Using faecal DNA to investigate the diet of the snakes, *Psammophis crucifer* and *Psammophylax rhombeatus*

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Keywords

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Abstract

Knowledge of the feeding ecology of an organism helps us to better understand predator-prey relationships and aspects of species biology, ecology and life-history traits. Understanding the feeding ecologies of snakes is challenging because snakes are generally secretive and often difficult to observe when foraging in the wild. Traditionally, studies attempting to quantify the diets of snakes relied on observing direct predation events, dissecting dead specimens, or microscopy of gut and stomach contents to identify prey species. However, investigations using traditional methods can result in an incomplete understanding of prey utilised by particular snakes. Analysis of prey DNA in snake faeces is a useful method to obtain accurate information on diet. Here, I present the results of the molecular analysis of faeces from wild-caught and captive-fed *P. crucifer* and *P. rhombeatus*. DNA was extracted from the faeces, and through the use of PCR primers, cloning, and Sanger sequencing, fragments of *CYTB* and *16S rRNA* was amplified within the faecal remains of both wild-caught individuals from Koeberg Private Nature Reserve, South Africa, and, subsequently, captive-fed individuals. Prey DNA was identifiable in 75% of samples from captive-fed individuals using prey-specific primers. In a subsample of these positive samples, prey DNA was recovered in 58% of cloned and sequenced samples with the use of universal primers. While I was able to identify prey items to species-level from faeces collected in captivity, I found that predator DNA was prominent. Detectability of prey remained unchanged regardless of the length of the fragments amplified. This study established no significant difference between 156 bp and 478 bp. In some cases, larger DNA fragments remain intact in these predators' faeces. Furthermore, in one faecal sample collected from a wild-caught *P. rhombeatus*, I identified DNA from *Tetradactylus seps*, which is the first known instance of this prey in the diet of this snake species. Snake identity and days since the last *P. geitje* meal positively contributed to the detection of prey DNA. Detection of prey was possible in fresh faeces collected within days, and in some cases, in older faeces collected after a month. The average day when prey detection decreased was after 30 days. Overall, detection probabilities varied between individual snakes and species. The approaches developed are applicable to investigate snake diets, as they offer greater insight into the predators feeding ecology. The non-invasive approach employed provides an opportunity to overcome the prejudices of traditional dietary analysis.

Declaration

I declare that **"Using faecal DNA to investigate the diet of the snakes, *Psammophis crucifer* and *Psammophylax rhombeatus"*** is my own work, that it has not been submitted for any degree or examination at any university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Kim Jennilee Scholtz   **Date:** February 2022

Signature: ……..Scholtz…………….

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Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

1.1.   Overview

Ecosystem functioning is largely maintained by the combined effects of species interacting with each other and their environments. In the face of ongoing global loss of biodiversity, understanding these patterns are crucial to predict how species will respond to disturbances and changing conditions within their environments, and how these responses may affect ecosystem health. Accordingly, a longstanding interest in the field of community ecology has been to study and elucidate the complex relationships in nature that arise as a result of interactions between species competing for limited resources (Morin 2009). Chief among such interactions are predation, whereby one population of individuals becomes the resource of another or several others, and competition, whereby species compete for the same food resources. These interactions bind species together in the struggle for existence and can result in evolutionary adaptations. An important goal in community ecology is therefore to understand the feeding and trophic ecologies of organisms and to relate these to broader ecosystem functioning (Arditi & Ginzburg 2012).

Comprehensive knowledge of the feeding ecology of an organism helps us better understand species interactions and predator-prey relationships (Brown & Gillooly 2003). Knowledge of what an organism consumes can tell us more about a predator's fundamental resources and their potential impact on prey populations (Gittleman & Harvey 1982; Sunquist & Sunquist 1989). Furthermore, understanding the food habits of animals may provide information useful for understanding several aspects of species biology, ecology, and life-history traits (Pianka 1986). Dietary information can also provide us with insight into whole community structure and functions (Estes et al. 2011). Moreover, it can be used to address potential competitive interactions among sympatric species (Wiens 1993; Mitchell & Banks 2005; Barends & Maritz 2021) and explain ecological divergence among species (Goodyear & Pianka 2008).

