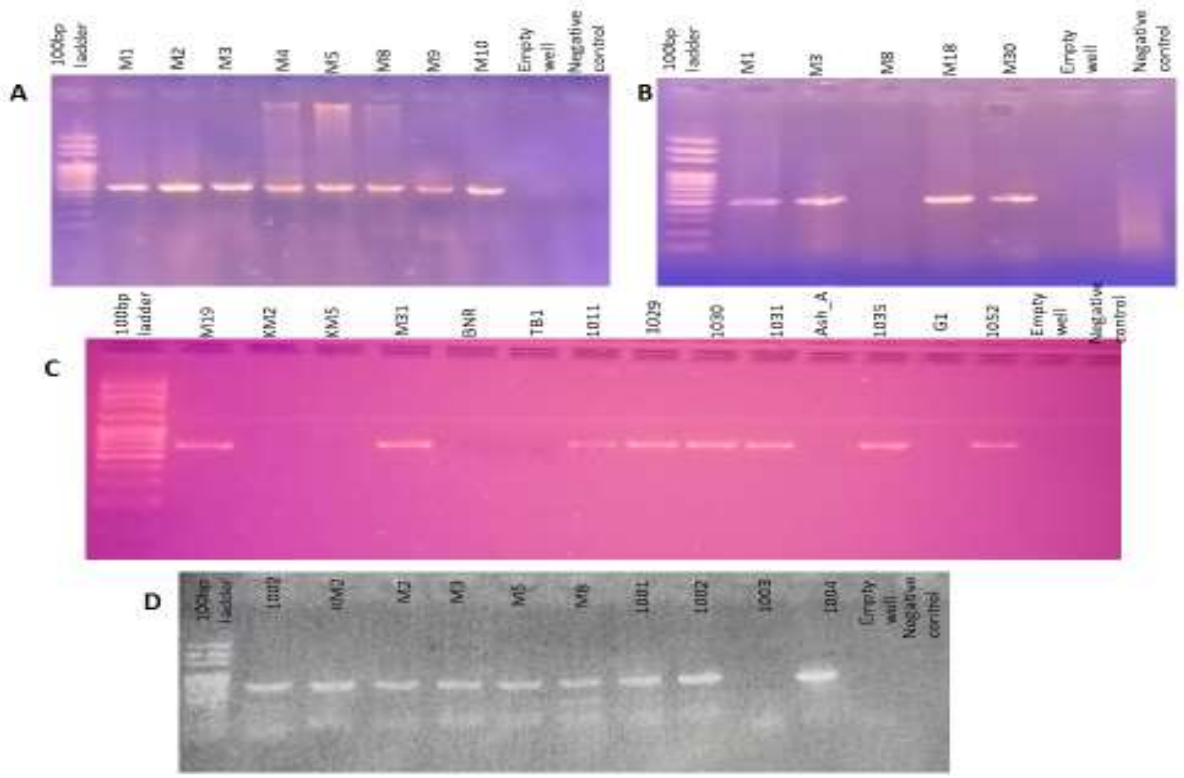


Figure 3.1. Agarose gel (2%) electrophoresis pictures showing amplification of various DNA regions. The 16S rRNA (A), D-loop (B), COI (C) and cyt *b* (D) regions.

Additional DNA sequences from 46 species generated from amplification of the 16S rRNA, COI, D-loop and cyt b were also added in order to generate sequence alignments and the specimen data was deposited into Barcode of Life Database (BOLD) (Table 3.2).

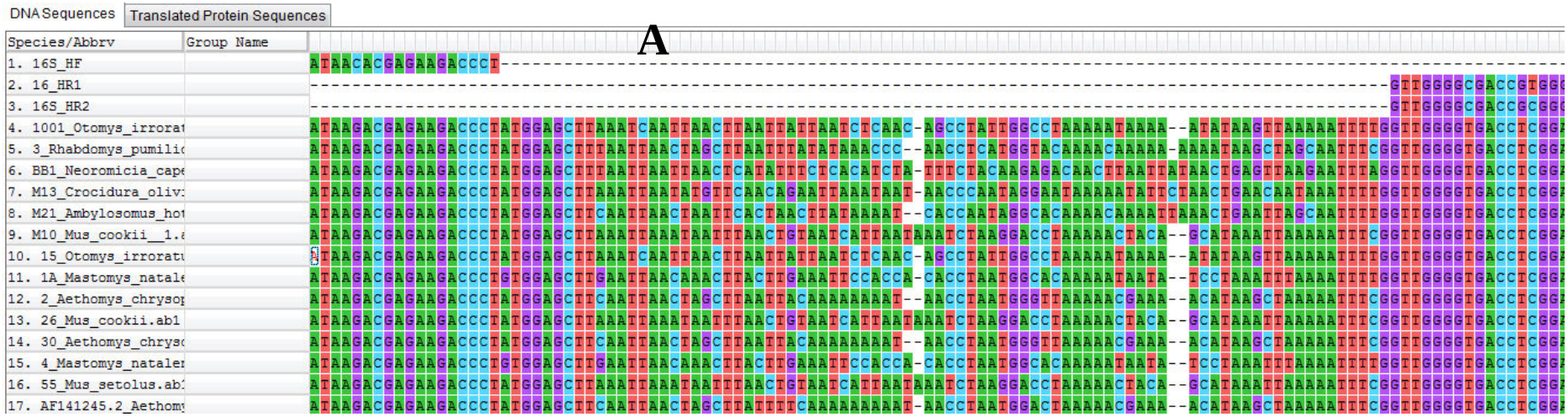
Table 3.2. List of small mammals generated for sequence alignments and the BOLD voucher numbers used for sequence alignments to test the efficacy of available metabarcoding primers.

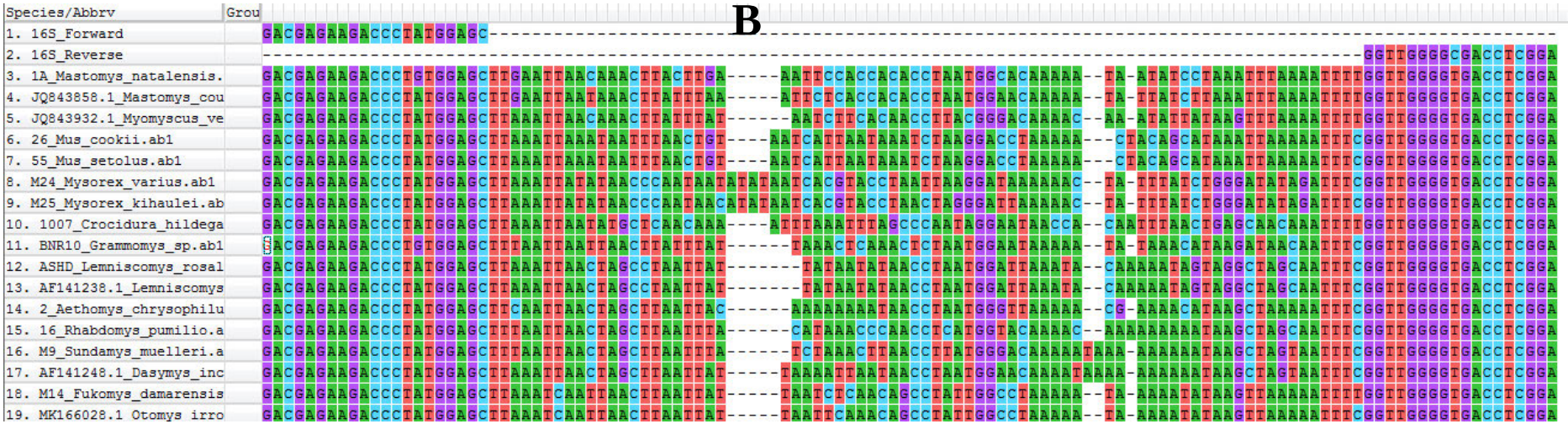
Order	Family	Species	Common name	BOLD voucher number
Afrosoricida	Tenrecidae	*Echinops telfairi*	Lesser hedgehog tenrec	UKZN004-20
	Chrysochloridae	*Amblysomus hottentotus*	Hottentot golden mole	UKZN021-20, UKZN030-20
Carnivora	Canidae	*Vulpes charma*	Cape fox	KALNC007-20
	Felidae	*Felis nigripes*	black-footed cat	BFC001-21 - BFC008-21
Chiroptera	Vespertilionidae	*Neoromicia capensis*	Cape serotine bat	KALNC009-20 - KALNC015-20
		Nycticeinops schliefferii	Schlieffen's twilight bat	DMKZN007-20
Eulipotyphla	Soricidae	*Crocidura oliveri*	African giant shrew	UKZN013-20
		Myosorex varius	Forest shrew	BBNR011-21, BBTK003-20, BBTK005-20, BBTK007-20 - BBTK009-20, BBTK021-20, BBTK022-20, BBTK021-20 - BBTK026-20, UKZN023-20, UKZN024-20, UKZN026-20
		Mysorex howelli	Howell's forest shrew	UKZN022-20
		Crocidura cynae	reddish-gray musk shrew	UKZN020-20
		Crocidura hildgardea	Hildegarde's shrew	BBTK006-20
		Crocidura hirta	lesser red musk shrew	DMKZN002-20
		Crocidura flavescens	Greater red musk shrew	BBNR002-21
		Crocidura olivieri	African giant shrew	UKZN013-20, UKZN018-20, UKZN029-20, UKZN031-20
		Crocidura shantungensis	Asian lesser white-toothed shrew	UKZN028-20
		Suncus lixus	greater dwarf shrew	DMKZN005-20
		Mysorex cafer	dark-footed mouse shrew	BBTK010-20, BBTK011-20, BBTK017-20
Macroscelidea	Macroscelididae	*Mysorex kihaulei*	Kihaule's mouse shrew	UKZN025-20
Rodentia	Bathyergidae	*Elephantalus edwardii*	Cape elephant shrew	UKZN006-20, UKZN007-20

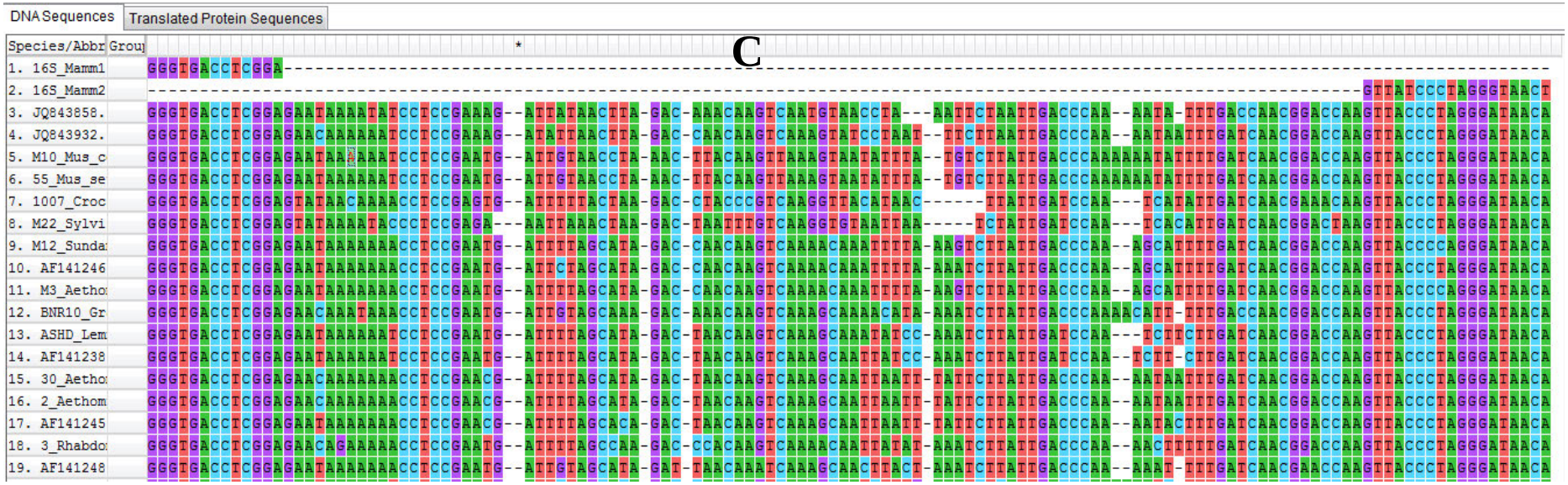
	Crptomys hottentotus	African mole-rat	UKZN017-20
Cricetidae	*Phodopus sungorus*	white dwarf hamster	UKZN001-20
	Neotoma magister	Allegheny woodrat	UKZN019-20
	Neotoma cinera	bushy-tailed woodrat	UKZN002-20, UKZN034-20
Caviidae	*Cavia porcellus*	guinea pig	UKZN002-20
Muridae	*Otomys irroratus*	southern African vlei rat	BBNR013-21, BBTK001-20, UKZN014-20, UKZN027-20, UKZN032-20
	Mastomys natalensis	Natal multimammate mouse	BBNR003-21 - BBNR005-21, BBNR008-21, DMKZN006-20, UKZN005-20, UKZN008-20
	Sundamys muelleri	Müller's giant Sunda rat	UKZN009-20, UKZN011-20, UKZN012-20
	Mus cookii	Cook's mouse	UKZN016-20
	Gerbilliscus leucogaster	bushveld gerbil	KALNC001-20
	Desmodillus auricularis	Cape short-eared gerbil	KALNC002-20, KALNC003-20, KALNC005-20, KALNC006-20, KALNC008-20
	Rhadbomys pumilio	four-striped grass mouse	BBTK004-20
	Rhadbomys dilectus	mesic four-striped grass rat	BBNR012-21, BBNR014-21
	Mus setulosus	Peters's mouse	BBTK010-20
	Aethomys chrysophilus	red veld rat	BBNR009-21
	Aethomys namaquensis	Namaqua rock rat	UKZN003-20
	Lemniscomys rosalia	single-striped grass mouse	BBNR006-21, UKZN035-20
	Mus munitoides	African pygmy mouse	BBNR007-21, BBNR010-21, DMKZN003-20
	Rodentia spp.	-	UKZN010-20,
	Rattus norvegicus	common rat	UKZN033-20
	Grammomys dolichurus	Woodland thicket rat	BBNR001-21
	Grammomys spp.	-	UKZN003-20
	Aethomys spp.	-	BBNR009-21
	Hydromys chrysogaster	water-rat	KALNC004-20
Nesomyidae	*Saccostomus campestris*	South African pouched mouse	DMKZN001-20
	Steatomys pratensis	Fat mouse	DMKZN004-20
	Dendromus mystacalis	Chestnut climbing mouse	DMKZN008-20

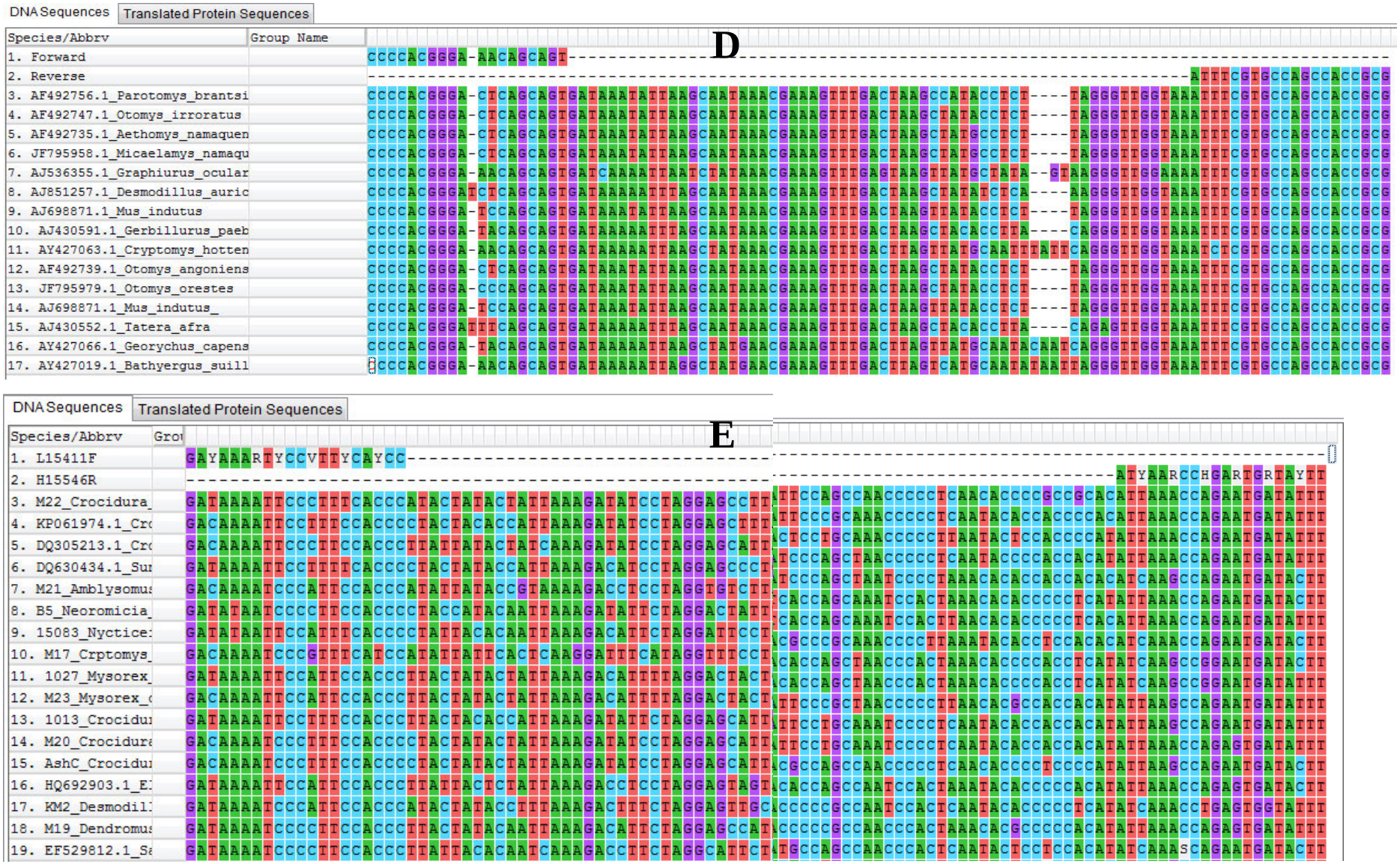
3.4.3 Data analyses

Each of the markers used had varying number of sequences and species used for calculating K2P genetic distances (Figure 3.2 and Figure 3.3). The COI region using the illmlCOIintF and jgHCO2198 primer pair (Geller *et al*. 2013; Leray *et al*. 2013) consisted of 195 sequences and 65 species. The 16S rRNA region using the 16S-HF and 16S-HR1 primer pair (Horreo *et al*. 2012) consisted of 60 sequences and 20 species. The 16S rRNA region using the partial 16S rRNA primer pair (Karlsson and Holmlund 2007) consisted of 75 sequences and 25 species. The 16S rRNA region using the 16Smamm1 and 16Smamm2 primer pair (Taylor 1996) consisted of 87 sequences and 29 species. The 12S rRNA region using the primer 12SV5 and 12SV5B2 primer pair (Riaz *et al*. 2011) consisted of 120 sequences and 40 species. The 12S rRNA region using the partal 12SrRNA primer pair (Karlsson and Holmlund 2007) consisted of 75 sequences and 25 species. The cyt b region made up of the primer pair L15411F and H15546R consisted of 126 sequences and 42 species. The COI illmlCOIintF and jgHCO2198 had the highest number of representative sequences and species, while the 16S rRNA primers, 16S-HF and 16S-HR1 had the lowest. Figure 3.2 shows the alignment of each of the primers used for the study across representative species.




B
Species/Abbrv
Grou
1. 16S_Forward
2. 16S_Reverse
3. 1A_Mastomys_natalensis.
4. JQ843858.1_Mastomys_cou
5. JQ843932.1_Myomyscus_ve
6. 26_Mus_cookii.ab1
7. 55_Mus_setolus.ab1
8. M24_Mysorex_varius.ab1
9. M25_Mysorex_kihaulei.ab
10. 1007_Crocidura_hildega
11. BNR10_Grammomys_sp.ab1
12. ASHD_Lemniscomys_rosal
13. AF141238.1_Lemniscomys
14. 2_Aethomys_chrysophilu
15. 16_Rhabdomys_pumilio.a
16. M9_Sundamys_muelleri.a
17. AF141248.1_Dasymys_inc
18. M14_Fukomys_damarensis
19. MK166028.1 Otomys irro


DNA Sequences
Translated Protein Sequences
C
Species/Abbr
Grou
1. 16S_Mamm1
2. 16S_Mamm2
3. JQ843858.
4. JQ843932.
5. M10_Mus_c
6. 55_Mus_se
7. 1007_Croc
8. M22_Sylvi
9. M12_Sunda
10. AF141246
11. M3_Aetho
12. BNR10_Gr
13. ASHD_Lem
14. AF141238
15. 30_Aetho
16. 2_Aethom
17. AF141245
18. 3_Rhabdo
19. AF141248


DNA Sequences
Translated Protein Sequences
Species/Abbrv
Group Name
D
1. Forward
2. Reverse
3. AF492756.1_Parotomys_brantsi
4. AF492747.1_Otomys_irroratus
5. AF492735.1_Aethomys_namaquen
6. JF795958.1_Micaelamys_namaqu
7. AJ536355.1_Graphiurus_ocular
8. AJ851257.1_Desmodillus_auric
9. AJ698871.1_Mus_indutus
10. AJ430591.1_Gerbillurus_paeb
11. AY427063.1_Cryptomys_hotten
12. AF492739.1_Otomys_angoniens
13. JF795979.1_Otomys_orestes
14. AJ698871.1_Mus_indutus_
15. AJ430552.1_Tatera_afra
16. AY427066.1_Georychus_capens
17. AY427019.1_Bathyergus_suill
DNA Sequences
Translated Protein Sequences
Species/Abbrv
E
1. L15411F
2. H15546R
3. M22_Crocidura_
4. KP061974.1_Cr
5. DQ305213.1_Cr
6. DQ630434.1_Su
7. M21_Amblysomus
8. B5_Neoromicia_
9. 15083_Nyctice
10. M17_Crptomys_
11. 1027_Mysorex_
12. M23_Mysorex_
13. 1013_Crocidu
14. M20_Crocidura
15. AshC_Crocidu
16. HQ692903.1_E
17. KM2_Desmodil
18. M19_Dendromus
19. EF529812.1_S

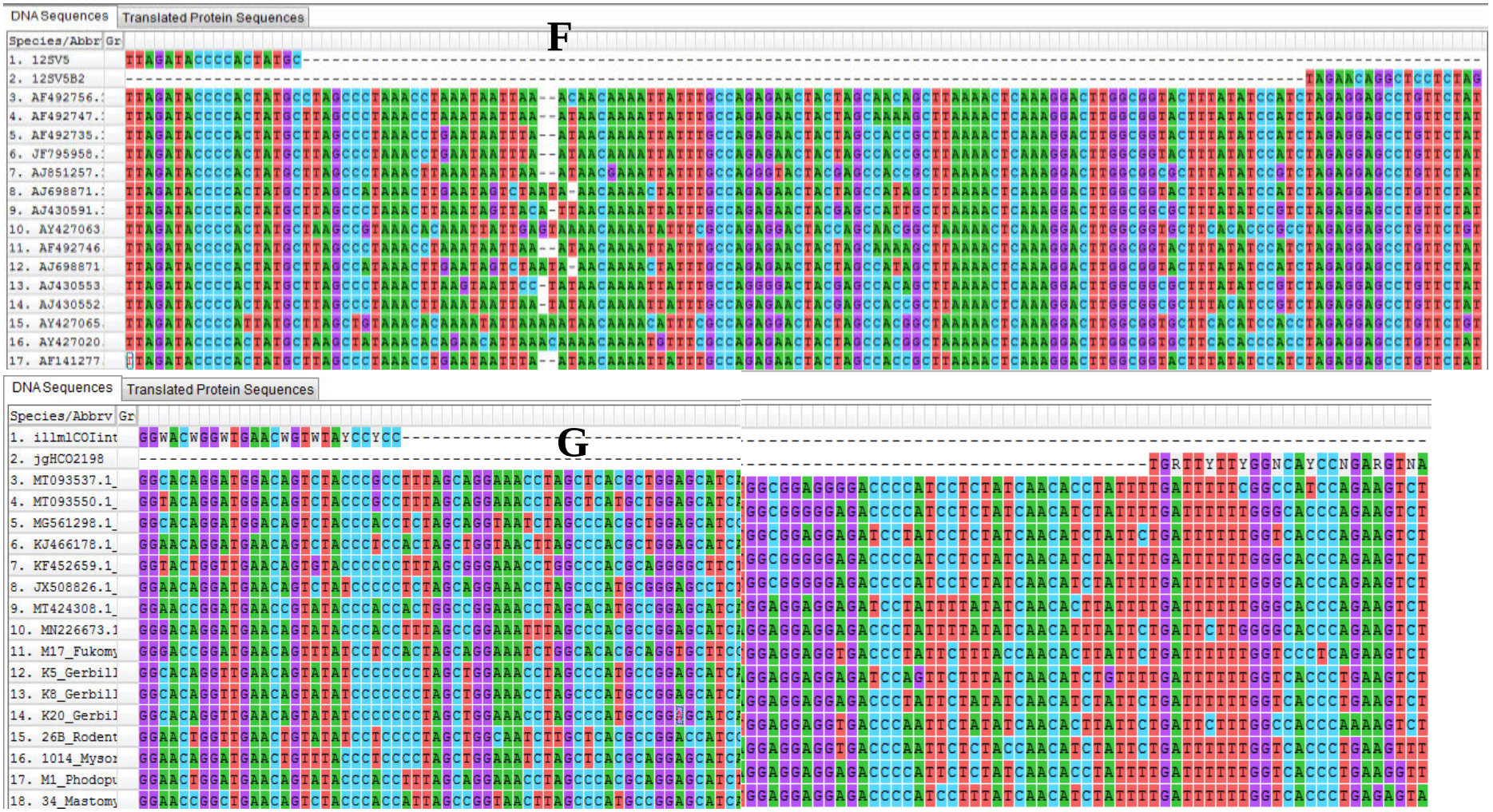
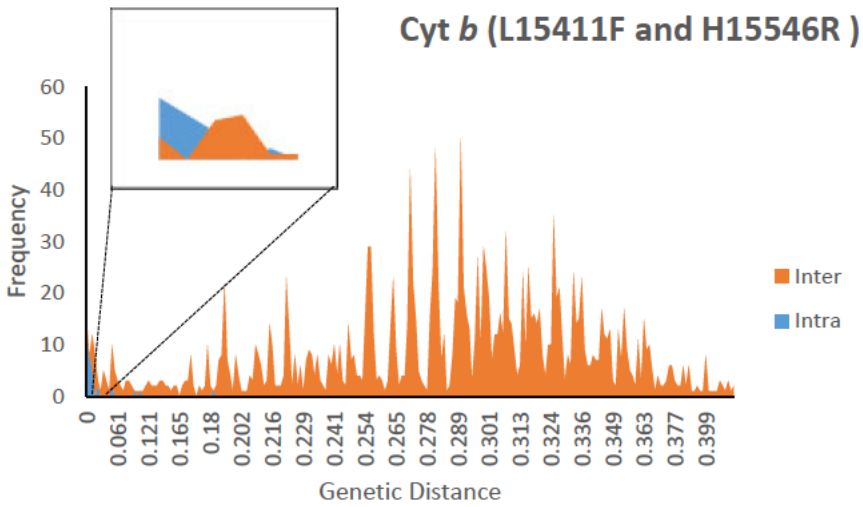


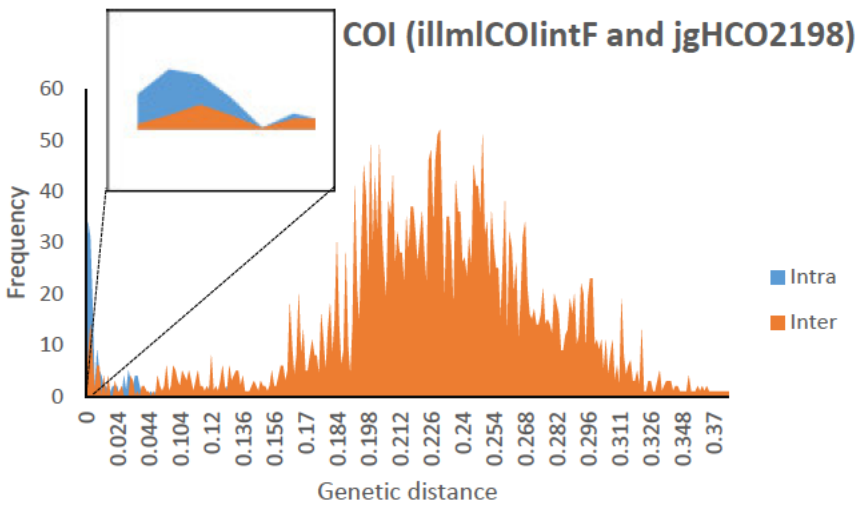
Figure 3.2. Sequence alignments of the various primers used in this study as viewed in MEGA v7.0.26 the16FH1 and 16FHR1 primer pair (A), the partial 16S rRNA forward and reverse primer pair (B), the 16mamm1 and 16 mamm2 primer pair (C), the partial 12S rRNA forward and reverse primer pair (D) the L15411F and H15546R primer pair (E) the 12SV5 and 12SV5B2 primer pair (F) and the illmlCOIintF and jgHCO2198 primer pair (G).

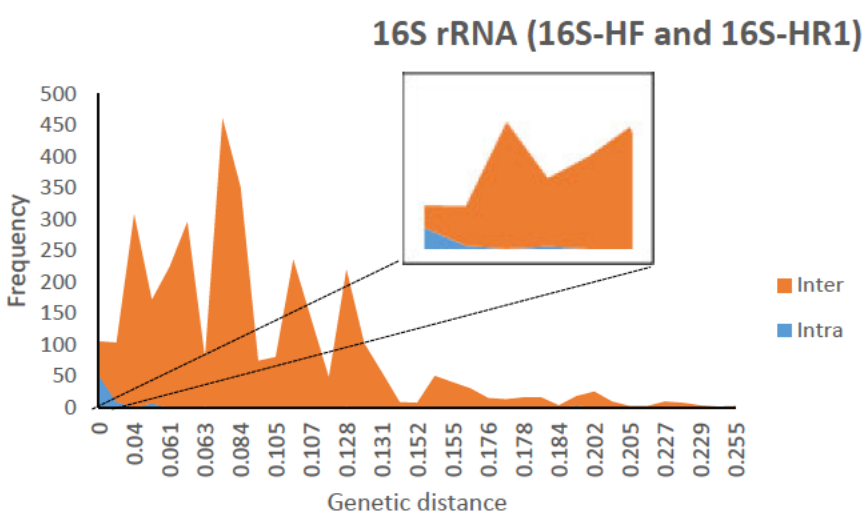
The frequency of the K2P pairwise genetic distance values was distributed between 0.000 and 0.452 (Figure 3.3). The K2P pairwise distance values between species were greater than within species for all the markers studied. The genetic distances between the markers varied. The lowest genetic distance (0.198) was found using the partial 12S RRNA primers (Karlsson and Holmlund 2007). The cyt b primers (Galan et al. 2012) had the highest genetic distance (0.452).

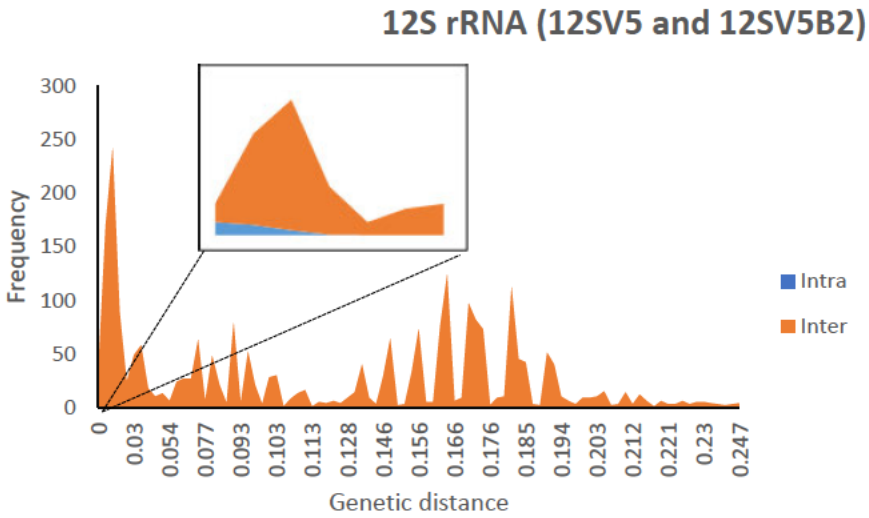
Table 3.1 shows the J-M values for the markers ranged from the lowest 1.23153 for the cyt b L15411F and H15546R primer pair (Galan et al. 2012) to the highest 1.933603 for the COI illmlCOIintF and jgHCO2198 primer pair (Geller et al. 2013; Leray et al. 2013). Three of the seven primer pairs tested exceeded the J-M cut-off value of 1.414 (Dabboor et al. 2014; Trigg and Flasse 2001b). The other four markers, the 16S rRNA, 16S-HF and 16S-HR1 (Horreo et al. 2012), and the cyt b, L15411F and H15546R (Galan et al. 2012), the partial 16S rRNA (Karlsson and Holmlund 2007), and the partial 12S rRNA (Karlsson and Holmlund 2007) were below the specified J-M threshold value. The COI illmlCOIintF and jgHCO2198 primer pair (Geller et al. 2013; Leray et al. 2013), the 12S rRNA 12SV5 and 12SV5B2 primers (Riaz et al. 2011) and the 16S rRNA 16Smamm1 and 16Smamm2 primers (Taylor 1996) were above the J-M threshold. The rest of the primers were below the specified J-M threshold. The J-M value for the Uni-MinibarR1 and Uni-MinibarF1 COI primer pair (Meusnier et al. 2008) could not be calculated because there were too many mismatches between the primer and the DNA sequences. The exact position of the primers could not be determined to identify which DNA sequences the primer would amplify and, therefore, be used to calculate genetic distances.

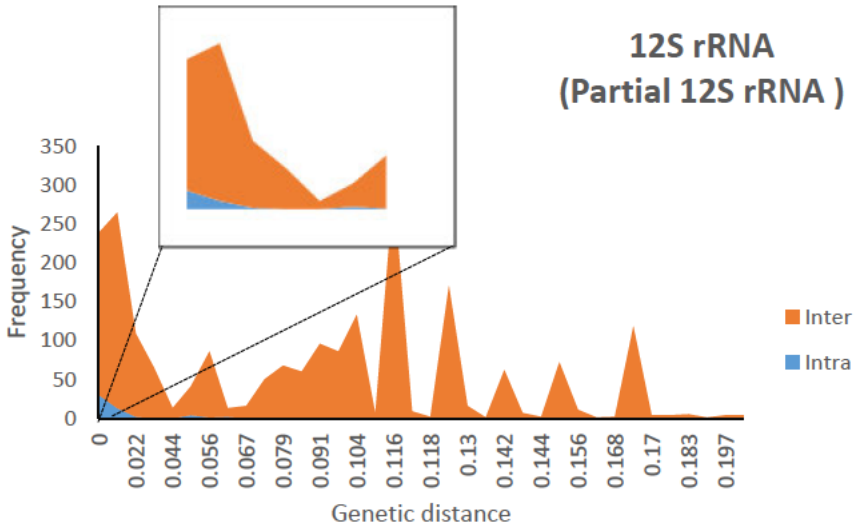
For all the markers studied, there was no distinct barcode gap between the inter- and intraspecific genetic distances (Figure 3.3). For the majority of the markers the COI, 16S rRNA and cyt *b* (Galan *et al.* 2012; Geller *et al.* 2013; Horreo *et al.* 2012; Karlsson and Holmlund 2007; Leray *et al.* 2013), there was slight overlap between and within species at lower genetic distances. However, for the other markers 12S rRNA and 16S rRNA (Karlsson and Holmlund 2007; Riaz *et al.* 2011; Taylor 1996), the interspecific variation completely overlapped intraspecific variation even at low genetic distances.

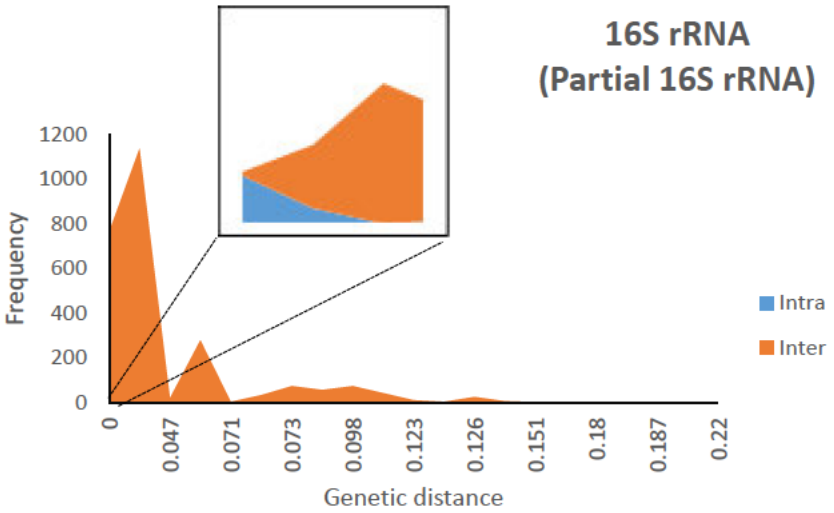

Cyt b (L15411F and H15546R)
Frequency
Genetic Distance
Inter
Intra


COI (illmlCOIintF and jgHCO2198)
Frequency
Genetic distance
Intra
Inter


16S rRNA (16S-HF and 16S-HR1)
Frequency
Genetic distance
Inter
Intra


12S rRNA (12SV5 and 12SV5B2)
Frequency
Genetic distance
Intra
Inter





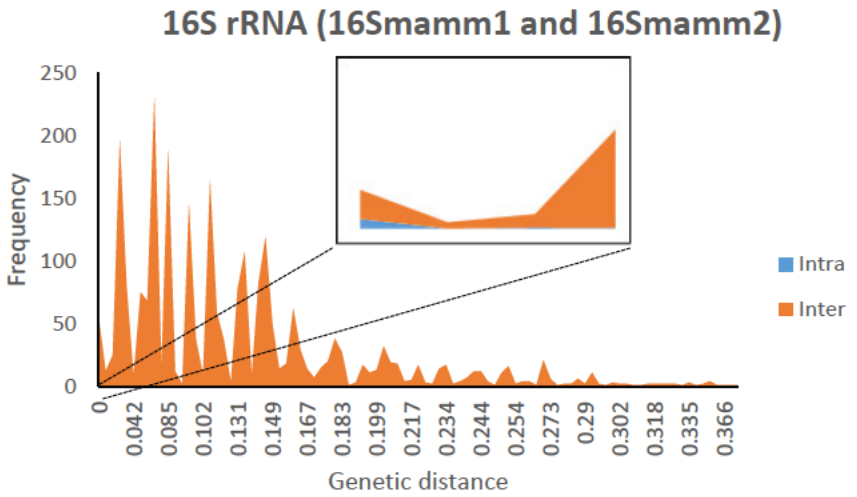


Figure 3.3. Frequency distributions of intra- and interspecific pairwise K2P distances calculated using the selected mini-barcode region as indicated in each heading. None of the markers have a distinct barcode gap that separates the intra- and interspecific genetic distances.

3.5 Discussion

The mitochondrial mini-barcode markers analysed in this study were tested on a taxonomically diverse set of small mammal species available in DNA reference libraries. These markers, covering different portions of the mitochondrial genome, were analysed in order to determine their efficacy in species identification and delineation of South African small mammals that predominantly make up the diet of small South African carnivores. An *in silico* study such as this one, aids in validating the performance of primers intended for ecological studies, where resources are limited by the unavailability of mock samples in the wild (Elbrecht and Leese 2017).

The J-M value for the Uni-MinibarR1 and Uni-MinibarF1 COI primers (Meusnier *et al.* 2008) could not be calculated. This was because the primers had too many mismatches with the available DNA sequences. Therefore, could not possibly anneal to the DNA sequences of the selected South African small mammals. The COI region primer binding sites are not highly conserved (Deagle *et al.* 2014). This makes specific primers unlikely to amplify any species outside of the intended taxon (Sambo *et al.* 2018). These results are similar to other studies that have shown poor COI metabarcoding success, where 95% of the species studied could not be assigned to a taxonomic level (Sikder *et al.* 2020). Other studies have shown that widely accepted fish COI primers were unsuitable for their intended study (Kumar *et al.* 2022). Another example is the poor performance of the L499 and H2123d (Clarke *et al.* 2017) primer pair when evaluated for the Dipteran family (Van Houdt *et al.* 2010). This was probably because the primers were intended for fruit flies and could, therefore, not be extended beyond that taxa. Primers designed for small taxa and lacking degeneracy tend not to work when applied to different taxa, ecosystem, or even geographic location (Elbrecht and Leese 2017).