Competition for food, or other resources, can lead to the exclusion of species and result in their displacement, and eventual extinction (MacArthur 1972; Schoener 1974; Moulton & Pimm 1986). One way in which species reduce such competitive interactions is through resource partitioning. Resource partitioning, first described by Schoener (1965) and expanded upon in Schoener (1974; 1977), refers to scenarios in which species with similar ecological

requirements avoid competing with one another by using different resources. This can promote coexistence in ecological communities as species are less likely to outcompete each other, as dictated by the competitive exclusion principle (Hardin 1960). Species often reduce competition for food by partitioning prey, either by foraging for different organisms or foraging for the same organisms in different spaces or at different times (Luiselli 2006a). This can result in character displacement and the adaptive evolution and fixation of novel traits that can allow species to develop behavioural, morphological or physiological adaptations that facilitate their coexistence with other competing species (Brown & Wilson 1956). In order to study competition or niche partitioning at fine spatial scales precise diet data are needed. However, high resolution diet data are often unavailable or difficult to obtain and may require feasible new approaches (Maritz et al. 2021b).

Until now, our understanding of the dietary components of species has been limited to identification of prey by direct observation or indirect observation like social media, videography, faecal material, stomach contents, regurgitates, and microscopy. Each of these methods varies in its appropriateness and effective ability to quantify predator-prey interactions (Symondson 2002; King et al. 2008). Furthermore, these methods often rely on hard body parts as key identifiers (Tollit & Thompson 1996; Cottrell & Trites 2002; Hume et al. 2004). Although these methods have offered insight into broadly identifying the diets of animals, they are often limited to identifying prey at the order and family levels (McKeand 1998; Jarman et al. 2002b; Kvitrud et al. 2005; Clusella-Trullas & Botes 2008). As such, research needs to be fuelled into method development that can identify animal diets effectively. Fortunately, improvements in alternative, indirect methods for the quantification of prey consumed by predators over the last few years, including PCR-based species tests (Hill et al. 2001; Kiesling et al. 2002), faecal DNA analysis (Deagle et al. 2009) and DNA metabarcoding (Ahrens et al. 2007) have meant the development of such methods has gained popularity. Several studies (Symondson 2002; Kvitrud et al. 2005; King et al. 2008) advocate that these methods may provide estimates of animals at a fine-scale taxonomic resolution.

Although the diet of some species has been studied in-depth (Shine et al. 1998, 2006a, 2006b; Luiselli & Angelici 2000; Luiselli et al. 2002; Luiselli 2003; Webb et al. 2001; Akani et al. 2003; Layloo et al. 2017; Smith et al. 2019; Greuel 2019; Maritz et al. 2021a, 2021c; Barends & Maritz 2021), the diets of most African snakes remain incompletely sampled. In this thesis, my primary focus was to develop improved methods for sampling snake diets. This dietary

information can likely be used in future to answer questions related to niche partitioning, dietary preference, seasonal changes, and rate of resource utilisation.

1.2. Snake feeding ecology

The diets of snakes and their selection of prey often shape prey community dynamics. Understanding how different snakes select their prey could enable ecologists to predict their impact on specific prey populations. Such information can be used to study ecological correlates that contribute to the evolution of specific preferences for certain prey in different snakes. For example, Gans (1952, 1961) reported that most snakes are able to consume prey larger than their own heads. This ability enables them to feed on prey items that vary in size, type, and shape. The dietary breadth of snakes offers critical information regarding the interactions these species have with other taxa. For example, Shine (1991) showed that variations in feeding patterns by generalist predators are attributable to the types of prey encountered in different habitats. Moreover, in several colubrid species, local specialisation correlated with the abundance of prey taxa that occurred in the area (Kephart 1982). By knowing which taxonomic groups snakes in different habitats consume, we can predict how changes in prey populations could affect the snake species occupying those areas.

Unlike endothermic predators snakes have: (1) evolved a variety of ecological and morphological mechanisms that allow them to locate, capture, and consume large prey relative to their own body mass (Pough 1983; Luiselli 2006a), and (2) they sometimes exhibit ontogenetic changes in habitat use, prey preferences, or foraging behaviour (Lind & Welsh 1994). As a result, answering questions that relate to why different species of snakes select certain prey types or prefer prey of differing sizes (Shine 1991; Rodríguez-Robles & Greene 1999; Rodríguez-Robles 2002) can help to further our understanding of their natural histories and their roles within ecosystems. Moreover, answering questions about the patterns of geographic variation and ontogenetic change in snake diets (Daltry et al. 1998; de Queiroz 2001; Clark 2002), their digestive physiology (Alexander et al. 2012), behavioural aspects of prey capture (Greene & Burghardt 1978), and interspecific competition for prey (Luiselli et al. 2006a, 2006b), can provide several insights into their community ecology.

Studies of the feeding habits of snakes have also revealed that some species adapt to changes in the availability of suitable prey to some extent. For example, several species of snakes

exhibit ontogenetic variations in food selection, where adults consume different prey to juveniles (Mushinsky et al. 1982; Lind & Welsh 1994; Daltry et al. 1998; Clark 2002; Ford & Hampton 2009). Similarly, several snake species alter their diets in response to fluctuations in prey abundance driven by differences in habitats or seasonal shifts (Madsen & Shine 2000; de Queiroz 2001; Madsen et al. 2006; Brown & Shine 2007). These studies support the idea that when food resources are limited, snakes can shift their diet to more abundant prey to prevent starvation and limit competition. However, the alternative can also occur. Madsen & Shine (1996) found that water pythons (*Liasis fuscus*) in tropical Australia tend to migrate with their preferred prey of dusky rats during wet seasons. Later, Brown et al. (2002) found that pythons that remained exhibited poor body conditions and low feeding rates (Brown et al. 2002).

The abovementioned studies shed light on the food habits and feeding ecology of many snake species. However, few studies have looked at how diet varies among individuals or populations (Luiselli et al. 2007). Locally abundant snakes provide an opportunity to gather comprehensive information on their feeding ecology (Mushinsky & Hebrard 1977; Gregory 1978, 1984; Kephart & Arnold 1982) such as prey preference and digestive physiology that can be useful for investigating co-occurrence patterns, and mechanisms facilitating resource partitioning.

1.2.1. Case study: two psammophiid snake species

In southern Africa, snake community dynamics are under-studied. This is somewhat surprising given the species richness of snakes in the region (~170 species; Bates et al. 2014), several of which occur sympatrically and are morphologically and ecologically similar. One group, the psammophiids (family: Psammophiidae) is a prime example of this. *Psammophylax rhombeatus* (spotted skaapsteker; Figure 1.1A) and *Psammophis crucifer* (cross-marked sand snake; Figure 1.1B) represent ideal candidate species for studies of competition for food (Figure 1.2A; Cottone & Bauer 2010; Bates et al. 2014). The snake family Psammophiidae (Kelly et al. 2008; Keates et al. 2019) is a group of about 40 to 50 species in which most have slender body forms. Most psammophiids are diurnal and fast-moving, although some have been reported as arboreal while others burrow in the sand. Furthermore, psammophiids occupy relatively open habitats in which they actively hunt their prey (Branch 1998). The geographical distributions of the two species overlap (Figure 1.2A;

Cottone & Bauer 2010; Bates et al. 2014) and they share similarities in their feeding ecology (Cottone & Bauer 2010).

Figure 1.1 Snake species utilised in this study. **(A)** Spotted skaapsteker (*Psammophylax rhombeatus*) and **(B)** Cross-marked sand snake (*Psammophis crucifer*).

Previous studies have shown that many species of *Psammophis* and *Psammophylax* utilise similar prey resources (Shine et al. 2006b; Cottone & Bauer 2010). This has mainly been through museum based dietary studies (Bates 1985; Van Wyk 1988; Butler 1993; Kark et al. 1997; Akani et al. 2002, 2003; Shacham 2004; Shine et al. 2006b; Cottone & Bauer 2008, 2009a, 2009b, 2010). Both *P. crucifer* and *P. rhombeatus* feed predominantly on mammals and reptiles but differ in the proportions at which they utilise these prey. These differences are likely due to differences in the sizes and geography of these species. Although patterns of dietary overlap and differentiation in prey use have been observed, a thorough investigation into these snakes' ecology is needed.

In this study, I performed a fine-scale assessment of prey identification of these snakes at my chosen study site (see below), because while a large summation of the diet of a species (Table 1.1) across its geographic range (Figure 1.2A) offers important information towards its overall ecology, it is not necessarily ideal for fine-scale assessments of prey use at particular sites. Moreover, to understand predator-prey dynamics, precise details of specific habits need to be investigated. For example, Hopcraft (2005) found that at a fine-scale, predators foraged in areas where more prey could be caught but at a broad-scale predators shift their ranges to accommodate seasonal movement of prey. This shows that the scale of investigation matters, as it can tell us about the distribution of predators and prey in relation to factors such as food availability and habitat type (Dorfman & Kingford 2001). Fine-scale studies are particularly