Primer success is most often determined by base degeneracy and the availability of a reference dataset (Elbrecht and Leese 2017). The COI Uni-MinibarR1 and Uni-MinibarF1 primers (Meusnier *et al.* 2008) lacked degeneracy and had the highest number of mismatches. This seems to be the reason why most studies redesign the available COI primers to be taxa specific (Geller *et al.* 2013; Govendor *et al.* 2019; Hoareau and Boissin 2010; Krosch *et al.*

2020; Leray *et al.* 2013). The other set of COI illmlCOIintF and jgHCO2198 primers (Geller *et al.* 2013; Leray *et al.* 2013) tested in this study were highly degenerate and could be used for identification of small South African mammal species because of the relative abundance of available DNA sequences (Siziba and Willows-Munro 2024). However, it is important to note that highly degenerate COI primers can amplify beyond the intended taxa (Elbrecht *et al.* 2018a; Elbrecht and Leese 2017). Which is a concern when working with eDNA found in scat that often contains microbes, parasites (Naqib *et al.* 2019; Oehm *et al.* 2011; Thuo *et al.* 2019), and inhibitors (Carvalho *et al.* 2017; Miya *et al.* 2015).

Based on J-M values, the preferred markers for a metabarcoding study for identification of South African small mammals would be 12S rRNA 12SV5 and 12SV5B2 primers (Riaz *et al.* 2011), the 16S rRNA 16Smamm1 and 16Smamm2 primers (Taylor 1996), the cyt *b,* L15411F and H15546R (Galan *et al.* 2012) and the COI illmlCOIintF and jgHCO2198 primer pair (Geller *et al.* 2013; Leray *et al.* 2013). For the COI gene region, the other alternative markers (Meusnier *et al.* 2008) would not amplify, so therefore, the other alternative marker (Geller *et al.* 2013; Leray *et al.* 2013) would be the marker of choice. For the cyt *b* gene region, the L15411F and H15546R (Galan *et al.* 2012) are the only cyt b markers available in the literature. As such, they would be used as part of a multi-marker approach to improve species resolution (Zhang *et al.* 2018a), even if the J-M value is not ideal.

The 18S rRNA and 28S rRNA (Fonseca *et al.* 2011; Machida and Knowlton 2012) were not included as part of this study because they have been shown to evolve slower than other mitochondrial gene regions and therefore tend to underestimate species diversity (Derycke *et al.* 2010; Hillis and Dixon 1991; Machida and Tsuda 2010; Tautz *et al.* 2003). *In silico* studies such as this one are invaluable for assessing the efficacy of available primers for specific metabarcoding studies.

In conclusion this work highlights the importance of filling in the gaps in DNA reference libraries and makes up the base framework for metabarcoding and application of eDNA in diet studies in previously understudied ecosystems.

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CHAPTER 4

A method for non-invasive individual genotyping of black-footed cat (*Felis nigripes*)

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4.1 Abstract

The black-footed cat (*Felis nigripes*) is endemic to the arid regions of southern Africa. One of the world's smallest wild felids, the species occurs at low densities and is secretive and elusive, which makes ecological studies difficult. Genetic data could provide key information such as estimates on population size, sex ratios, and genetic diversity. In this study, we test if microsatellite loci can be successfully amplified from scat samples that could be non-invasively collected from the field. Using 21 blood and scat samples collected from the same individuals, we statistically tested whether nine microsatellites previously designed for use in domestic cats can be used to identify individual black-footed cats. Genotypes recovered from blood and scat samples were compared to assess loss of heterozygosity, allele dropout, and false alleles resulting from DNA degradation or PCR inhibitors present in scat samples. The microsatellite markers were also used to identify individuals from scats collected in the field that were not linked to any blood samples. All the nine microsatellites used in this study were amplified successfully and were polymorphic. Microsatellite loci were found to have sufficient discriminatory power to distinguish individuals and identify clones. In conclusion, these molecular markers can be used to monitor populations of wild black-footed cats non-invasively. The genetic data will be able to contribute important information that may be used to guide future conservation initiatives.

Keywords: *Felis nigripes*, microsatellites, scats, fecal DNA, genotyping error, allelic dropout.

4.2 Introduction

The black-footed cat (*Felis nigripes* Burchell 1824) is the smallest wild cat species in Africa (Sliwa 2013). Endemic to the arid region of southern Africa, the species has the most restricted distribution of any African felid (Sliwa 2004; Sliwa *et al.* 2016). The species' core distribution is in South Africa although some populations are also found in Botswana, Namibia, and marginally into Angola and Zimbabwe (Wilson *et al.* 2016). Black-footed cats are listed as Vulnerable by the International Union for Conservation of Nature (IUCN) due to limited range and small population sizes (Sliwa *et al.* 2016). Due to its elusive, solitary, and nocturnal habit, much of the black-footed cat's ecology, behavior, and population dynamics remain unknown (Molteno *et al.* 1998; Sliwa 1994; Sliwa 2004; Sliwa 2006).

Traditional methods of studying carnivores are challenging to implement for black-footed cats because of low trapping success (Sliwa *et al.* 2010; Sliwa *et al.* 2018). Most studies of black-footed cats have been based on direct observations (Sliwa 1994; Sliwa 2004; Sliwa 2006) and camera traps (Sliwa *et al.* 2018). Direct observations are extremely time-consuming and laborious as the species is highly mobile and travels long distances (Sliwa 2004). Camera traps have been shown to have low efficacy for the species because individuals do not travel predictable routes marked by scent (Molteno *et al.* 1998; Sliwa 2006). In rare cases where the cats have been recorded, they often move too fast for behavior to be captured by camera (Sliwa *et al.* 2018). Despite these challenges, direct observations have aided in answering questions about the relationships of black-footed cats with other carnivores (Kamler *et al.* 2015), estimating home ranges and habitats (Sliwa 2004), social organization and seasonal prey preferences (Sliwa 1994; Sliwa 2006). However, significant knowledge gaps still exist in the black-footed cats' population estimates in the wild. To this end, DNA data could contribute towards the conservation of the species by identifying individuals and possible population estimates. Recent approaches such as metabarcoding have been used in diet studies in carnivores (Forin-Wiart *et al.* 2018b) and could also provide additional crucial ecological information on the feeding behavior of black-footed cats.

A non-invasive approach to monitor populations of scarce species such as the black-footed cat, could be more sustainable and reliable (Banks and Piggott 2022; Piggott *et al.* 2008). DNA extracted from scats reduces the need to handle the animal (Ramon-Laca *et al.* 2015) and

could provide information on individual occupancy (Fernando *et al.* 2003), paternity and kinship (Constable *et al.* 2001; Wang *et al.* 2015), genetic diversity (Mengulluoglu *et al.* 2019), species identification and sexing (Dalén *et al.* 2004; Kurose *et al.* 2005), identifying hybrids (Adams *et al.* 2003), and estimating population sizes (Banks *et al.* 2002; Eggert *et al.* 2003; Piggott *et al.* 2006). DNA amplification from scat material can be challenging as DNA may be degraded, leading to low amplification success (Ramon-Laca *et al.* 2015; Taberlet *et al.* 1996) or may contain genotyping errors due to allelic dropout and false alleles (Fernando *et al.* 2003; Taberlet *et al.* 1996). Microsatellite loci are routinely used in analysis of pedigree, genotyping and parentage (Balloux and Lugon-Moulin 2002; Constable *et al.* 2001; Estoup *et al.* 1998; Reis *et al.* 2008; Vigilant *et al.* 2001), and have been used on scat samples in a number of studies (Bourgeois *et al.* 2019; Kurose *et al.* 2005). However, with felids most studies are biased towards large felids such as snow leopard (*Panthera uncia*) (Janečka *et al.* 2008) and leopard (*Panthera pardus)* (Mondol *et al.* 2009).

In this study, we test the utility of a suite of microsatellite markers as a molecular tool for the individual identification and monitoring of black-footed cats using DNA extracted from scats. As well as to identify which microsatellite loci were the most informative with low genotyping errors. We compared the genotypes obtained from DNA extracted from scats to genotypes obtained from high-quality DNA extracted from blood taken from the same individual to assess any possible error rates.

4.3 Materials and methods

4.3.1 Sample collection and DNA extraction

Blood and scat samples were collected from Benfontein Nature Reserve in Kimberley, Northern Cape Province, South Africa. Blood samples were taken from eight individual cats; four individuals had corresponding scat samples. These matched scat samples were collected while the cats were handled during radio-collaring events. Two scats were collected per individual cat from which blood was collected, except from one cat where seven scat samples were collected. The rest (n = 70) were scat samples collected using a trained detection dog-handler team (Long *et al.* 2007; MacKay *et al.* 2008). The training followed general guidelines recommended for conservation detection dogs (DeMatteo *et al.* 2019; Reed *et al.* 2011).

Blood and scat samples were collected from the 3rd of March 2020 to the 9th of July 2020 as part of an ongoing research project of the Black-footed Cat Working Group (Sliwa *et al.* 2021). Samples were collected under the following permits: Northern Cape Department of Environment and Nature Conversation FAUNA 1218/2016, FAUNA 0636/2021. Blood samples were collected and stored onto Whatman FTA Elute (Sigma-Aldrich, St Louis, Missouri, USA) cards. Scat samples were collected and stored in falcon tubes with 96% ethanol. Both blood and scat samples were placed in -80°C freezers for long-term storage at the McGregor Museum, Northern Cape, South Africa.

DNA from blood samples was extracted using the E.Z.N.A® Tissue DNA kit (Omega BioTek, Georgia, USA) following the standard protocol. DNA from scat samples was extracted from 0.22g of material scraped from the outer layer of each scat using the Qiagen QIAmp Fast DNA stool kit (Qiagen Inc., Hilden, Germany) following the standard protocol. All DNA extracts were stored at -20°C until PCR amplification of microsatellite loci.

4.3.2 Sex determination and microsatellite genotyping

All samples were sexed using a nested PCR protocol, with the first reaction using the primers RG4 and RG7 (Griffiths and Tiwari 1993) and the second PCR using Carni-SRY2 and SRY-CR1 primers (Kurose *et al.* 2005). The first PCR amplifications were performed using 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension of 72°C for 10 mins. The second PCR was the same as the first, except the number of cycles was increased to 45. The PCR products were run on a 1% TBE agarose gel and visualized on the GelDoc™ EZ Imager (Biorad, Johannesburg, South Africa). The presence of a 135bp band indicated the presence of the partial SRY gene and identified the individual as male. The absence of a band indicated that the sample came from a female. If no band was present, the PCR was repeated with increased DNA concentration and a positive control from one of the blood samples was included to confirm the absence of a band.

Nine unlinked autosomal feline microsatellites (FCA_008, FCA_023, FCA_026, FCA_043, FCA_058, FCA_088, FCA_126, FCA_132, FCA_149; (Menotti-Raymond *et al.* 1997; Menotti-Raymond and O'Brien 1995) were screened for use in this study. These markers were initially designed for domestic cats (Menotti-Raymond *et al.* 1999) but have been used previously in

black-footed cats (Mattucci *et al.* 2018). All forward primers were labeled with a fluorescent dye on the 5' end. Amplifications were performed in 12.5µL reactions containing 6.25µL TEMPase Multiplex Taq (Amplicon, Odense, Denmark), 4µL dH_2O, and 0.25µL each of the forward and reverse primer. Amplifications were performed with an initial denaturation step at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 15 secs, annealing at 57°C for 30 secs, extension at 68°C for 30 secs and a final extension of 68°C for 5 mins. Amplicons were sent to the Central Analytical Facility (CAF) at Stellenbosch University, South Africa, for fragment analyses using an ABI PRISM™ 3500XL Genetic Analyzer (Life Technologies, Applied Biosystems, Warrington, UK) and a standard GeneScan™ ROX500™ (Applied Biosystems) internal size standard. The software package Geneious v8 (https://www.geneious.com) was used for genotype scoring. All scat samples were re-genotyped up to five times through PCR amplifications, and fragment analysis genotype scores were compared throughout to check for consistency. No samples were discarded for comparison purposes. To further validate the use of the microsatellite markers in black-footed-cat samples, each marker was used to perform a BLASTn search against the black-footed cat genome (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_028533295.1/) in order to test for primer mismatches and to validate the consistency of the microsatellite motif.

Data analyses

4.3.3 Genotyping comparison: Blood versus scat

The genotypes recovered from blood and scat from the same individuals (N=4) were compared for each microsatellite locus. The genotypes obtained from the same individual were scored as either matching (same genotype for blood and scat), not matching (different genotypes), or unreadable (either blood or scat had no readable data). The PCR success rate for each locus was assessed based on the total number of successful PCRs, allelic dropout, and false alleles by comparing the blood and scat sample. We also compared null allele frequency, number of alleles, and the polymorphic information content (Piche *et al.* 2010) of each locus.

4.3.4 Assessing utility of microsatellite loci

The presence of null alleles was estimated using the expectation maximization (EM) algorithm (Dempster *et al.* 1977) in FreeNA (Chapuis and Estoup 2007) for both blood and scat samples. The polymorphic information content (Piche *et al.* 2010) was estimated for each locus in Cervus v3.0.7 (Kalinowski *et al.* 2007). PIC values higher than 0.6 were considered highly informative (Mateesu *et al.* 2005). The minimum number of microsatellite loci needed to accurately identify individual cats was assessed by ranking loci by PIC values and systematically excluding loci with lower PIC values until the panel could no longer detect identity. Allelic richness (Ar) was estimated using the rarefaction method in FSTAT v2.9.3.2 (Goudet 1995) to account for the difference in sample size between the blood and scat samples. The discriminatory power of each locus was assessed by calculating the probability of identity (P_{ID}) and probability of exclusion (P_{E2}- excluding a putative parent pair) for each locus in GenAlEx v6.503 (Peakall and Smouse 2012). Overall P_{ID} and P_{E2} were also calculated using data from all loci. The P_{ID} was calculated using a conservative approach, assuming that a black-footed cat was homozygous for the most common allele at each locus (Butler 2005). Deviation from Hardy-Weinberg equilibrium (HW *p*-value) was estimated in Cervus.

4.3.5 Individual identification

Assignment of scats to individual cats was done using GIMLET v1.3.3 (Valière 2002) by identifying identical genotypes and comparing genotypes to a set of reference genotypes. GIMLET allows the selection of specific genotypes to be used as a reference to identify similar genotypes in the rest of the dataset, even when some loci are not amplified. This is particularly useful when using degraded DNA that does not always successfully amplify across all loci. Genotypes amplified from the blood samples were used as reference genotypes. As a compliment to the GIMLET analyses, Principal Coordinates Analyses (PCoA) was conducted in GenAlEx (Peakall and Smouse 2012), and identity analyses were conducted in Cervus (Kalinowski *et al.* 2007). The last two analyses do not take into account reference samples and can be biased by missing data.

Loci were ranked from lowest to highest PIC value, and the probability of identity was calculated for the entire loci set. Thereafter, the probability of identity was recalculated after excluding the locus with the lowest value PIC marker until only one locus remained. These

results were plotted to determine the minimum number of loci needed for individual identification.

4.4 Results

4.4.1 Sample collection, DNA extraction, sex determination, and microsatellite genotyping

A total of 70 scat samples were collected in the field. Sixty-six of these samples were found by detection dogs, and the other four were identified as possible black-footed cat scat by field staff. DNA from all the blood and scat samples were successfully extracted, and all nine microsatellite loci were amplified. All the scat samples linked to blood samples were correctly identified as male or female. The rest of the field-collected scats were identified as either male or female depending on the presence or absence of a 135bp PCR product. The amplification success of individual loci varied even when amplified on high molecular weight DNA extracted from blood.

Table 4.1. Microsatellite loci comparison between black-footed cat (*Felis nigripes*) blood and the corresponding scat samples showing missing data (allelic dropout = ADO), null alleles, and incorrect genotypes (false alleles = FA) across nine loci amplified. The - denotes genotypes where blood samples were not analyzed. Each blood and corresponding scat sample have a similar name. The scat sample has a numerical value attached to the name, indicating how many scat samples were collected per individual cat.

Sample	Sex	Sample type	Missing data (ADO)	Null alleles in scat sample	Incorrect genotype reads in scat sample (FA)
Ca	Male	Blood	0.000	-	-
Sa	Female	Blood	11.111	-	-
Pu	Male	Blood	0.000	-	-
Te	Male	Blood	0.000	-	-
Du	Female	Blood	0.000	-	-
Du1	Female	Scat	44.444	11.111	33.333
Du2	Female	Scat	44.444	11.111	33.333

Ha	Male	Blood	0.000	-	-
Ha1	Male	Scat	11.111	0.000	0.000
Ha2	Male	Scat	0.000	11.111	11.111
Ka	Female	Blood	0.000	-	-
Ka1	Female	Scat	22.222	0.000	22.222
Ka2	Female	Scat	44.444	0.000	22.222
Ka3	Female	Scat	44.444	0.000	22.222
Ka4	Female	Scat	22.222	11.111	33.333
Ka5	Female	Scat	11.111	55.560	22.222
Ka6	Female	Scat	22.222	33.333	33.333
Ka7	Female	Scat	22.222	44.444	33.333
Ph	Male	Blood	0.000	-	-
Ph1	Male	Scat	11.111	11.111	22.222
Ph2	Male	Scat	22.222	22.222	0.000

4.4.2 Blood versus scat genotypes

Genotypes produced from blood and scats collected from the same individual were compared for consistency (Figure 4.1). The sex of the individual was correctly determined for all scat and blood samples (Table 4.1). Comparison of heterozygosity indices (Table 4.2) found that scat samples generally showed reduced heterozygosity, reflecting the impact of allelic dropout. Table 4.1 shows that the majority of the scat samples had between 1 to 3 fewer alleles when compared to genotypes generated from the blood samples. Loci FCA_043 showed an equal number of alleles between blood and scat samples, while three of the markers (FCA_026, FCA_058, and FCA_149) showed an increase in the number of alleles in the scat samples.

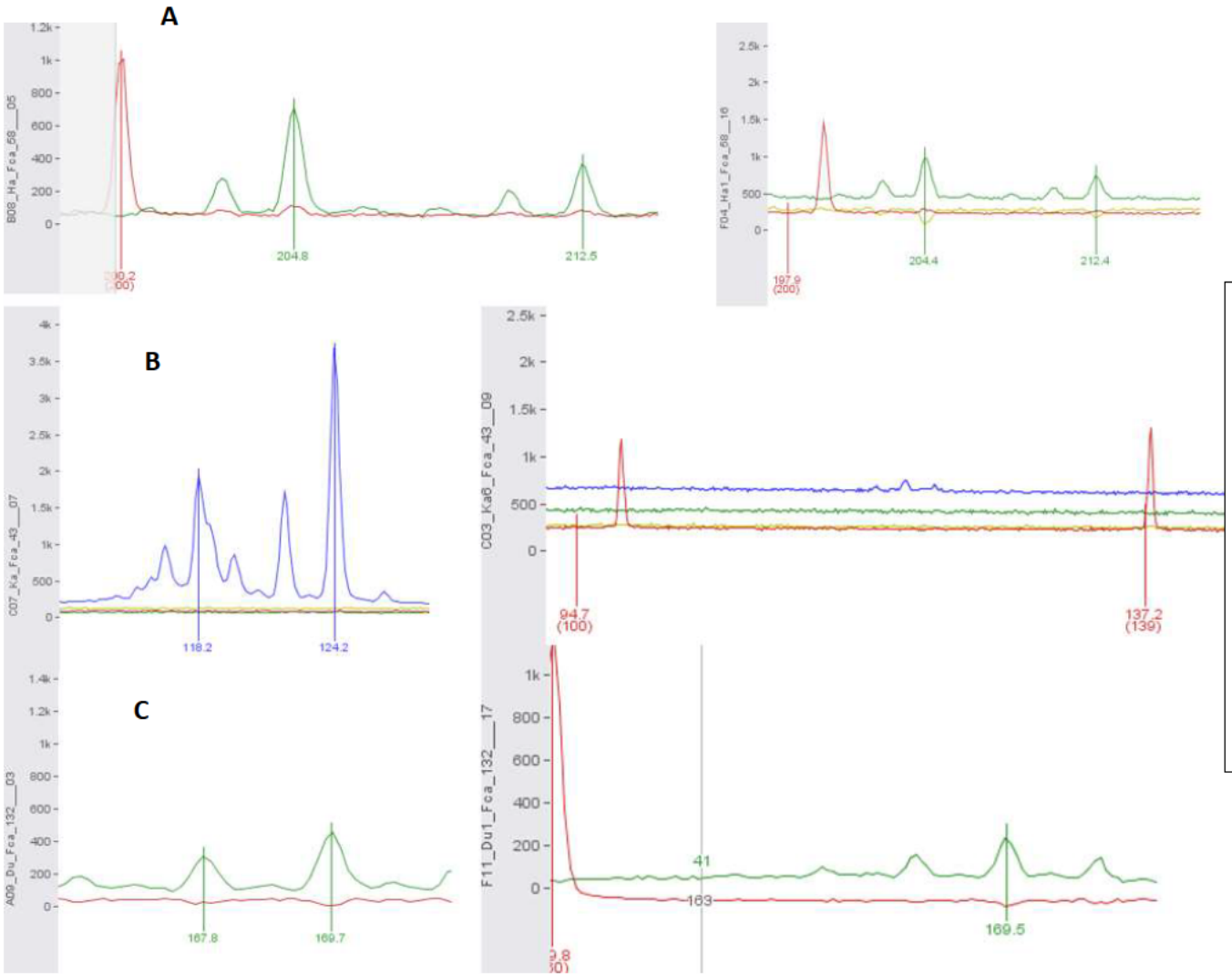

Figure 4.1. Electropherograms of blood (left) and scat (right) samples of black-footed cat using the microsatellite plugin of Geneious v8.1.9.

A - Blood and scat samples with exact Heterozygous genotypes.

B - Heterozygous genotype in the blood sample and a null allele in the scat sample.

C - Loss of heterozygosity in the scat sample and a heterozygous genotype in the blood sample

4.4.3 Assessing utility of microsatellite loci

Most markers, when analyzed for both blood and scat samples, were highly informative, except for loci FCA_023, FCA_043, and FCA_058 (Table 4.2, see PIC values). When analyzing the scat samples, the same markers (FCA_023, FCA_043, and FCA_058) were considered uninformative together with markers FCA_126 and FCA_132 based on low PIC values. When using non-invasive samples, five markers were highly informative and were sufficient to discriminate individual cats. Marker FCA_008, FCA_023, FCA_058, and FCA_088 had an equal number of alleles in both blood and scat samples. Marker FCA_126 and FCA_132 showed a lower number of loci in the scat samples when compared to the blood samples. Loci FCA_026 and FCA_043 showed one more allele in the scat samples than in the blood samples. Blood samples had an average of four alleles, compared to scat samples that had three (Table 4.2).

The allele frequencies for the blood samples ranged from 3.000 (FCA_132) to 6.893 (FCA_088). For the scat samples, allele frequencies ranged from 1.989 (FCA_132) to 6.658 (FCA_058) (Table 4.2). Only four loci showed null allele frequencies above 0.1 (FCA_008, FCA_058, FCA_132, FCA_149). The mean number of alleles per locus ranged from 2 (FCA_043, FCA_058, FCA_126, and FCA_132) to 6 (FCA_088). Eight of the nine markers showed high levels of heterozygosity, and only one locus (FCA_132) showed heterozygote deficiency.

Performing a BLASTn search of the microsatellite primers against the black-footed cat genome showed that all the nine microsatellite markers had a dinucleotide $(CA)_n$ motif. The loci were distributed across five of the 19 chromosomes. Namely, chromosome three (FCA_023 and FCA_149), chromosome five (FCA_043), chromosome seven (FCA_8 and FCA_088), chromosome 12 (FCA_026), and chromosome 16 (FCA_058, FCA_132). Only one of the primers had primer mismatches. FCA_008 mismatched to a position on chromosome nine.

All the loci had 100% amplification success in the blood samples except FCA_132, which had an amplification success of 87.5% (Table 4.2). Except for FCA_46, none of the markers had a 100% amplification success in the scat samples. Amplification success for the rest of the eight markers in the scat samples ranged from 61.54% (FCA_149) to 92.31% (FCA_026 and FCA_058). Amplification success for the scat samples was above 70% for eight of the nine markers.

Heterozygosity values for the blood samples ranged from 0.25 (FCA_023) to 0.93 (FCA_088). For the scat samples, heterozygosity values ranged from 0.29 (FCA_058) to 0.81 (FCA_088). In the blood samples, marker FCA_023 was the only marker with low genetic diversity. In the scat samples, marker FCA_023 as well as FCA_058 had low diversity values. Heterozygosity values for blood samples were higher than those for scat samples except for markers FCA_008 and FCA_023 (Table 4.2).

Table 4.2. Primer and loci details, BLASTn results per chromosome (Ch), the number of primer mismatches and genetic diversity estimates per locus from the free-ranging black-footed cat (*Felis nigripes*) data used in this study. Heterozygosity (He), PIC (Polymorphic information content), Null allele frequency, NA (Number of Alleles), and Ar (Allelic richness) values are given for blood and scat separately.

Locus	Primer sequence (5'-3')	Motif	No. primer mismatch	Chr	Amplification Success (%)	He	PIC	Null allele frequency	NA	Ar
FCA_008	F- ACTGTAAATTTCTGAGCTGGCC	CA	9	7	Blood: 100.000	Blood: 0.750	Blood: 0.712	Blood: 0.107	Blood: 5.000	Blood: 5.436
	R- TGACAGACTGTTCTGGGTATGG				Scat: 76.920	Scat: 0.779	Scat: 0.709	Scat: 0.335	Scat: 5.000	Scat: 3.615
FCA_023	F- ACTGTAAATTTCTGAGCTGGCC	CA	-	3	Blood: 100.000	Blood: 0.250	Blood: 0.511	Blood: 0.000	Blood: 3.000	Blood:3.699
	R- TGACAGACTGTTCTGGGTATGG				Scat: 84.620	Scat: 0.333	Scat: 0.163	Scat: 0.073	Scat: 3.000	Scat: 2.714
FCA_026	F- ACTGTAAATTTCTGAGCTGGCC	CA	-	Twelve	Blood: 100.000	Blood: 0.750	Blood: 0.671	Blood: 0.000	Blood: 4.000	Blood:3.743
	R- TGACAGACTGTTCTGGGTATGG				Scat: 92.310	Scat:0.659	Scat: 0.611	Scat:0.000	Scat: 5.000	Scat: 5.615
FCA_043	F- ACTGTAAATTTCTGAGCTGGCC	CA	-	5	Blood: 100.000	Blood: 0.857	Blood: 0.668	Blood: 0.000	Blood: 4.000	Blood:4.492
	R- TGACAGACTGTTCTGGGTATGG				Scat: 100.000	Scat: 0.766	Scat: 0.530	Scat:0.000	Scat: 5.000	Scat: 4.124
FCA_058	F- ACTGTAAATTTCTGAGCTGGCC	CA	-	Sixteen	Blood: 100.000	Blood: 0.607	Blood: 0.359	Blood: 0.000	Blood: 2.000	Blood: 3.742
	R- TGACAGACTGTTCTGGGTATGG				Scat: 92.310	Scat: 0.290	Scat: 0.151	Scat:0.221	Scat: 2.000	Scat: 6.658
FCA_088	F- ACTGTAAATTTCTGAGCTGGCC	CA	-	7	Blood: 100.000	Blood: 0.929	Blood: 0.786	Blood: 0.000	Blood: 6.000	Blood: 6.893
	R- TGACAGACTGTTCTGGGTATGG				Scat: 84.620	Scat: 0.814	Scat: 0.619	Scat:0.000	Scat: 6.000	Scat: 4.485
FCA_126	F- ACTGTAAATTTCTGAGCTGGCC	CA	-	7	Blood: 100.000	Blood: 0.750	Blood: 0.605	Blood: 0.000	Blood: 4.000	Blood: 4.692
	R- TGACAGACTGTTCTGGGTATGG				Scat: 76.920	Scat: 0.542	Scat: 0.368	Scat: 0.082	Scat: 2.000	Scat: 2.000
FCA_132	F- ACTGTAAATTTCTGAGCTGGCC	CA	-	Sixteen	Blood: 87.500	Blood: 0.607	Blood: 0.605	Blood: 0.000	Blood: 4.000	Blood: 3.000
	R- TGACAGACTGTTCTGGGTATGG				Scat: 84.620	Scat: 0.416	Scat: 0.318	Scat:0.167	Scat: 2.000	Scat: 1.989
FCA_149	F- ACTGTAAATTTCTGAGCTGGCC	AC	-	3	Blood: 100.000	Blood: 0.750	Blood: 0.746	Blood:0.001	Blood: 5.000	Blood: 4.443
	R- TGACAGACTGTTCTGGGTATGG				Scat: 61.540	Scat: 0.508	Scat: 0.718	Scat: 0.254	Scat: 5.000	Scat: 6.000

The PID values in Table 4.3 ranged from 0.06 (FCA-88) to 0.39 (FCA_058) for the blood samples and from 0.36 (FCA_088) to 3.97 (FCA_132) for scat samples. PIDSib values ranged from 0.36 (FCA_088) to 0.61 (FCA_088) for the blood samples and from 0.40 (FCA_149) to 0.85 (FCA_058) for the cat samples (Table 4.3).

Table 4.3. Probability of Identity for unrelated individuals, Probability of Identity for Siblings, Expected number of individuals with the same genotype, and Probability of Exclusion between blood and fecal scats of black-footed cat (*Felis nigripes*) across nine microsatellite loci.

	Microsatellite loci								
Source	FCA_ 008	FCA_ 023	FCA_ 026	FCA_ 043	FCA_ 058	FCA_ 088	FCA_ 126	FCA_ 132	FCA_ 149
P_{ID} Blood (*n*=4)	0.169	0.634	0.169	0.101	0.283	0.062	0.169	0.301	0.193
P_{ID} Scat (*n*=8)	0.152	0.289	0.069	0.149	0.045	0.186	0.407	0.497	0.088
PID_{Sib} Blood	0.464	0.799	0.464	0.400	0.555	0.359	0.464	0.575	0.470
PID_{Sib} Scat	0.440	0.542	0.369	0.443	0.343	0.491	0.630	0.706	0.392
P_{OE} Blood	0.678	2.537	0.678	0.402	1.131	0.246	0.678	1.204	0.771
P_{OE} Scat	1.219	2.310	0.548	1.191	0.362	1.486	3.259	3.973	0.701
P_{E2} Blood	0.677	0.281	0.677	0.772	0.519	0.831	0.677	0.519	0.519
P_{E2} Scat	0.677	0.519	0.871	0.772	0.898	0.772	0.281	0.281	0.831

For the blood samples, the highest ranking PIC marker was FCA_088, and for scat samples, this was FCA_149. Both blood and scat samples recovered FCA_058 as the locus with the lowest PIC. Plotting the PID values against the locus combinations shows that five markers are sufficient for individual analysis of black-footed cat individuals in blood and scat samples (Figure 4.2). The combination of the five markers based on PIC was the same in both blood and scat samples (FCA_008, FCA_026, FCA_043, FCA_088, and FCA_149). The differences in blood samples (Figure 4.2A) and scat samples (Figure 4.2B) are the order in which the markers are excluded based on their PIC values (Table 4.1).

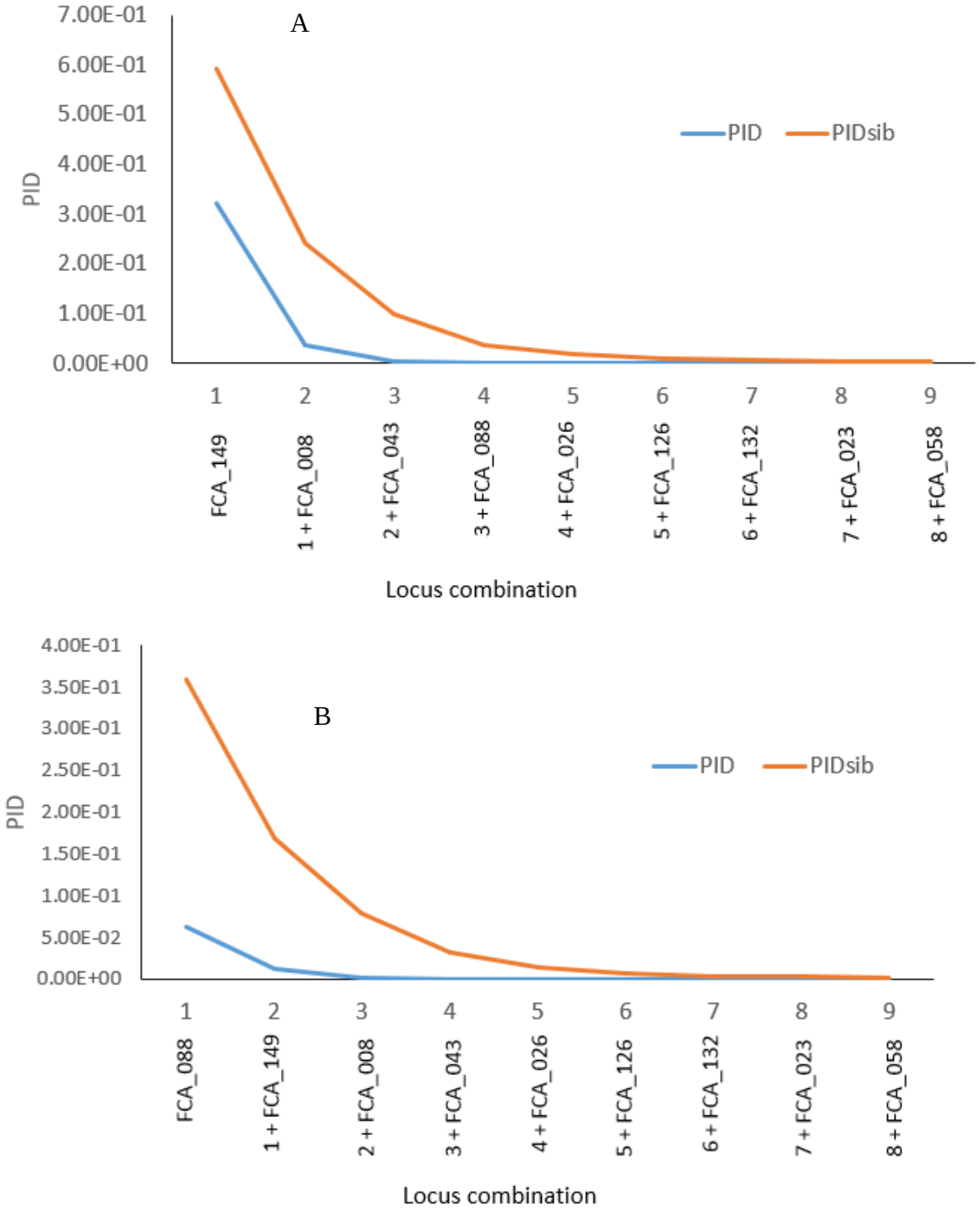

Figure 4.2. Probability of identity and probability of sib identity (A - Blood, B - Scat) for each locus and for increasing combinations for the nine loci. Each number represents the combination of the previous microsatellite locus combination.

4.4.4 Individual identification

Analysis of the scat samples that sniffer dogs obtained showed that out of 70, 35 were identified as black-footed cat based on their allele sizes. Individual analyses showed that the majority of the scat samples belonged to four individual cats already identified in the blood samples. An additional five cats, which were not in the reference genotype, were identified based on their genotypes. Thirteen scat samples were assigned to the same blood sample as the cat identified as Kazi (Ka), ten were assigned to the same blood sample as Hamba (Ha), and three of the scats were assigned to the same blood sample as Durga (Du). The rest of the scats were identified as six separate individuals.

4.5 Discussion

This study investigated the possibility of individual identification of black-footed cats using microsatellite markers and DNA collected from scat samples in the wild. This data was validated by comparing how amplification success, heterozygosity, allelic dropout, and probability of identity of the microsatellite markers are affected using DNA collected from blood and scat samples from the same individual cats. The data indicates that scat samples are a reliable source of microsatellite genotyping, as shown by similar studies that compared genotyping between blood and scat samples of American bison (*Bison bison*) (Forgacs *et al.* 2019), koala (*Phascolarctos cinereus*) (Schultz *et al.* 2018), red wolf (*Canis rufus*) (Adams and Waits 2008), Mojave desert tortoise (*Gopherus agassizii*) (Mitelberg *et al.* 2019), common bottlenose dolphins (*Tursiops truncatus*) (Parsons 2001), Asian elephants (*Elephas maximus*) (Fernando *et al.* 2003), and red deer (*Cervus elaphus*) (Valière *et al.* 2017).

A previous study on captive black-footed cats using pre-developed microsatellite markers for domestic cats used hair tufts collected in a Polish zoo (Mattucci *et al.* 2018). However, collecting hair or fresh scats is not always feasible with wild populations (Giambattista and Gentile 2018; Piggott *et al.* 2008). Unlike the previous study, this study validates the practicality of using DNA from scats collected in the wild for individual analysis by comparing error rates between blood and scat samples. The less than 100% amplification success is most likely due to the age of the scats sampled. This data was validated through a study by Schlutz *et al*. (2018), which showed that 70% of 14-day-old scats had high-quality DNA for individual

genotyping analyses. The majority of mismatches in the dataset resulted from allelic dropout, which has been identified in various studies as a drawback of using scat samples (Fernando *et al.* 2003; Johnson and Haydon 2007).

Marker FCA_058, which had the maximum allele size (>200bp), also showed the highest allelic dropout and lowest heterozygosity in scats. This further supports the assumption that DNA degradation was the leading cause of lack of amplification. All of the cats identified as heterozygotes using the blood samples for this marker (FCA_058) were identified as homozygotes using the scat samples. This data correlates with studies that have shown that longer alleles of heterozygous loci tend to have higher levels of allelic dropout in scat samples as a result of degraded DNA (Gerloff *et al.* 1995; Goossens *et al.* 1998). A study by Pompanon *et al*. (2005) showed that drop-out rates ranged from 0.2 – 15% per locus and this number increased significantly when more loci were used (Johnson and Haydon 2007). When used for population structure and genetic diversity, this marker will significantly underestimate the level of genetic diversity (Gagneux *et al.* 1997; Pompanon *et al.* 2005; Waits and Leberg 2000). Loci with shorter amplicons have higher amplification and lower error rates and are, therefore, more ideal for scat samples (Broquet *et al.* 2006; Frantzen *et al.* 1998). Marker FCA_023 and FCA_058 showed the lowest PIC values. This means that in downstream applications, they are the first markers to be excluded when analyzing paternity, kinship, or even the identification of an individual. For the other markers with low heterozygosity or high null allele values, the data was improved by repeating the PCRs. This study showed a loss in heterozygosity between blood and scat samples. This data correlates to other studies that have compared genotypes between blood and scat samples (Forgacs *et al.* 2019; Johnson and Haydon 2007). However, the higher rates of genotyping failure in this study could be due to a significantly small sample size and the unavailability of scat samples for all the corresponding blood samples.

BLASTn results showed that the primer motif was conserved across both domestic and black-footed cats. Locus FCA_008 was the only marker with a primer mismatch and the only marker with a null allele value higher than 0.00 in the blood samples. Primer mismatches have been shown to decrease the thermal stability of primer-template matches thus reducing the PCR yield significantly (Kwok *et al.* 1990; Stadhouders *et al.* 2010). Primer mismatches were

attributed to only one of the markers; therefore, null alleles in the scat samples could be a result of low DNA yields as a result of DNA degradation in the field (Manning *et al.* 2022).

A comparison of null alleles between blood and scat genotypes showed that markers FCA_026, FCA_043, and FCA_088 had a zero null allele value in both blood and scat individuals. Markers FCA_023, FCA_126, FCA_132 had low null allele frequencies. These six markers would be the better choice for population genetics studies than the other markers that have higher null alleles. Black-footed cats have low population sizes and are endemic to southern Africa, unlike other wildlife species. Therefore, these markers would have a higher probability of identity when compared to other species with larger populations. Null alleles can result from low DNA template quality, mutations in the flanking region, or differential amplification of size variant alleles. The source of null alleles can be assessed through the comparison of alleles (Chapuis and Estoup 2007; Dakin and Avise 2004) and sequencing of the primer annealing sites (Callen *et al*. 1993).

Identifying individuals using blood samples showed that seven markers were highly informative (PIC value higher than 0.6). Analysis of the scat samples, however, showed that only five markers were highly informative. This would be a direct result of allelic dropout in those markers that would result in a reduced PIC value. This is to be expected from non-invasive samples. This study, therefore, provides an accurate guideline on the best markers to use when studying non-invasive samples. Other studies have shown that as few as three markers were sufficient to discriminate individuals accurately (Parsons 2001).

Individual identification of black-footed cats using the older popular programs such as Cervus (Kalinowski *et al.* 2007) and Colony (Jones and Wang 2010) greatly overestimated the number of individual cats. This is because these programs do not take into account errors introduced by allelic dropout through direct comparison and are not ideal for non-invasive genetic sampling. GIMLET (Valière 2002) showed the best results when used across the same individuals with both blood and scat samples. This is mainly because GIMLET is adapted to analyzing genotypes obtained from non-invasive samples and accommodates error rates between the reference genotypes (blood samples) and the genotypes required for analysis (scat genotypes).

This study highlights the need to assess markers in low-quality samples before use in scat samples with a larger dataset, especially for endangered or threatened species where a non-invasive approach is ideal. Once these markers are verified, as with this study, they can be used in other studies, such as population structure and genetic diversity (Schwartz *et al.* 2007). A paired sample-based genotyping method such as this one provides the most accurate validation of scat DNA markers and other low-quality DNA sources such as hair. Amplification was possible from all the nine markers used. However, some markers amplified better than others. Marker performance can be affected by various reasons, such as the allelic range, DNA degradation, or PCR artifacts. In most cases, the actual cause of reduced marker performance cannot be accurately determined. As such, an assessment of markers in scat DNA to show which markers are most reliable is necessary before using the markers in larger datasets. This improves the estimation of allelic dropout and false allele error rates through a reference dataset (Johnson and Haydon 2007).