important for species that have relatively low dispersal ability, such as snakes (Capizzi & Luiselli 1996). Such local scale studies may show different patterns of prey use than commonly observed on a broad-scale. Here, fine-scale use of prey was investigated at Koeberg Private Nature Reserve (Figure 1.2B).
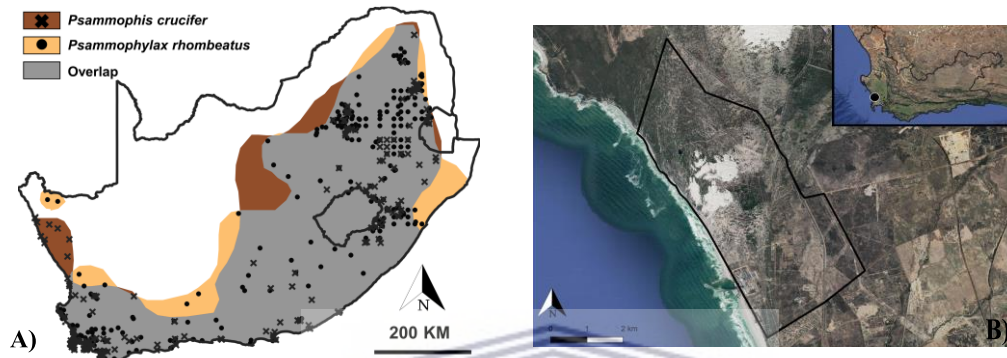
Figure 1.2 Geographical distribution and location of the two study species. **(A)** Geographical distributions of *Psammophylax rhombeatus* and *Psammophis crucifer* across South Africa, Lesotho and Eswatini. Occurrences of *P. rhombeatus* (505) and *P. crucifer* (253) were obtained from GBIF. Polygons of distribution ranges were obtained from Roll et al. 2017. Final distribution map was produced in QGIS v2.3. **(B)** Geographical location of Koeberg Private Nature Reserve along the West Coast of South Africa.

Table 1.1 Dietary summary of *Psammophis crucifer* and *Psammophylax rhombeatus*. Sources include published literature, Facebook, iNaturalist, Flickr, iSpot and YouTube. Values represent the total number of prey items recorded for each species.