This study, like other studies that shows that without careful assessment of markers skewed population estimates may be reported (Forgacs *et al.* 2019). However, because studies involving rare or endangered species rely on non-invasive samples, it is prudent to use studies such as this one to verify the reliability of each marker used and possibly select only the most reliable markers. This greatly reduces skewed estimates of genetic diversity (Taberlet *et al.* 1996). Our pilot study can be used as a reference to monitor the growth of the black-footed cat population in the Benfontein area. Similar studies have been used to monitor brown bear (*Ursus arctos*) populations after reintroduction in the Italian Alps (De Barba *et al.* 2005).

Data availability statement

All the data used in this research is available within the manuscript. Additional data is available on figshare https://figshare.com/s/a6bb2663a502140e41e0.

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CHAPTER 5

DNA metabarcoding to determine the diet of the elusive black-footed cat *Felis nigripes*

5.1 Abstract

The study of the diet of predators provides key insight into overall ecosystem health and provides information such as population persistence and competition with coexisting predators, which is vital for the conservation management of threatened carnivores. However, for smaller carnivores such as black-footed cats, diet analyses can be complicated due to their elusive nature and low population densities which make traditional methods of diet assessment challenging. DNA metabarcoding of scats has been shown to be an efficient and accurate method of identifying prey taxa across a wide range of carnivore species. Using scats collected in the field and a multi-marker DNA metabarcoding approach, this study analysed the diet of black-footed cats. Sixty-five prey items were identified consisting of thirty-five families and twenty-two orders. The diet of black-footed cat consisted of twenty-five mammals, twenty-three birds, 8 carnivores, 4 reptiles and amphibians, 3 fish and 2 invertebrates. Mammal species made up the majority of the prey items, while our data suggests that invertebrates were not routinely consumed. Across 12 individual black-footed cats, small mammals in the rodent family were evenly distributed. However, some prey items were not consistent across all the 12 individual cats. The Chiroptera species were only found in four of the cats. Some species could not be identified because the reference DNA species were absent in the available DNA repository libraries. Better resolution of prey items can be obtained by extending the DNA sequence libraries.

Keywords: scats, eDNA, dietary scat analysis, non-invasive, high-throughput sequencing

5.2 Introduction

Knowledge derived from the study of predator diets is essential to determine the role of the predator in an ecological niche (Ritchie *et al.* 2012), evaluate the possible factors, if any, that limit population persistence, and determine any competition for resources with other

carnivores (Hammerschlag *et al.* 2019). This data feeds directly into the design of effective management and conservation strategies (Soule *et al.* 2003). As a consequence, diet assessment is considered an active area of research (Thuo *et al.* 2019), and there are a range of methods that can be used to determine the dietary components of predators. Traditional methods, such as direct observations of carnivores in the wild, are time-consuming (Marcolin *et al.* 2020; Weiskopf *et al.* 2016) and not suitable for rare and elusive carnivores. Scat samples have been used to determine wildlife distribution (Kovach *et al.* 2003; Palomares *et al.* 2002), species identification (Fernandes *et al.* 2008; Kurose *et al.* 2005; Reed *et al.* 1997), individual analysis and population estimates (Adams and Waits 2008; Coetzer *et al.* 2017), parentage (Bernatchez and Duchense 2000), as well as diet (Berry *et al.* 2017; Deagle *et al.* 2005; Parsons *et al.* 2005). Identification of prey through morphological analysis of remains in scat samples often requires taxonomic expertise, which can make accurate identification of prey taxa challenging (Alheit and Scheibel 1982; De Sousa *et al.* 2019; Scholz *et al.* 1991; Sheppard and Harwood 2005) especially for closely related prey taxa (Da Silva *et al.* 2019; Keller 1998). Soft-bodied prey is also easily digested, and some species can be missed, leading to incomplete dietary information (Pompanon *et al.* 2012; Symondson 2002).

High-throughput sequencing (Alberdi *et al.* 2019; Kircher and Kelso 2010) methods such as DNA metabarcoding of scats are considered an efficient and accurate non-invasive option for diet analyses (Chapple and Ritchie 2013; Deagle *et al.* 2005; Pompanon *et al.* 2012). DNA metabarcoding of scats allows for the simultaneous identification of both the predator and prey species consumed (Monterroso *et al.* 2018; Shao *et al.* 2021a; Shao *et al.* 2021b). For example, metabarcoding of scat samples was used for the parallel identification of bat predators and their prey (Galan *et al.* 2017). Furthermore, DNA metabarcoding has been shown to successfully identify highly degraded prey items as well as rare prey items (Harper *et al.* 2020; Nielsen *et al.* 2018; Traugott *et al.* 2020). DNA metabarcoding has been used successfully to determine the diet of a variety of animals (Klare *et al.* 2011; Monterroso *et al.* 2018; Shao *et al.* 2021b) ranging from badger and fox (Nørgaard *et al.* 2021), leopard cat (Shehzad *et al.* 2012), leopard (de Jesus 2021), otter (Buglione *et al.* 2020), Pyrenean desman (Gillet *et al.* 2015), fish (Traugott *et al.* 2020), to scampi (van der Reis *et al.* 2018). The ability of scat DNA metabarcoding to identify a broad range of dietary taxa has been used to assess the diet of generalist predators and omnivores (Tercel *et al.* 2021). Scat DNA metabarcoding

can however not replace traditional methods, such as direct observation, as DNA-based analyses cannot provide information on the size or life stage of prey taxa (Harper *et al.* 2020).

The success of DNA metabarcoding is heavily dependent on the availability and completeness of DNA reference libraries (Nielsen *et al.* 2018; Traugott *et al.* 2020), the choice of metabarcoding primers (Ficetola *et al.* 2010), and the application of a threshold to remove low abundance reads (Deagle *et al.* 2019). In some cases, a multi-marker approach may be more informative because primers may not amplify consistently across all prey taxa, and using a single universal primer can influence what prey species will be identified (Da Silva *et al.* 2019). For carnivores that have a simple diet, such as snow leopards, that feed on about ten prey items (Weiskopf *et al.* 2016), a single primer pair may be sufficient (Browett *et al.* 2021). However, for carnivores with a broad prey range, multiple primers amplifying different regions of the genome are recommended to reveal the complete diet (Da Silva *et al.* 2019; Quéméré *et al.* 2021).

Black-footed cat *Felis nigripes* (Burchell 1824) is the smallest felid endemic to southern Africa (Sliwa 2004; Sliwa *et al.* 2016). It is known as a generalist predator and often a scavenging predator with a diet mainly composed of small mammals, birds, and invertebrates. The diet composition of black-footed cats had previously been described using visual observations of the cat in the wild (Sliwa 1994; Sliwa 2006). These studies tend to be tedious and time-consuming, covering long distances in order to adequately assess the target prey (Sliwa 2006) and often more difficult for nocturnal small carnivores (Symondson 2002) and generalist predators (Sheppard and Harwood 2005). Additionally, thorough taxonomic knowledge is necessary to accurately identify the consumed prey items (Corse *et al.* 2010). DNA metabarcoding could be an alternative for assessing the diet of black-footed cats using scats collected in the wild. Comparing the results obtained from a combination of non-invasive sample collection and high-throughput sequencing to studies using visual observations can be used to test the application of DNA metabarcoding to diet assessments. The results can be used to determine if DNA metabarcoding will provide better resolution of prey items than visual observations.

DNA metabarcoding as a tool to study the diet of various carnivores is well documented, but the use of this genetic tool in African carnivores is still limited. This study aimed to use

metabarcoding of DNA extracted from scat to determine the diet of the elusive black-footed cat. The study also compared diet between sexes and the distribution of the various prey items across different individual cats. Comparison was made between previous diet studies that did not use a molecular DNA approach to determine if DNA metabarcoding showed improved resolution.

5.3 Materials and methods

5.3.1 Sample collection, DNA extraction, individual identification, and sexing

Black-footed cat scats were collected in the Benfontein Nature Reserve near Kimberley, Northern Cape Province, South Africa (28°50'S, 24°50'E). This area lies in the center of the known distribution of black-footed cats (Nowell and Jackson 1996) and has been an active site for black-footed cat research including diet analyses (Sliwa 1994; Sliwa 2006). The study area (60 km^2) encompassed a variety of arid vegetation communities (Sliwa 1996) including the elements of 3 major biomes: Kalahari thornveld, pure grassveld, and the Karoo (Acocks 1988). Black-footed cats, like other small cats, rarely deposit scats in visible or predictable sites (Molteno *et al.* 1998). In this study, scat samples were collected using a trained detection dog-handler team (Long *et al.* 2007; MacKay *et al.* 2008). The training of dogs to detect black-footed cat scat followed general guidelines recommended for conservation detection dogs (DeMatteo *et al.* 2019; Reed *et al.* 2011). Scat samples were collected from the 3rd March 2020 to the 5th October 2020 as part of an ongoing research project of the Black-footed Cat Working Group (Sliwa *et al.* 2021). Scats were preserved in the field using 90% ethanol. Permits for collection of samples was provided by the Northern Cape Department of Environment and Nature Conversation (FAUNA 1218/2016, FAUNA 0636/2021). Eighty-three potential black-footed cat scats were collected in the Benfontein Nature Reserve, Kimberley, Northern Cape, South Africa. Thirteen of the scats were collected directly from the cats, sixty-six were obtained through the use of detection dogs, and the remaining four samples were identified as possible black-footed scats by field staff. DNA from the scats was extracted using Qiagen QIAmp Fast DNA stool kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions.

The area surveyed could have included the territories of a number of cats. Scats were assigned to individuals using a panel of nine unlinked autosomal feline microsatellites (Menotti-Raymond and O'brien 1995; Menotti-Raymond *et al*. 1997). These markers were initially designed for domestic cats (Menotti-Raymond *et al*. 1999) but have been used previously in black-footed cats (Mattucci *et al*. 2018) including scats (Siziba *et al*. 2024). All forward primers were labeled with a fluorescent dye. Amplifications were performed in 12.5µL reactions containing 6.25µL TEMPase Multiplex Taq (Amplicon, Odense, Denmark), 4µL dH_2O, and 0.25µL each of the forward and reverse primer. Amplifications were performed with an initial denaturation step at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 15 secs, annealing at 57°C for 30 secs, extension at 68°C for 30 secs and a final extension of 68°C for 5 mins. Amplicons were sent to the Central Analytical Facility (CAF) at Stellenbosch University, South Africa, for fragment analyses using an ABI PRISM™ 3500XL Genetic Analyzer (Life Technologies, Applied Biosystems, Warrington, UK) and a standard GeneScan™ ROX500™ (Applied Biosystems) internal size standard. The software package Geneious v8 (https://www.geneious.com) was used for genotype scoring. All scat samples were re-genotyped up to five times, and genotypes were compared to check for consistency. Assignment of scats to individual cats was done using GIMLET v1.3.3 (Valière 2002), which takes into account allelic drop-out associated with degraded DNA often extracted from scat samples (Siziba *et al*. 2024). Scats belonging to the same cat were pooled into equimolar concentrations of 30 µl per individual.

All the scat samples were assigned to sexes using sex-determination markers (Griffiths and Tiwari 1993; Kurose *et al.* 2005). The presence of a PCR product identified each individual as male. Individuals with no bands were repeated to confirm the absence of a PCR product. Confirmation of the absence of a PCR identified the individual as female.

5.3.2 PCR assay and metabarcoding sequencing

An assay comprising four metabarcoding primers (Table 5.1) across 12 Individual black-footed cats (Siziba *et al.* 2024) was set up using a HotStarTaq Plus Master Mix Kit (Qiagen, USA). The cycling conditions were made up of an initial denaturation of 95 °C for 5 minutes, 30 cycles of 95°C for 30 secs, 53°C for 40 secs, 72°C for 1 min, and a final elongation step at 72°C for 10 mins. These primers had previously been tested in silico for their efficacy in identifying

possible species that make up the diet of black-footed cat (Chapter 4). After amplification, PCR products were visualised using a 2% TBE agarose gel to determine amplification success, size, and intensity of each band. Samples were multiplexed using unique dual indices and pooled together in equal proportions relative to their DNA concentrations and molecular weight. The samples were purified using Ampure XP beads (Beckman Coulter, Indianapolis, USA) and used to prepare an Illumina DNA library at the Molecular DNA (MR DNA) Research Lab (Shallowater, Texas, USA)**.**

Table 5.1. List of primers used for scat metabarcoding to identify prey species of black-footed cat (*Felis nigripes*).

Gene	Taxonomic group	Primer name	Primer sequence (5' – 3')	Amplicon length (bp)	Reference
16S rRNA	Mammal	16Smamm1	CGGTTGGGGTGACCTCGGA	140	Taylor 1996
		16Smamm2	GCTGTTATCCCTAGGGTAACT		
12S rRNA	Vertebrate	12SV5	TTAGATACCCCACTATGC	90	Riaz *et al*. 2011
		12SV5B2	TAGAACAGGCTCCTCTAG		
COI	Universal	illmlCOIintF	GGWACWGGWTGAACWGGTWTAYCCYCC	330	Geller *et al.* 2013
		jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA		Leray *et al*. 2013
Cyt *b*	Rodent	L15411F	GAYAAARTYCCVTTCAYCC	136	Galan *et al*. 2012
		H15546R	AARTAYCAYTCDGGYTTRAT		

5.3.3 Data filtering and Operational Taxonomic Unit (OTU) analyses

Paired sequence reads were imported into Geneious Prime v2023.1.2 and trimmed using the BBDuk plugin Q30 (Kearse *et al.* 2012) to remove sequencing primers and adapters. The amplicons were merged using the BBMerge plugin tool, and reads that could not be merged because they were too short after quality trimming were discarded. Reads that were too long, possibly due to contamination, were also discarded. Chimeric reads with the expected amplicon length were removed from the remaining reads using the UCHIIME plugin. Sequence reads were clustered according to similarity into Operational Taxonomic Units (OTUs) using USEARCH (Edgar 2010) using a 97% similarity threshold. OTUs with less than 100 sequences were deleted to remove low abundant potentially erroneous sequences. Paired sequences

were also filtered into zOTUs (zero-radius OTUS) to reduce the loss of biological information. zOTUS identify all sequences, even those with a single difference, unlike OTUS, which groups sequences that have 97% similarity.

5.3.4 Taxonomic Assignment

Amplicons that passed the quality filtering process were used to perform a BLASTn search against the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database [accessed July 2023] (Benson *et al.* 2015) using BLASTn (Basic Local Assignment Search Tool) (Altschul *et al.* 1990) with the default parameters and a reward of 1.

Each species identified through DNA metabarcoding was confirmed to make up the diet of black-footed cat by checking the presence of each species in the same geographic region as the predator using the Global Biodiversity Information (GBIF) website [accessed February 2024). The search parameters were made up of a derived dataset of occurrences in the continent of Africa, in the country South Africa and in the administrative area of the Northern Cape. Either the species name, genus, family or order were used to confirm the presence of the prey item in the Northern Cape.

5.4 Results

5.4.1 Sample collection, DNA extraction, individual identification, and sexing

DNA was successfully extracted for each scat sample and amplified using the nine microsatellite loci. The scats were identified as either male or female, depending on the presence or absence of a 135bp PCR product. Seven black-footed cats were identified as female, and the other five as male

5.4.2 PCR assay and metabarcoding sequencing

Three of the four primer pairs were successfully amplified and sequenced. The cyt *b* primer pair (Galan *et al.* 2012) was unsuccessful in the metabarcoding sequencing. The primer failed to amplify the extracted DNA even after multiple troubleshooting attempts such as decreasing the annealing temeperature, increasing the primer and DNA concentration and

addition of cofactors such as $MgCl_2$. The cyt *b* primers could therefore not be used for further downstream analyses.

5.4.3 Data filtering and Operational Taxonomic Unit (OTU) analyses

A total of 9 157 066 reads were sequenced, 2 051 054 were obtained from the COI region, 3 001 704 from the 16S rRNA region, and 4 104 308 from the 12S rRNA region. The paired reads were trimmed, merged, and made up a total of 1 420 468 reads. COI region made up of 123 978, 16S rRNA had 80 481 reads, and 12S rRNA had 1 216 049 reads.

5.4.4 Taxonomic Assignment

GenBank was used to provide taxonomic identification for each OTU. The confidence level of the sequence assignments was modified from 97% and above of the query sequence (Razgour *et al.* 2011). During the BLASTn process, single nucleotide substitutions can be attributed to individual variation (Parsons *et al.* 2005). The prey list for each black-footed cat was compiled using information from all three markers based on percentage similarity to the query sequence as specified by Edgar, (2010). Taxonomic assignment was classified as species (> 97%), unclassified genus (95 – 97 %), unclassified family (90 – 94%), unclassified order (85 – 89%), unclassified class (80 – 84%), unclassified phylum (77 – 79%), and, unknown (< 77%). As well as the occurrence of each pre item as per GBIF Species identification per gene region was collated as indicated in Table 5.2. The diet of black-footed cat comprised of a total of 64 species belonging to 22 orders and 35 families. Forty five species were confirmed to the species level and 14 species were confirmed to the genus level. Five species could not be assigned to either genus or species level due to lower than required percentage similarities (< 95%) and were therefore assigned to either family or order. The prey items that could not be assigned to either species or genus were from the family Geomydidae, Strigidae and Phasianidae. The remaining prey items that could not be assigned to either species, genus or family level were of the order Squamata and Pelecaniformes.

The diet of black-footed cat was made up of 33 mammals, 23 birds, 2 invertebrates, 3 fish and 4 reptiles and amphibians. The majority of the black-footed cat diet was made up of 22 small mammal rodent species and birds (Table 5.2).

Table 5.2. Species list of prey items of black-footed cat (*Felis nigripes*) identified through DNA metabarcoding of the 12S rRNA, 16S rRNA and COI gene regions and GBIF.

Order	Family	Scientific name	Common name	Gene region
Large mammals				
Artiodactyla	Bovidae	*Bos indicus*	Cattle	12S rRNA, 16S rRNA
		Tragelaphus spp.	Antelope	12S rRNA
	Suidae	*Potomochoerus larvatus*	Bushpig	12S rRNA, 16S rRNA, COI
Small mammals				
Rodentia	Muridae	*Desmodillus auricularis*	Cape short-eared gerbil	12S rRNA
		Gerbilliscus vallinus	Bushy-tailed hairy-footed gerbil	12S rRNA
		Gerbilliscus leucogaster	Bushveld gerbil	12S rRNA, 16S rRNA, COI
		Gerbilliscus afra	Cape gerbil	12S rRNA
		Gerbilliscus paeba	Hairy-footed gerbil	12S rRNA, 16S rRNA
		Mus indutus	Desert pygmy mouse	12S rRNA, 16S rRNA, COI
		Rhabdomys bechuanae	Muroid rodent	COI
		Rhabdomys pumilio	Four-striped grass mouse	12S rRNA, 16S rRNA
		Mastomys coucha	Southern multimammate mouse	12S rRNA, 16S rRNA
		Thallomys nigricauda	Black-tailed tree rat	12S rRNA
		Otomys unisulcatus	Bush vlei rat	12S rRNA
		Paratomys brantshii	Brants's whistling rat	12S rRNA, 16S rRNA
		Micaelamys namaquensis	Namaqua rock rat	12S rRNA, 16S rRNA
	Scuiridae	*Xerus inauris*	Cape ground squirrel	12S rRNA
		Praxerus cepapi	Smith's bush squirrel	12S rRNA
	Nesomyidae	*Malaconthrix typica*	Gerbil mouse	12S rRNA
		Saccostomus campestris	South African pouched mouse	12S rRNA
Eulipotyphla	Soricidae	*Crocidura cynae*	Reddish-gray musk shrew	12S rRNA
		Atelerix frontalis	Southern African hedgehog	12S rRNA, 16S rRNA, COI
Afrosoricida	Chrysochloridae	*Chrysochloris asiatica*	Cape golden mole	12S rRNA, 16S rRNA

Chiroptera	Mollossidae	*Tadarida aegyptiaca*	Egyptian free-tailed bat	12S rRNA
	Vespertilionidae	*Neromicia capensis*	Cape serotine bar	12S rRNA
Carnivores				
Carnivora	Herpestidae	*Suricata suricatta*	Meerkat	12S rRNA, 16S rRNA, COI
		Cynictis penicillata	Yellow mongoose	12S rRNA
		Herpestes spp.	Mongoose	16S rRNA
		Mungos mungo	Banded mongoose	12S rRNA
	Viverridae	*Genetta* spp.	Genet	12S rRNA, 16S rRNA, COI
	Mustelidae	*Poecilogale albinucha*	African Striped Weasel	12S rRNA
		Mellivora capensis	Honey badger	12S rRNA, 16S rRNA
		Ictonyx striatus	African polecat	12S rRNA
Birds				
Passeriformes	Alaudidae	*Chersomanes albofasciata*	Spike-heeled lark	16S rRNA
	Muscicapidae	*Calliope* spp.	Rubythroat	12S rRNA
		Myrmecocichla formicivora	Ant-eating chat	16S rRNA
	Cistcolidae	*Cisticola* spp.	Cisticola	16S rRNA
	Remizidae	*Anthoscopus minitus*	Cape penduline tit	12S rRNA
	Hirundinidae	*Hirundo rustica*	Barn swallow	12S rRNA
	Pycononotidae	*Pycnonotus nigricans*	African red-eyed bulbul	12S rRNA
	Phylloscopidae	*Phylloscopus trochilus*	Willow warbler	12S rRNA, 16S rRNA
	Passeridae	*Passer* spp.	Sparrow	16S rRNA
	Locusteliidae	*Bradypterus baboecala*	Little rush warbler	12S rRNA
Accipitriformes	Accipitridae	*Haliaeetus vocifer*	African fish eagle	12S rRNA
		Accipiter spp.	Hawk	12S rRNA
		Buteo spp.	Buzzard	12S rRNA
		Milvus spp.	Kite	12S rRNA
		Gyps coprotheres	Cape vulture	16S rRNA
		Necrosyrtes monachus	Hooded vulture	16S rRNA, COI
Strigiformes	Strigidae	-	Unidentfied owl*	12S rRNA
Caprimulgiformes	Caprimulgidae	*Caprimulgus* spp.	Nightjar	12S rRNA

Procellariformes	Procellaridae	*Ardenna* spp.	Shearwater	12S rRNA
Apodiformes	Apodidae	*Aerodramus* spp.	Swiflet	12S rRNA
Pelecaniformes	-	-	Unidentfied waterbird*	12S rRNA
Gallioformes	Phasianidae	-	Unidentified landfowl*	12S rRNA, 16S rRNA
Podicipediformes	Podicipedidae	*Tachybaptus ruficollis*	Little grebe	12S rRNA
Invertebrates				
Scorpiones	Scorpionidae	*Opistophthalmus* spp.	Burrowing scorpion	COI
Scolopendromorpha	Cryptopidae	*Scolopendra morsitans*	Red-headed centipede	COI
Reptiles and amphibians				
Testudines	Geoemydidae	-	Unidentified turtle*	12S rRNA
	Pelomedusidae	*Pelomedusa* spp.	African turtle	12S rRNA
Squamata	-	-	Unidentified snake*	12S rRNA
Anura	Microhylidae	*Phrynomantis annectens*	Marbled rubber frog	16S rRNA
Fish				
Anguilliformes	Anguillidae	*Anguilla mossambica*	African longfin eel	12S rRNA
Cichliformes	Cichilidae	*Orechromis mossambicus*	Mozambique tilapia	16S rRNA
		Tilapia sparrmanii	Banded tilapia	12S rRNA

Based on GBIF occurrence results the unidentified owl species could either be *Ptilopsis grantii* (southern white-faced owl), *Otus senegalensis* (African scops owl), *Bubo africanus* (spotted eagle-owl), *Claucidium perlatum* (pearl-spotted owlet) or *C. capense* (African barred owlet). The unidentified water birds could either be any of the occurring ten species. Namely, *Bostrychia hagedash* (hadada ibis), *Ardea cinera* (grey heron), *A. goliath* (giant heron), *A. brachyrhyncha* (yellow-billed egret), *Bubulcus inis* (cattle egret), *Scopus umbretta* (hamerkop), *Threskiornis aethiopicus* (African sacred ibis), *Platelea alba* (African spoonbill), *Pecelecanus onocrotalus* (great white pelican) or *Plegadis falcinellus* (glossy ibis).

The unidentified landfowls could either be *Pternistis adspersus* (red-billed spurfowl), *Scleroptila afra* (grey-winged francolin), *Coturnix cortunix* (common quail), *Fancolinus africanus* (grey-winged francolin), *F. swainsonii* (Swainson's francolin), *F. adspersus* (red-billed francolin) or *F. capensis* (Cape Francolin). The unidentified turtles could either be *Chersina angulate* (angulate tortoise), *Stigomocheyl pardalis* (leopard tortoise) or *Psammobates tentoris* (tent tortoise). The unidentified snake is the most difficult to determine which species possibly be the prey items as there are about 60 species of snakes recorder in the same region as black-footed cat used in this study.

The 12S rRNA was the most informative DNA region for diet assessment, with the largest number (49) of birds and small mammal species. Although COI identified the largest number of families, the majority of them were not informative of the diet of black-footed cat but instead consisted of parasites, fungi, and mites. The 16S region had the lowest representative species but still showed higher dietary components for some orders than the COI region (Figure 5.1).

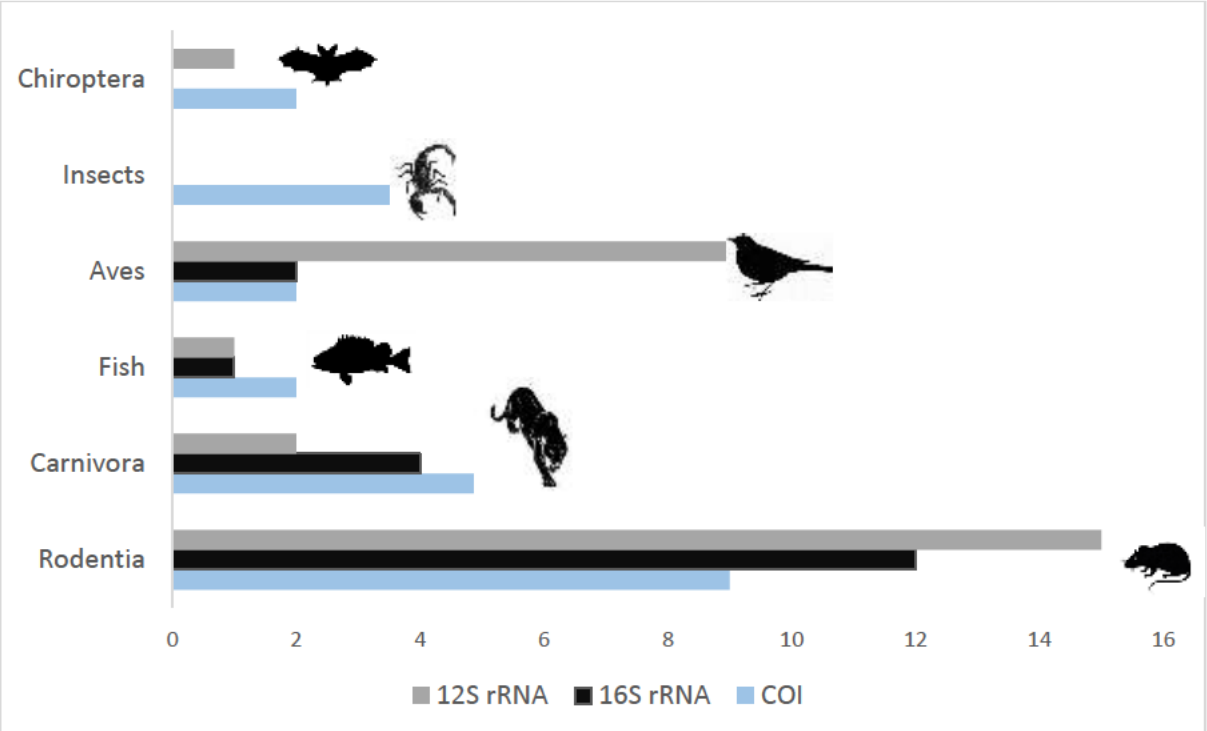


Figure 5.1. Different food groups of black-footed cat (*Felis nigripes*) diet shown by the different gene region.

The 12S rRNA identified the largest number of species that make up the diet of black-footed cats. The 12S rRNA region identified thirty-eight species, and 8 and 3 species for the 16S rRNA and COI gene regions respectively. Ten species were identified simultaneously by both 12S rRNA and 16S rRNA, one specie was identified by the 16S rRNA and COI, while all three gene regions identified 6 species simultaneously. No species were identified by both 12S rRNA and COI (Figure 5.2).

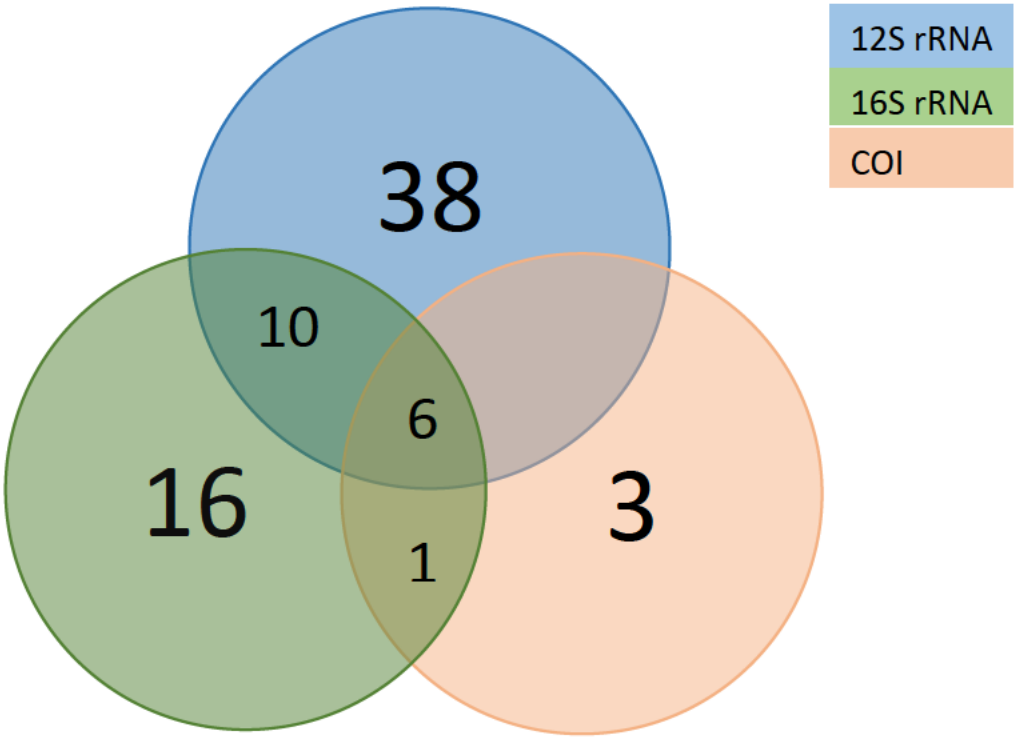
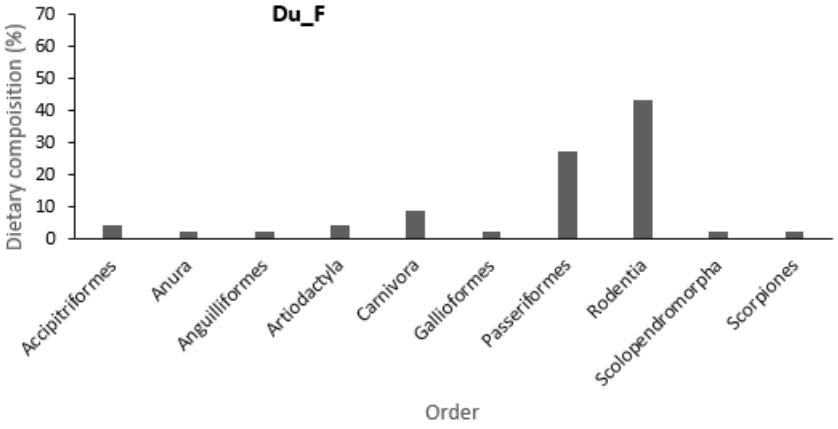


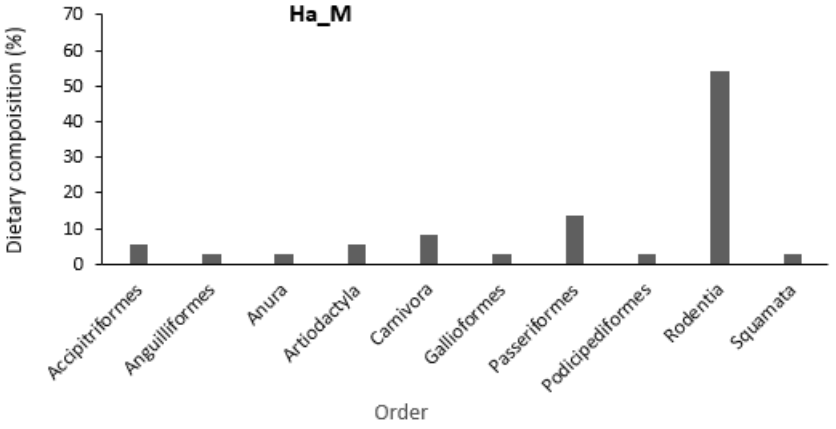
Figure 5.2. Venn diagram showing correlation between the different gene regions in identification of species that make up the diet of black-footed cat (*Felis nigripes*).

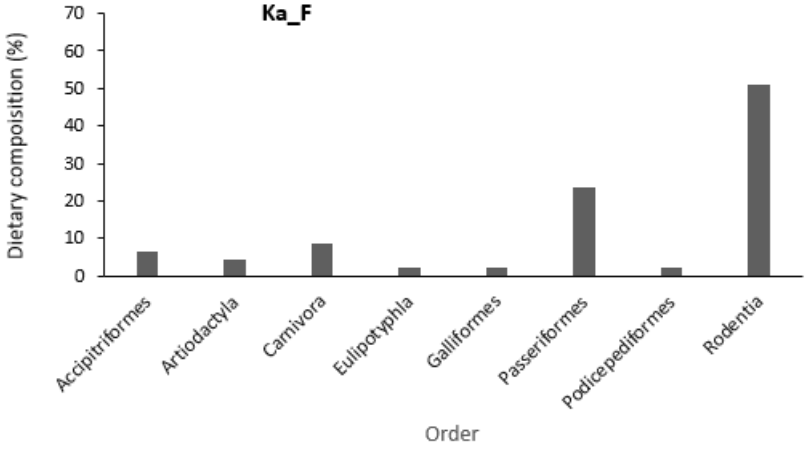
The diet of individual black-footed cats ranged from seven (Individual Sc8 and Individual PO4) to thirteen different prey orders (Individual Sc13). Five orders Accipitiformes. Artiodactyla, Carnivora, Passeiformes, and Rodentia were found in all 12 black-footed cats. Across all black-footed cats, small mammals of the order Rodentia comprised the majority (~50%) of prey items. In some of the black-footed cats, in addition to the Rodentia order, small mammals also included Chiroptera (PO2, PO4, Sc5A, and Sc50), Eulipotyphla (Ka, PO2 and Sc10), and Afrosoricida (Sc10) orders (Figure 5.2). Birds comprised the second largest dietary profile (~20%), with the orders Accipitriformes and Passeiformes found in all 12 cats. Some bird

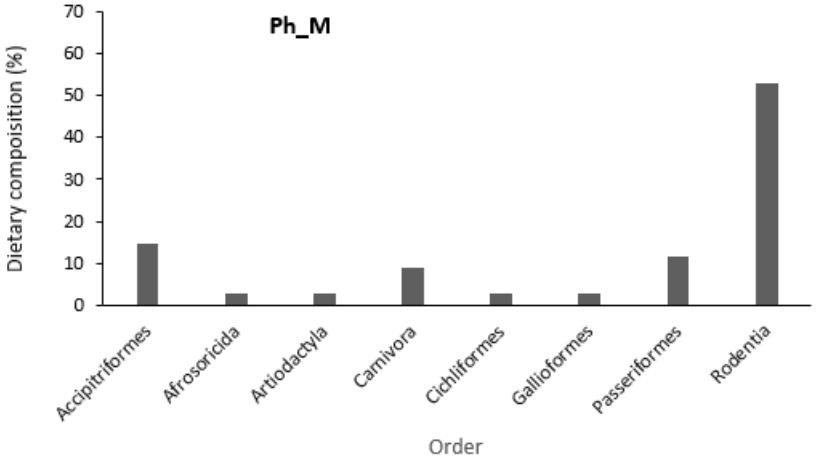
orders, such a Gallioformes, were found in nine of the black-footed cats (Du, Ha, Ka, Ph, PO2, Sc5A, Sc8, Sc11, and Sc13). The Carnivora order was present in all 12 individual cats and ranged from 2% to 13% of the total prey items per individual. For some orders distribution of prey items across the 12 individuals was disproportionate. Seven orders were only found in only one of the twelve cats. For example, the Caprimulgiformes (Sc13), Pelecaniformes (Sc13), Procellariformes (Sc5A), Scolopendromorpha (Du), Scorpiones (Du), Squmata (Ha) and Strigiformes (Sc13) .Some orders were only represented in two of the black-footed cat individuals, such as, Anura (Du and Ha) and Cichliformes (Ph and Sc13) (Figure 5.3).

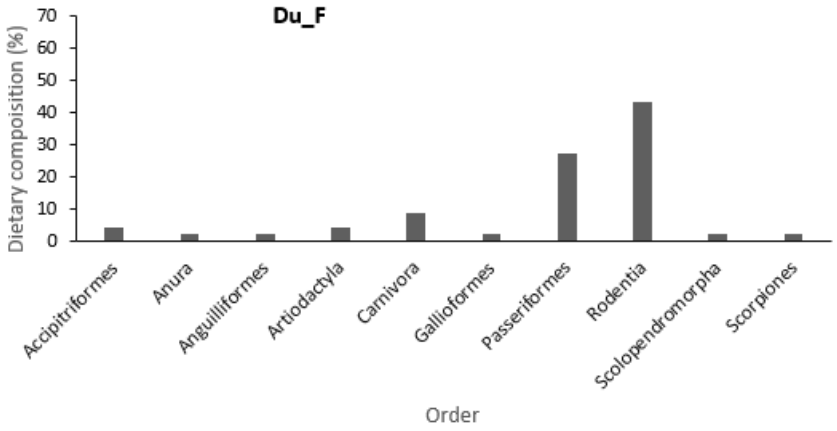
The black-footed cats consisted of seven females and five males. Dietary distribution was not evenly distributed between the sexes. The female dietary profile had a wider dietary range consisting of more species than the males (Figure 5.4). Fourteen different orders of species were shared between the males and females. However, the females had seven additional orders that were not found in any of the males, while the males that had only one orders not found in the females.