Prey item	*P. crucifer*	*P. rhombeatus*	References
REPTILES			
Cordylidae			
Chamaesaura anguina	2	0	Branch and Bauer 1995; Cottone and Bauer 2010
Chamaesaura aenea	1	0	Cottone and Bauer 2010
Cordylus vittifer	1	0	Maritz and Maritz 2020[a]
Cordylus cordylus	1	0	Asche 2017[b]
Scincidae			
Acontias orientalis	1	0	Cottone and Bauer 2010
Trachylepis capensis	5	0	Cottone and Bauer 2010; Maritz and Maritz 2020[a]
Trachylepis homalocephala	2	1	Cottone and Bauer 2010; Maritz and Maritz 2020[a]
Trachylepis striata	1	0	Cottone and Bauer 2010
Trachylepis sulcate	1	0	Cottone and Bauer 2010
Trachylepis varia	9	1	Cottone and Bauer 2010; Maritz and Maritz 2020[a]
Trachylepis variegata	2	0	Cottone and Bauer 2010
Trachylepis punctatissima	1	9	De Waal 1977; Maritz and Maritz 2020[a]
Trachylepis sp.	1	1	Broadley 1966; Cottone and Bauer 2010
Unidentified scincid	1	2	Cottone and Bauer 2010
Gekkonidae			
Hemidactylus mabouia	1	0	Jade Vianello 2021[c]
Afrogecko porphyreus	4	0	Cottone and Bauer 2010; Maritz and Maritz 2020[a]; Winter 2020[d]
Lygodactylus bradfieldi	1	0	Cottone and Bauer 2010
Lygodactylus capensis	1	0	Cottone and Bauer 2010
Panaspis wahlbergi	1	1	Cottone and Bauer 2010; Maritz and Maritz 2020[a]
Goggia lineata	1	0	FitzSimons 1935
Chondrodactylus bibronii	1	0	Haagner and Branch 1993a
Pachydactylus geitje	0	1	Branch and Bauer 1995
Pachydactylus maculatus	5	1	Cottone and Bauer 2010; Nicolau et al. 2020[e]
Pachydactylus affinis	0	1	Cottone and Bauer 2010
Pachydactylus capensis	0	1	Cottone and Bauer 2010
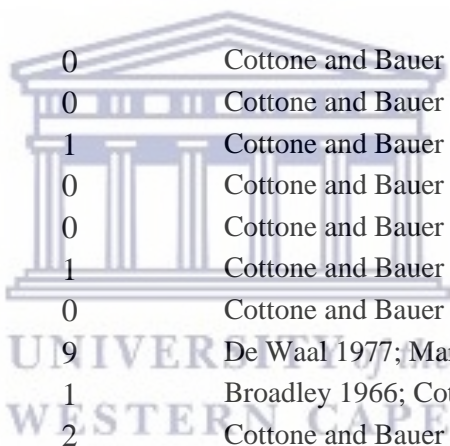
Prey item	*P. crucifer*	*P. rhombeatus*	References
Gekkonidae cont.			
Pachydactylus sp.	1	1	Cottone and Bauer 2010
Unidentified gekkonid	1	0	FitzSimons 1962
Lacertidae			
Meroles knoxii	1	1	Cottone and Bauer 2010
Nucras lalandii	2	0	De Waal 1977; Parbhoo-Mohan 2012[f]
Nucras livida	1	0	Cottone and Bauer 2010
Pedioplanis namaquensis	1	0	Cottone and Bauer 2010
Unidentified lacertid	6	1	De Waal 1977; Van Wyk 1988; FitzSimons 1962; Cottone and Bauer 2010; Louisef 2019[g]
Gerrhosauridae			
Gerrhosaurus flavigularis	2	0	Cottone and Bauer 2010; Van Wyk 2020[h]
Gerrhosaurus typicus	1	0	Cottone and Bauer 2010
Colubridae			
Philothamnus natalensis	1	0	Maritz and Maritz 2020[a]
Lamprophiidae			
Duberria lutrix	2	0	Cottone and Bauer 2010; Maritz and Maritz 2020[a]
Psammophiidae			
Psammophylax rhombeatus	2	0	Schönland 1895; Maritz and Maritz 2020[a]
Psammophis notostictus	1	0	Berning[i] 2021
Psammophis sp.	1	0	Cottone and Bauer 2010
Chamaeleonidae			
Bradypodion ventrale	0	1	Cottone and Bauer 2010
Agamidae			
Agama atra	0	2	Van Wyk 1988
Agama sp.	1	0	Maritz and Maritz 2020[a]
Pipidae			
Xenopus laevis	1	1	Rebelo 2016[j]; Maritz and Maritz 2020[a]
Hyperoliidae			
Kassina senegalensis	0	2	Cottone and Bauer 2010
Pyxicephalidae			
Cacosternum boettgeri	1	4	De Waal 1977; Cottone and Bauer 2010; Haagner et al. 1990

Prey item	*P. crucifer*	*P. rhombeatus*	References
Pyxicephalidae cont.			
Cacosternum nanum	0	1	Cottone and Bauer 2010
Strongylopus bonaespei	0	2	Cottone and Bauer 2010
Strongylopus fasciatus	0	4	Cottone and Bauer 2010
Strongylopus wageri	0	1	Cottone and Bauer 2010
Strongylopus grayii	0	4	Broadley 1977; Cottone and Bauer 2010
Amietia fuscigula	0	6	Cottone and Bauer 2010; Phillipskop Mountain Reserve 2015[k]
Amietia delalandii	1	0	Maritz and Maritz 2020[a]
Unidentified pyxicephalid	0	1	Cottone and Bauer 2010
Brevicepitidae			
Breviceps namaquensis	0	1	Cottone and Bauer 2010
Breviceps sp.	3	5	FitzSimons 1962; Cottone and Bauer 2010; Maritz and Maritz 2020[a]; Spottiswoode 2020[l]; Bracher 2020[m]
BIRDS			
Ploceidae			
Ploceus capensis	0	1	Maritz and Maritz 2020[a]
MAMMALS			
Soricidae			
Crocidura silacea	0	1	Cottone and Bauer 2010
Suncus varilla	0	1	Van Wyk 1988
Unidentified soricid	0	33	Van Wyk 1988; FitzSimons 1962; Herp Island 2015[n]
Nesomyidae			
Dendromus melanotis	0	4	Cottone and Bauer 2010
Malacothrix typica	0	1	Van Wyk 1988
Muridae			
Mus domesticus	0	2	Cottone and Bauer 2010
Mus minutoides	0	2	Cottone and Bauer 2010
Mus sp.	0	1	Cottone and Bauer 2010
Rattus	0	3	Van Wyk 1988
Rhabdomys pumilio	0	1	Maritz and Maritz 2020[a]
Unidentified murine	0	3	FitzSimons 1962; De Waal 1977; Cottone and Bauer 2010
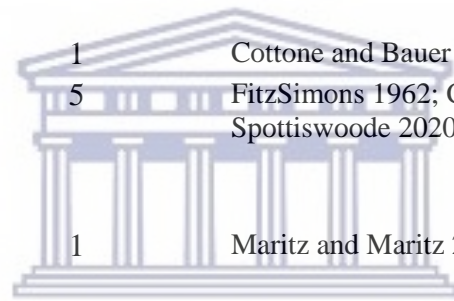
Prey item	*P. crucifer*	*P. rhombeatus*	References
Macroscelididae			
Elephantulus myurus	0	1	Van Wyk 1988
Unidentified macroscelid	0	14	Cottone and Bauer 2010; Steenhouder 2015[o]; Steenhouder 2013[p]; De Waal 1977; Maritz and Maritz 2020[a]
ARTHROPODS			
Scorpiones	0	1	Cottone and Bauer 2010
INSECTA			
Coleoptera	1	1	Cottone and Bauer 2010
Orthoptera	0	1	Cottone and Bauer 2010
Unidentified insect	0	1	Cottone and Bauer 2010