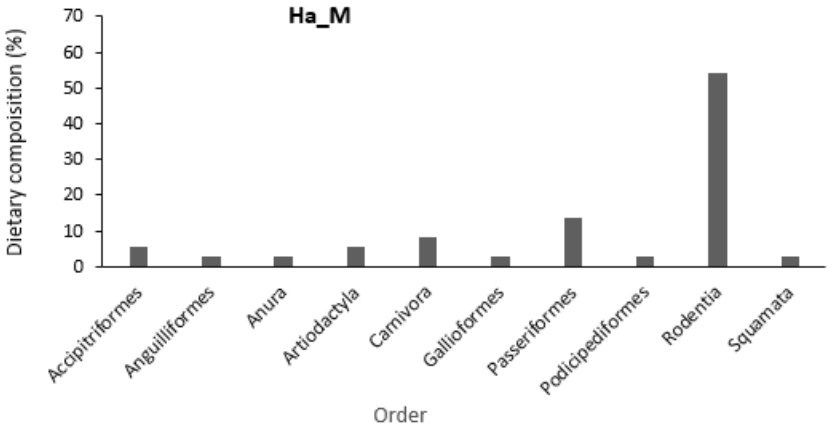

Du_F
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Anura
Anguilliformes
Artiodactyla
Carnivora
Gallioformes
Passeriformes
Rodentia
Scolopendromorpha
Scorpiones
Order

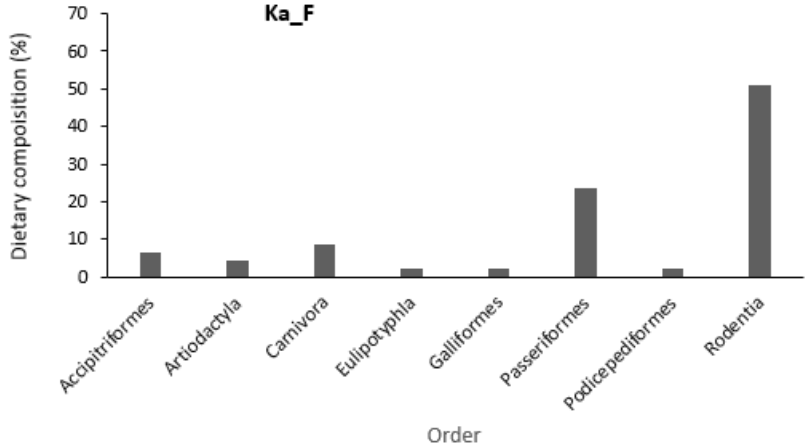

Ha_M
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Anguilliformes
Anura
Artiodactyla
Carnivora
Gallioformes
Passeriformes
Podicipediformes
Rodentia
Squamata
Order

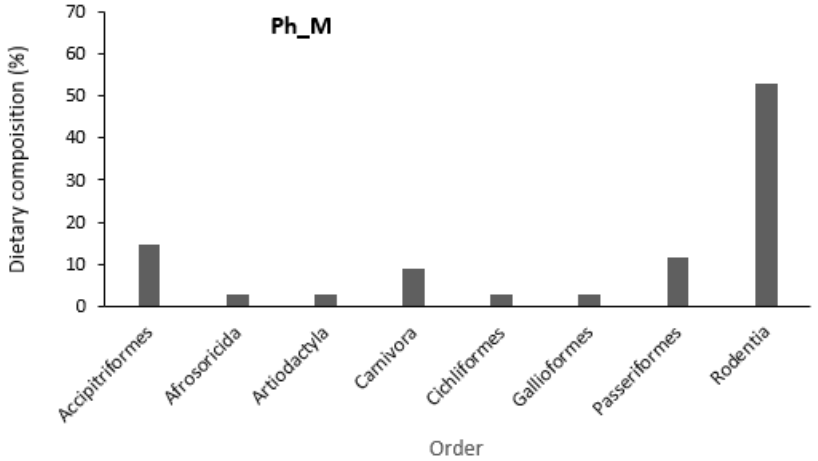

Ka_F
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Artiodactyla
Carnivora
Eulipotyphla
Galliformes
Passeriformes
Podicepediformes
Rodentia
Order


Ph_M
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Afrosoricida
Artiodactyla
Carnivora
Cichliformes
Gallioformes
Passeriformes
Rodentia
Order


Du_F
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Anura
Anguilliformes
Artiodactyla
Carnivora
Galliioformes
Passeriformes
Rodentia
Scolopendromorpha
Scorpiones
Order


Ha_M
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Anguilliformes
Anura
Artiodactyla
Carnivora
Galliioformes
Passeriformes
Podicipediformes
Rodentia
Squamata
Order


Ka_F
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Artiodactyla
Carnivora
Eulipotyphla
Galliformes
Passeriformes
Podicepediformes
Rodentia
Order


Ph_M
Dietary compoisition (%)
70
60
50
40
30
20
10
0
Accipitriformes
Afrosoricida
Artiodactyla
Carnivora
Cichliformes
Galliioformes
Passeriformes
Rodentia
Order

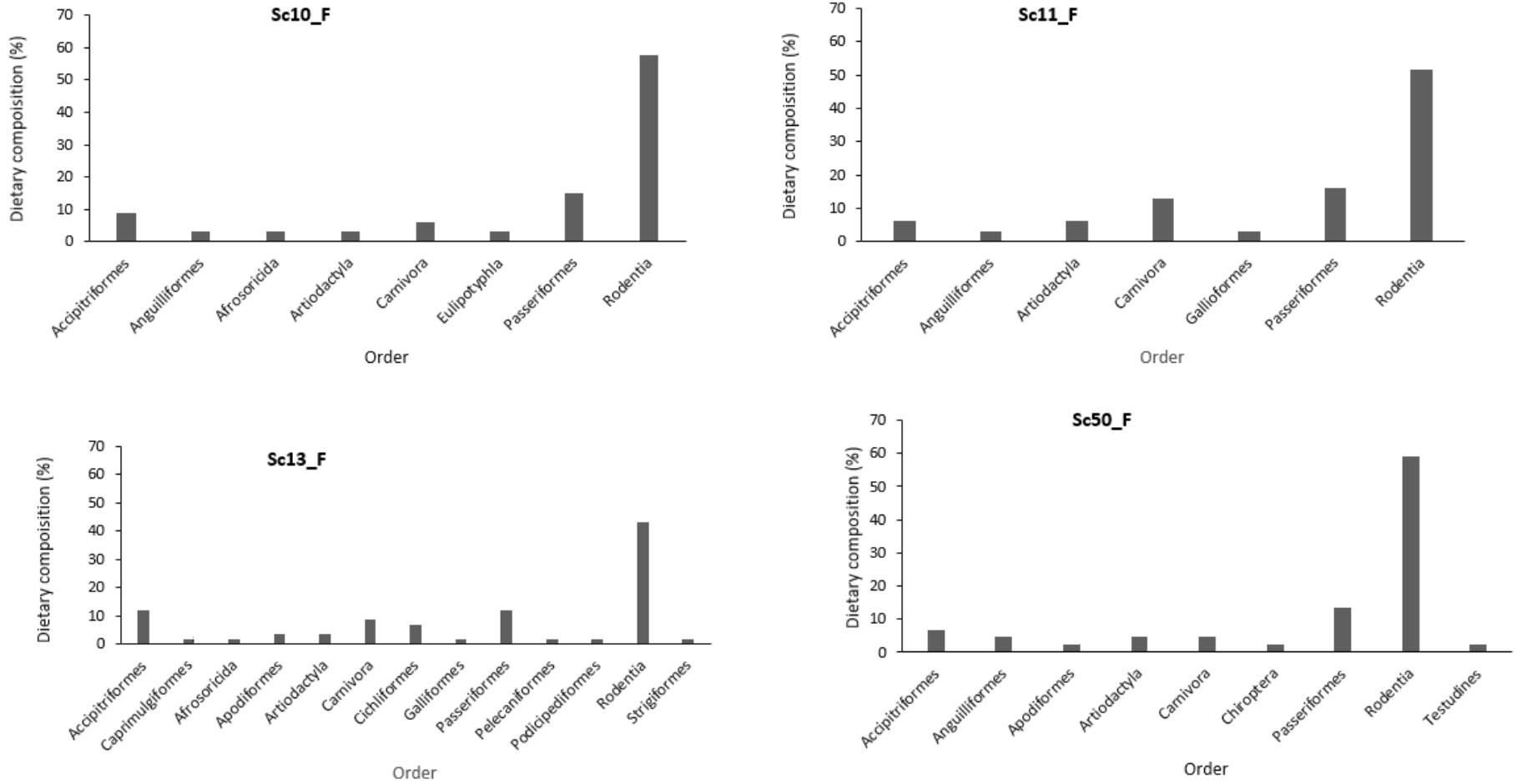


Figure 5.3. Bar graphs showing dietary profile per individual black-footed cat (*Felis nigripes*) across different orders of prey items. The F denotes female, and the M denotes male.

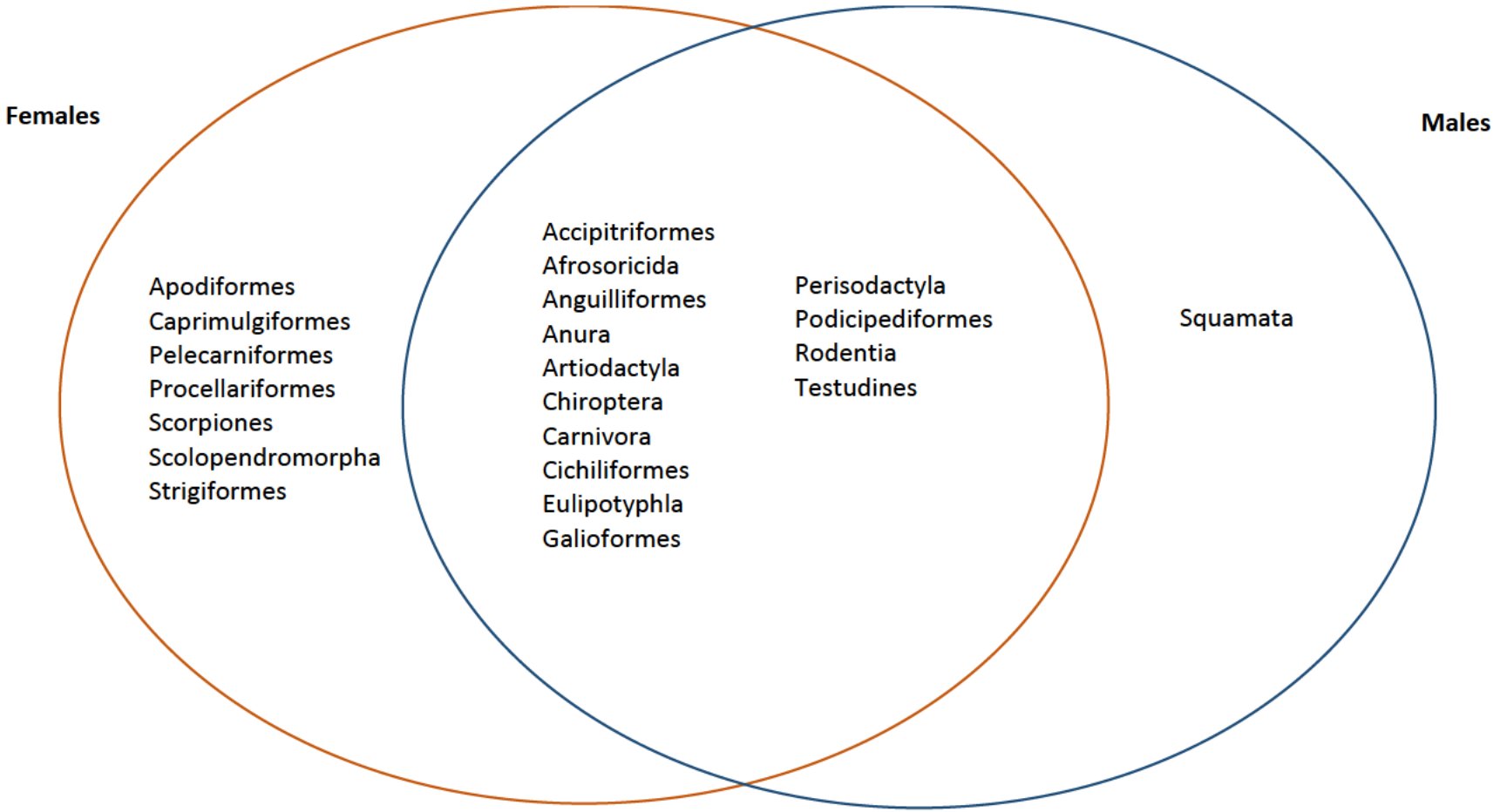


Figure 5.4. Venn diagram showing differences and similarities of the prey order in female and male diet composition of black-footed cats (*Felis nigripes*)

5.5 Discussion

This is the first study that investigates and describes the diet of black-footed cats using DNA metabarcoding of scats. Previous studies of the diet of black-footed cat used traditional approaches through direct observations of the cats as they fed in the wild (Sliwa 2006; Wilson *et al.* 2016). Our study validates and expands on the prey range of black-footed cats. This study is similar to other studies where a DNA-based approach is used to validate and identify a broader prey base (Gillet *et al.* 2015) when compared to previous studies (Bertrand 1993; Bertrand 1994; Castién and Gosálbez 1995; Santamarina 1993; Santamarina and Guitian 1988). Our study demonstrates that DNA metabarcoding is an effective method of determining black-footed cat diet through non-invasive collection of scats in the wild. This DNA metabarcoding approach may also be applied in studying the diets of other carnivores using the same modified primers with no prior knowledge of both the predator and possible prey items.

Comparison of this study that used DNA metabarcoding and a previous study by Sliwa 2006, showed that DNA metabarcoding had a higher prey species (65 species) compared to visual observations (49 species). DNA metabarcoding identified more mammals (33 species) than visual observations (14 species). The number of birds identified was almost equal, but DNA metabarcoding identified more birds (23 species) than visual observations (21 species). Visual observations however, identified more invertebrates six species compared to the two identified by DNA metabarcoding, as well as more reptiles and amphibians. DNA metabarcoding identified four reptiles and amphibians, while visual observations identified eight.

The most usual prey items found in this study were fish species. The black-footed cat has no previous records of using fishing as a hunting technique. In fact, the cat has been recorded to get its moisture needs from prey. Therefore it is most likely that the presence of fish in the black-footed cat diet is as a result of secondary predation. Secondary predation is when a predator consumes another predator that has prey in its stomach contents (Sheppard *et al*. 2005). This is one of the limitations of DNA metabarcoding in that it cannot differentiate method of feeding between primary predation, secondary predation, or scavenging

(Pompanon *et al*. 2012; Liu *et al*. 2021). The diet of black-footed cat contains birds that are known to feed on fish such as the African fish eagle. This is the most likely way that the cat could have consumed fish. Secondary predation has been recorded in other diet studies of carnivores such as sharks (de Bruyn *et al*. 2021).

Like previous metabarcoding diet studies, this study has been hindered by lack of adequate reference barcodes (Berry *et al.* 2017; Siziba and Willows-Munro 2024). Several species were unidentified at the species level due to unavailable DNA sequences in reference libraries. However, the distinct advantage of DNA metabarcoding is that once reference libraries are updated the data can be reanalysed (Berry *et al.* 2017) and additional prey items can be identified.

Using a single set of primers focusing on specific prey groups may produce biased results (Clarke *et al.* 2014; Piñol *et al.* 2015). Therefore, multiple primers may often minimize such bias and produce a more comprehensive result (Alberdi *et al.* 2018; Quéméré *et al.* 2021). Some studies have cautioned against using the COI DNA region for metabarcoding studies because it is a protein-coding gene lacking sufficient conserved regions (Deagle *et al.* 2014; Ficetola *et al.* 2010). The authors chose to use the COI metabarcoding primers as an alternative for the failed cyt *b* markers (Pers. Communication). Although the COI identified the highest number of species, the majority of them were not prey taxa and therefore could not be used to address the study objective. The main reason for this could be because the COI illmlCOIintF and jgHCO2198 primers (Geller *et al*. 2013; Leray *et al*. 2013) were highly degenerate and produce a 330bp PCR product. Species-specific primers have been shown to have better resolution than degenerate primers when applied in diet assessments studies (Deagle and Tollit 2007). Longer length metabarcodes (> 250bp) reduce the successful amplification of degraded prey items that have shorter lengths (Symondson 2002; Deagle *et al.* 2006; Troedson *et al*. 2009) by preferential amplification of fresh undegraded DNA from parasites and microbes present in the scats (Acinas *et al.* 2005; Clarke *et al.* 2014). Studies have found that degenerate primers tend to amplify gut parasites and symbionts (Pompanon *et al*. 2012). We would thus not recommend using this particular set of markers for diet assessment.

It has been suggested that the 12S rRNA and 16S rRNA mitochondrial regions in mammals is more conserved that the cyt *b* (Fernandes *et al.* 2008; Kitano *et al.* 2007). This could explain why the 12S rRNA and 16S rRNA had better amplification and species resolution that the cyt *b* and the COI. DNA metabarcoding has not been widely adapted for use in diet studies as expected (Alberdi *et al*. 2019; Liu *et al*. 2021). This could explain why the available metabarcoding markers are still limited in their application for specific metabarcoding studies.

Metabarcoding has been applied without use of blocking primers to simultaneously identify predators and their diet (Galan *et al*. 2012). Blocking primers are a set of primers that are often used in diet assessments to prevent the amplification and subsequent sequencing of predator DNA in order to improve amplification of possible rare prey items (Kumari *et al*. 2019; Vestheim and Jarman 2008). In contrast to other molecular diet studies (Kumari *et al.* 2019; Shao *et al.* 2021b; Xiong *et al.* 2017), we did not use blocking primers. Previous studies of black-footed cat diet have shown that the cats often feed on prey items that are also in the Canivora order (Sliwa 2006; Wilson *et al.* 2016). Since blocking primers may also block prey sequences of phylogenetically related taxa (Piñol *et al.* 2015; Piñol *et al.* 2014; Robeson *et al.* 2018), thus biasing the representatio of prey items (Pompanon *et al*. 2012), we decided against their use. Not using blocking primers can improve the accuracy of prey detection and identify predators simultaneously (Harper *et al.* 2020; Piñol *et al.* 2014).

Although the scats were collected across different seasons, seasonal dietary shifts could not be assessed because the scats were pooled together as per individuals regardless of season. However, subtle differences were noted between male and female cats. The females had a much wider dietary range than males. Seven orders of prey items were identified only in the female cats, while only one order (Squamata) was present only in the male cats. Unlike the study by Sliwa *et al*. 2006, our study noted that larger mammals were not restricted to male cats. For example, the larger antelope species, such as *Tragelaphus* spp. and the bush pig were identified from both male and female black-footed cats. Given how large these species are it seems likely that these were scavenged. Small rodent mammals and birds seemed to make up the majority of diet across all cats. Some prey items were only restricted to some individuals but not across all cats. It is important to note, however, that DNA metabarcoding of scats is the dietary representation at a given time. For this study, however, the scats were

collected over different seasons and pooled together. The authors are therefore confident that this gives a realistic dietary profile of black-footed cats.

5.6 References

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CHAPTER 6

Intestinal parasites of black-footed cat *Felis nigripes* detected using scat metabarcoding

6.1 Abstract

Intestinal parasites found in domestic cats are well documented, but limited information is available for wild felids, especially wild African felids. Most studies have relied on the morphological description of parasites and their eggs, which may underestimate the true diversity of parasite species in infected hosts. This study used results obtained from DNA metabarcoding of scat samples to identify parasitic species (nematode, bacteria, and fungal species) infecting wild black-footed cats**.** Three nematode species belonging to the genera *Ancylostoma*, *Mammonogamus*, and *Strongyloid*e, one bacterial (*Citrobacter*), and one fungal species (*Cladosporium*) known to be parasitic in domestic cats were found. This study is the first description of nematodes, bacterial and fungal species in wild black-footed cats. This data confirms the presence of parasites in a wild African felid that had previously been documented only in domestic cats.

Keywords: nematodes, Felidae, COI, South Africa, hookworm, gapeworm, roundworm

6.2 Introduction

Africa's smallest felid, the black-footed cat (*Felis nigripes* Burchell 1824), is endemic to the Karoo's open, arid savannas, semi-arid shrub land, and the southwestern Kalahari of southern Africa (Wilson 2015). With core distribution in the northern Karoo of South Africa, the species has also been recorded in southern Botswana, eastern Namibia, southern Angola, and southern Zimbabwe (Sliwa 2004; Wilson *et al.* 2016). This restricted distribution and declining populations have meant that the black-footed cat is now listed as Vulnerable by the International Union for Conservation of Nature (IUCN) (Sliwa *et al.* 2016).

Infectious disease risk is essential in conservation initiatives (Ruggiero *et al.* 1994; Smith *et al.* 2009), especially with vulnerable or endangered species (Angerbjörn *et al.* 2013). Pathology

studies on radio-collared wild black-footed cat populations have previously highlighted disease as an important driver (16%) of mortality (Sliwa *et al.* 2022). Other diseases such as amyloidosis (Terio *et al.* 2008), feline panleukopenia virus (Lane *et al.* 2016), retinal atrophy (Oh *et al.* 2017), herpesvirus and calicivirus (Rivas *et al.* 2015) have all been recorded in captive black-footed cats. However, information on these diseases in natural populations is not available. Endo- and exo-parasites are known to be prevalent in wild felids (Millán *et al.* 2007; Wierzbowska *et al.* 2020), but to date, limited data is available on parasites infecting black-footed cats, with previous studies only focussing on exo-parasites such as ticks (Horak *et al.* 2010). Parasitic nematodes are of veterinary significance, and as a result, nematode studies of wild felids have recently received increased research attention (Stevanović *et al.* 2019; Traversa *et al.* 2021). Most of these pathogen studies have focused on domestic cats, particularly the nematodes that cause pulmonary diseases (Bulbul *et al.* 2020; Morelli *et al.* 2020). Fewer studies are available for wild cats as compared to domestic cats (Roeber *et al.* 2013). No studies have examined the prevalence of parasitic nematodes in wild populations of the elusive black-footed cat nor the associated risk of bridging infections between wild felids in areas that co-habit with domestic cats (Morelli *et al.* 2021; Traversa and Di Cesare 2016).

Parasitic nematodes have been shown to negatively impact the development and survival of wild felids and many other wild vertebrate species (Arneberg *et al.* 1996; Tompkins and Begon 1999). Traditional monitoring of gastrointestinal parasites in wild populations usually involves morphological identification of eggs, larvae, and parasitic adults from scat or intestinal samples (Diakou *et al.* 2021). Adult forms of parasites are often required for species-level identification (Gillespie 2006; Stien *et al.* 2002). For some taxa, such as nematodes, gut contents from the dead animal host is needed to identify the nematode community with high taxonomic accuracy (Gillespie 2006; Stien *et al.* 2002). DNA metabarcoding has been shown to provide a non-invasive, time- and cost-effective tool for monitoring parasite communities using DNA extracted from scats (Davey *et al.* 2021).

This study aims to use non-invasive DNA metabarcoding for the detection of parasitic nematodes (and other parasites) in a wild black-footed cat population. Information on the prevalence and diversity of parasites infecting the gastrointestinal tract of wild black-footed

cats could feed directly into conservation programs focussing on monitoring the health of wild population of this species.

6.3 Materials and methods

6.3.1 Sample collection, DNA extraction, individual identification, and sexing

Black-footed cat scats were collected in the Benfontein Nature Reserve near Kimberley, Northern Cape Province, South Africa (28°50'S, 24°50'E). This area lies in the center of the known distribution of black-footed cats (Nowell and Jackson 1996) and has been an active site for black-footed cat research. The study area (60 km^2) encompassed a variety of arid vegetation communities (Sliwa 1996), including the elements of three major biomes: Kalahari thornveld, pure grassveld, and the Karoo (Acocks 1988). Like other small cats, black-footed cats rarely deposit scats in visible or predictable sites (Molteno *et al.* 1998). This study collected scat samples using a trained detection dog-handler team (Long *et al.* 2007; MacKay *et al.* 2008). The training of dogs to detect black-footed cat scat followed general guidelines recommended for conservation detection dogs (DeMatteo *et al.* 2019; Reed *et al.* 2011). Scat samples were collected from the 3rd of March 2020 to the 5th of October 2020 as part of an ongoing research project of the Black-footed Cat Working Group (Sliwa *et al.* 2021). Scats were preserved in the field using 90% ethanol. Permits for sample collection were provided by the Northern Cape Department of Environment and Nature Conversation (FAUNA 1218/2016, FAUNA 0636/2021). DNA from the scats was extracted using the Qiagen QIAmp Fast DNA stool kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions.

The area surveyed could have included the territories of a number of different cats. Scats were assigned to individuals using a panel of nine unlinked autosomal feline microsatellites (FCA_008, FCA_023, FCA_026, FCA_043, FCA_058, FCA_088, FCA_126, FCA_132, FCA_149; Menotti-Raymond and O'brien 1995; Menotti-Raymond *et al*. 1997). These markers were initially designed for domestic cats (Menotti-Raymond *et al*. 1999) but have been used previously in black-footed cats (Mattucci *et al*. 2018) to identify individuals using a variety of sample types including scats (Siziba *et al*. 2024). All forward primers were labeled with a fluorescent dye. Amplifications were performed in 12.5µL reactions containing 6.25µL

TEMPase Multiplex Taq (Amplicon, Odense, Denmark), 4µL dH_2O, and 0.25µL each of the forward and reverse primer. Amplifications were performed with an initial denaturation step at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 15 secs, annealing at 57°C for 30 secs, extension at 68°C for 30 secs and a final extension of 68°C for 5 mins. Amplicons were sent to the Central Analytical Facility (CAF) at Stellenbosch University, South Africa, for fragment analyses using an ABI PRISM™ 3500XL Genetic Analyzer (Life Technologies, Applied Biosystems, Warrington, UK) and a standard GeneScan™ ROX500™ (Applied Biosystems) internal size standard. The software package Geneious v8 (https://www.geneious.com) was used for genotype scoring. All scat samples were re-genotyped up to five times, and genotypes were compared to check for consistency. Assignment of scats to individual cats was done using GIMLET v1.3.3 (Valière 2002), which takes into account allelic drop-out associated with degraded DNA often extracted from scat samples (Siziba *et al*. 2024).

Gender was assigned to black-footed cats from scat samples using a nested PCR with felid sex-determination markers. The first PCR protocol used the RG4 and RG7 primers (Griffiths and Tiwari 1993). The PCR protocol consisted of 35 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension of 72°C for 10 mins. The second PCR used the Carni-SRY2 and SRY-CR1 primers (Kurose *et al.* 2005). The second PCR protocol was the same as the first, with an increased 45 cycles. The PCR products were run on a 1% TBE agarose gel and visualized on the GelDoc™ EZ Imager (Biorad, Johannesburg, South Africa). The presence of a 135bp PCR product identified each individual as male. Individuals with no bands were repeated to confirm the absence of a PCR product. Confirmation of the absence of a PCR identified the individual as female.

6.3.2 PCR assay and metabarcoding sequencing

In this study, we used a portion of the cytochrome oxidase I (COI) mitochondrial gene. The sequence data were obtained as part of a multi-marker study to assess the diet of black-footed cats from scats. An assay comprising COI universal primers illmlCOIintF (Geller *et al.* 2013) and jgHCO2198 (Leray *et al.* 2013c) was set up using a HotStarTaq Plus Master Mix Kit (Qiagen, USA). The cycling conditions were made up of an initial denaturation of 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 53°C for 40 seconds, 72°C for 1 minute, and a final

elongation step at 72°C for 10 minutes. After amplification, the PCR products were visualised using a 2% agarose gel (Merck, Darmstadt, Germany) to determine amplification success, size, and intensity of each band. Samples were multiplexed using unique dual indices and pooled together in equal proportions relative to their DNA concentrations and molecular weight. The samples were purified using Ampure XP beads (Beckman Coulter, Indianapolis, USA) and used to prepare an Illumina DNA library at the Molecular DNA (MR DNA®) Research Lab (Shallowater, Texas, USA). Metabarcoding sequencing was done using cluster generation, paired-end sequencing by synthesis using the Illumina MiSeq platform SY-410-1003 using the MiSeq reagent kit v3 (San Diego, California, USA)**.**

6.4 Results

6.4.1 Sample collection, DNA extraction, individual identification, and sexing

Eighty-three black-footed cat scats were collected. Thirteen of the scats were collected directly from individual cats, sixty-six were obtained through the use of detection dogs, and the remaining four samples were identified as possible black-footed scat by field staff.

DNA was successfully extracted and genotyped using the nine microsatellite loci. Genotyping and comparison to a reference dataset identified twelve individual black-footed cats. Seven cats were identified as female, and the other five as male.

6.4.2 PCR assay and metabarcode sequencing

A total of 2 051 054 paired reads were generated through Illumina NGS. After trimming, merging, and quality filtering, 123 978 reads were used for BLASTn searches [date access 03 July 2023]. Reads with a sequence similarity above 97% were considered at the species level and, those below the specified threshold were listed only at the genus level (Edgar 2010).

Five species of veterinary concern were detected in the metabarcoding data (Table 6.1). *Ancylostoma tubaeforme, Mammomogamus ierie,* and *Strongyloides* spp. are parasitic nematodes. *Mammomogamus ierie* and *Strongyloides* spp. infect a wide range of vertebrate taxa (both domestic and wild), while *Ancylostoma tubaeforme* predominantly infects felines. *Ancylostoma tubaeforme* and *Strongyloides sp* both infect the small intestine. Sequence data

also revealed the presence of microbial pathogens, including *Citrobacter freundii* and *Cladosporium cladosporioides.*

Table 6.1. Family, order, and species of the pathogens detected through DNA metabarcoding of black-footed cat (*Felis nigripes*) scat. Sequence similarity indicates the percentage similarity of the query sequence and the BLASTn result. Sex denotes the sex of the black-footed cat where the pathogens were identified.

Order	Family	Species	Common name	Sequence similarity (%)	Sex	No. of infected cats	Pathogenesis
Rhabditida	Ancylostomitidae	*Ancylostoma tubaeforme*	Hookworm	98.4	Males and Females	2	Adult hookworms found in the small intestine can cause haemorrhaging, leading to poor weight gain and anaemia (Kalkofen 1987; Otranto and Deplazes 2019; Roelke *et al.* 1985).
Rhabditida	Syngamidae	*Mammomogamus ierie*	Gapeworm	97.3	Females	3	Parasite of the upper respiratory tract of a wide range of vertebrate taxa. Symptoms include nasal discharge, coughing, sneezing, and loss of

							weight (Cuadrado *et al.* 1980; Guilbride 1953; Lindquist and Austin 1981; Tudor *et al.* 2008).
Rhabditida	Strongylidae	*Strongyloides* spp.	Roundworm	96.8	Females	3	Obligate gastrointestinal parasite of vertebrates. Adults reside in the small intestine. Can cause diarrhoea, malabsorption, and bronchopneumonia in young animals (Bowman *et al.* 2002; Thamsborg *et al.* 2017).
Enterobacterales	Enterobacteriacea	*Citrobacter feundii*	Facultative anaerobic gram negative bacteria	97.0	Females	3	Can cause urinary tract infections, and are found in wounds, respiratory infections, meningitis, and infections (Wanger *et al.* 2017).

Capnodiales	Davidiellaceae	*Cladosporium cladosporioides*	Mould	100.0	Males and Females	4	Abundant mould found on surfaces. Is associated with cerebellar phaehyphomycosis, cutaneous, subcutaneous, and cerebral granulomas, abscesses, and systemic dissemination in domestic cats (Coldrick *et al.* 2007; Revankar *et al.* 2004; Velázquez-Jiménez *et al.* 2019).

Out of 12 cats, five did not have any of the bacteria or parasites identified in Table 6.1. *Cladosporium cladosporiodes* was the most prevalent pathogen, found in four black-footed cats, two female and four male cats. *Ancylostoma tubarforme* was found in two individuals, one male and one female cat. The rest of the pathogens *Citrobacter feundii*, *Mammomogamus ierie*, and *Strongyloides* spp was found in three cats, all female. There were no pathogens that were restricted only to the male cats.

6.5 Discussion

This study is the first report of pathogens of veterinary importance found in wild black-footed cats. The results of this study identified the presence of multiple intestinal parasites infecting wild black-footed cats through the DNA metabarcoding. Studies on pathogens infecting wild felids have been marginally understudied in wild African felids (Bokaba *et al.* 2022), and this study can aid in bridging those knowledge gaps.

Mammomogamus irei (Buckley 1934) was initially known to be from domestic cats (Červená *et al.* 2018b). Five of the thirteen known species are known to infect cats, making cats the most frequent hosts of the gapeworms (Červená *et al.* 2018b). Nematodes of the genus *Mammomogamus* (Ryzhikov 1949) have been described in wild felids and domestic cats either from nares (Buckley 1934; Guilbride 1953), nasopharynx (Cuadrado *et al.* 1980), and more recently, eggs in scats (Gattenuo *et al.* 2014). In *Leopardus pardalis* (Magnaval and Magdeleine 2004*;* Murray and Gardner 1997), *Prionailurus bengalensis* (Hasegawa 1992; Patton and Rabinowitz 1994), *P. tigris* and *P. pardus* (Patton and Rabinowitz 1994) and domestic cats (Krecek *et al.* 2010). Despite the numerous identifications of Mammonogamus infecting felids in other parts of the world, only one report of the nematode infecting cats exists in Africa (Lindquist and Austin 1981). However, *Mammomogamus* spp. host range has been shown to extend beyond carnivores in lowland gorillas (*Gorrilla gorilla gorilla*), forest elephants (*Loxodonta cyclotis*) (Červená 2017), some African herbivores (Červená 2018a), and birds (Červená *et al.* 2018b). As black-footed cats have been shown to feed on birds (Siziba 2024; Sliwa 1994) it is therefore not unusual for these pathogens to be found in the scats of the cats.

Hookworms that make up the genus *Ancylostoma* are considered the most pathogenic parasites of juvenile dogs and cats (Levine 1980). *Ancylostoma tubaeforme* has been described as a species parasitizing cats worldwide in areas where domestic cats are found (Bowman *et al.* 2002; Rep 1966). However, few studies document the presence of this parasite in wild felids. One such study has shown the presence of *A. tubarforme* in Persian leopards (*Panthera pardus tulliana*) in Iran (Youssefi *et al.* 2010). A study of the Iberian lynx (*Lynx pardinus*) in Spain showed that all juveniles under six months old were parasitized with Ancylostoma spp. This led to the consideration that ancylostomosis may play a role in morbidity and mortality of the already endangered Iberian lynx population (Vicente *et al.* 2004). Two separate studies in Spain showed that parasites were being co-transmitted between domestic cats and the lnyx because of shared haplotypes between the two species (Millán and Casanova 2007; Vicente *et al.* 2004). Genetic divergence studies using the cytochrome c oxidase subunit I gene (*Cox* I) showed that domestic cats in the nearby areas act as natural reservoirs for the parasites transmitted to lynx populations (Millán and Blasco-Costa 2012).

Strongyloides spp. have been identified as zoonotic parasites in wild carnivores (Otranto and Deplazes 2019; Thamsborg *et al.* 2017). Four species of Strongloidae have been identified in cats. Namely, *Strongyloides planiceps*, *S. stercoralis*, *S. tumefaciens*, and, *S. felis* (Wulcan *et al.* 2019). However, this study could not confirm which of the four species was present in the black-footed cat to the species level. This was because the percentage sequence similarity (96.8%) was below the species threshold for species identification, according to Edgar, (2010).

Estimates show that about 43% of zoonotic and bacterial infections naturally originate from carnivore hosts (Cleaveland *et al.* 2001). This statistic shows the health relevance of wild carnivores (Otranto and Deplazes 2019). *Citrobacter feundii* is a gram-negative bacteria often isolated from cats or dogs with Urinary Tract Infections (UTIs) and has been noted for its resistance to antibiotics (Harada *et al.* 2019; Moon *et al.* 2022). Feline phaeohyphomycotic cerebellitis has been reported in domestic cats (Velázquez-Jiménez *et al.* 2019). This study shows the possibility of feline phaeohyphomycotic cerebellitis in wild felids as the presence of *Cladosporium cladosporioides* is considered a differential diagnosis for central nervous system disease in cats (Velázquez-Jiménez *et al.* 2019).

It is important to note that use of a single marker approach such as the one used in this study could possibly underestimate the possible diversity of the pathogens infecting wild black-footed cats. Various studies have shown that a multi-marker approach is ideal for species identification in metabarcoding (Travadi *et al.* 2023; Zhang *et al.* 2018a). Other studies investigating pathogens in the wild have used not only the COI marker (Macheriotou *et al.* 2019; Tytgat *et al.* 2019) but the 18S rDNA (Aivelo *et al.* 2018; Treonis *et al.* 2018; Waeyenberge *et al.* 2019), cyt *b* (Eves-van den Akker *et al.* 2015) and ITS2 rDNA (Pafčo *et al.* 2018; Redman *et al.* 2019). However, ITS2 rDNA has been shown to be the most effective at species identification of parasitic nematodes (Avramenko *et al.* 2018; Avramenko *et al.* 2017). It is therefore advisable, for other studies of parasites in wild species to use a multi-marker approach in order to fully assess the possible parasites infecting each species.

It is unclear if the pathogens detected in the wild black-footed cats were transmitted from domestic cats or were naturally occurring in wild black-footed cat populations. In this study the majority of the parasitic nematodes were restricted to the female cats except for *Ancylostoma tubaeforme*. However, the sample size used in this study is too small to validate these results. Literature on parasitic nematodes in other wild felids with larger datasets has shown that the parasites were not restricted to a single sex (Krone *et al.* 2008; Lynsdale *et al.* 2020).

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CHAPTER 7

7.1 Conclusions and perspectives

The use of various molecular DNA approaches for studying the ecology of wildlife species has shown great potential worldwide. However, these studies have been limited in Africa, and especially on small carnivores. Studies making use of high-throughput sequencing on non-invasive samples to determine the diet free-ranging small carnivores are distinctly lacking. Non-invasive metabarcoding has become the preferred method when studying elusive or rare species. This study bridged these knowledge gaps by using a set of DNA-based methods on non-invasively collected scat samples to address key ecological questions using black-footed cats (*Felis nigripes*) as a case study.

The initial consideration in a metabarcoding study is the availability and abundance of DNA reference libraries in GenBank and BOLD. This is because the accuracy of species identification of metabarcoding diet assessments dependent on the current status of DNA sequence libraries. In order to determine the feasibility of a metabarcoding approach in assessing the diet of black-footed cats, in **Chapter 2** I reviewed the current status of DNA repository libraries of small mammals that are known to make up the majority of black-footed cat diet.

In **Chapter 2** I showed that small mammals make up to 193 species. Only 141 of the total number of species available have DNA sequences across either one of the five mitochondrial gene regions routinely used in metabarcoding. The COI region is considered the most common DNA region for barcoding of animal species, and it has the highest number of DNA barcodes. However, in terms of species coverage, the cytochrome *b* (cyt *b*) gene sequence was available for 59% of the known species while the COI gene was available for 34%. The 12SrRNA, D-loop, and 16S rRNA had 24%, 18%, and 15% species coverage, respectively. Species coverage across the different mitochondrial genes varied, which led to the determination that a multi-marker approach would be ideal for species resolution of South African small mammals.

In **Chapter 3**, I evaluated the available metabarcoding primers for their efficacy in amplification and subsequent species delineation of South African small mammals. The available markers in the literature had either been used for metabarcoding diet assessments in previous studies or for species identification in ancient DNA studies. These markers would be ideal for a non-invasive study using environmental DNA (eDNA) that is often degraded and where full-length barcodes would not be ideal.

My results showed that the available metabarcoding markers did not have a distinct barcoding gap as would be expected of markers that can robustly differentiate species. These results were not unexpected, as various studies have shown that marker performance differs across geographical locations and ecosystems (Bylemans *et al.* 2018; Zeale *et al.* 2011). This is usually a result of mutations in the primer binding sites that often limit the ability of the primer to amplify across species (Housley *et al.* 2006). The Jeffries-Matusita (J-M) values for some of the markers, however, was above the specified threshold of 1.414 (Dabboor *et al.* 2014; Trigg and Flasse 2001). In **Chapter 3** I determined that the ideal suite of primers for use in diet analysis of the black-footed cat were the 12S rRNA 12SV5 and 12SV5B2 primers (Riaz *et al.* 2011), 16S rRNA 16Smamm1 and 16Smamm2 primers (Taylor 1996), COI illmlCOIintF and jgHCO2198 (Geller *et al.* 2013; Leray *et al.* 2013) and cyt *b* L15411F and H15546R primers (Galan *et al.* 2012). Unfortunately, no D-loop metabarcoding primers were available in the literature and, therefore, were not included in this study. The lack of an adequate barcode gap in the currently available DNA barcode markers highlights the need for design and testing mini-barcode markers for South Africa's small mammal species. This has already been done for other taxonomic groups (Geller *et al.* 2013; Govendor *et al.* 2019; Hajibabaei *et al.* 2011; Leray *et al.* 2013).

In **Chapter 4** I tested if microsatellite loci can be successfully amplified from black-footed cat scat samples that could be non-invasively collected from the field. Using blood and scat samples collected from the same individuals, I also determined if these microsatellites could be used to individually identify cats. Twelve individual cats were identified, consisting of seven females and five males. The main limitation of this method is that other closely related wild felids tend to co-amplify with the same microsatellites as they were initially designed for domestic cats (Menotti-Raymond *et al.* 1999; Menotti-Raymond *et al.* 1997). However, to

overcome this limitation, only samples with genotypes within the specified genotypic ranges were used (Mattucci *et al.* 2018).

In **Chapter 5** the different individuals identified and the ideal primers were collectively used to set up a metabarcoding assay to determine the diet of black-footed cat in the Benfontein Nature Reserve in South Africa. The results were compared to those of a previous study that described the diet of black-footed cats using direct observations of following the cats in the wild (Sliwa 2006). DNA metabarcoding identified 32 mammalian prey taxa, whereas the study by Sliwa *et al*. (2006) identified only 14 mammals. Some items could not be assigned to any species, genus, order, or family because of the absence of the reference DNA in the repository libraries. However, the results from this study can always be re-analysed as the DNA sequence libraries improve.

The results from the diet assessment revealed the presence of pathogens of veterinary importance previously discovered in domestic cats. I presented these five pathogens in **Chapter 6**. Though well documented in domestic cats, hookworms, gapeworms, roundworms, bacteria, and mould had not been identified in wild populations of black-footed cats, or any other wild African felid. It is unclear if these pathogens are co-transmitted between domestic cats and black-footed cats. Previous studies have however shown that domestic cats act as natural reservoirs of pathogens that are transmitted to wild felids (Millán and Blasco-Costa 2012; Millán *et al.* 2007; Vicente *et al.* 2004).

The results from this Ph.D dissertation have shown that non-invasive metabarcoding, though highly lacking in African and, in particular, South African carnivores, can be successfully performed using available DNA reference libraries and metabarcoding primers. These results show improved resolution compared to direct observations and could be the preferred choice for ecological studies of wild carnivores. However, there is still a need to improve DNA reference libraries. Some small mammal DNA sequences are absent and can, therefore, not be identified, especially species that are endemic to southern Africa.

The results presented in this dissertation provide insights to the application of DNA metabarcoding in assessing the diet of black-footed cats in South Africa. These results can be applied to assess the diet of other South African carnivores and aid in identification of prey

species that had previously not been documented. Especially with small carnivores that have remained largely understudied. Additionally, these results provide information on the pathogens that could possibly contribute to the health and possible mortality in black-footed cat populations in the wild.

7.2 References

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