[a]https://doi.org/10.6084/m9.figshare.11920128.v2
[b]https://www.flickr.com/photos/7272097@N08/23934609088/in/photolist-2jm4vY6-2jewa41-Ct28pW-aVLb7n-2hrwJUT-diZzgn-pvA6zz-Qxzpsd-qq4SeS-7rQ7kT-7rU38U-4WgHRp-8yjYZf-am1NUE-wWqxCX-23YcDD3-23YcFHJ
[c]https://m.facebook.com/groups/PredationRecordsReptilesandFrogsSubSaharanAfrica/permalink/3561352127233948/
[d]https://www.inaturalist.org/observations/43313974
[e]https://m.facebook.com/groups/PredationRecordsReptilesandFrogsSubSaharanAfrica/permalink/3249011908467973/
[f]https://www.inaturalist.org/observations/10805453
[g]https://www.inaturalist.org/observations/34305042
[h]https://m.facebook.com/groups/PredationRecordsReptilesandFrogsSubSaharanAfrica/permalink/3208339619201869/
[i]https://m.facebook.com/groups/PredationRecordsReptilesandFrogsSubSaharanAfrica/permalink/4149583415077480/
[j]https://www.inaturalist.org/observations/11228804
[k]https://www.youtube.com/watch?v=TzrQ_qwxw5s
[l]https://www.inaturalist.org/observations/59156467
[m]https://www.ispotnature.org/communities/southern-; africa/view/observation/795280/snake-black-triangles-making-continuous-stripe-on-back
[n]https://www.youtube.com/watch?v=ZUUIb3eYLIc
[o]https://www.youtube.com/watch?v=DSugiogcP6k&t=13s
[p]https://www.youtube.com/watch?v=waxlP82AoC8
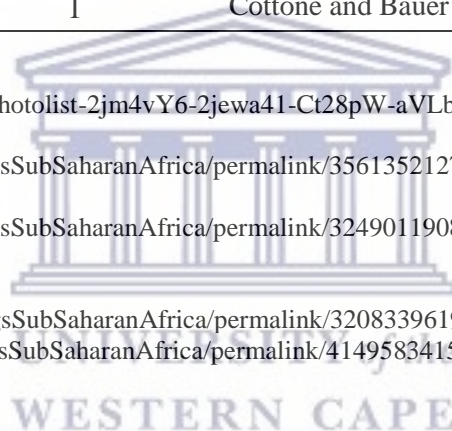
1.3. Approaches used to study feeding ecology

1.3.1. Invasive approaches

A range of methods for cataloguing prey items such as the examination of stomach contents (Vestjens & Hall 1977; Smuts 1979; Best & St-Pierre 1986; Christian 1982; Moteki et al. 2001; Meynier et al. 2008), faecal contents (Calver & Wooller 1982; Bigg 1985; Wachter et al. 2012), palpation of stomachs (MacArtney 1989; Brito 2004) and microscopy (Symondson 2002; Mumma et al. 2015) have been widely used across vertebrate taxa. Some of these methods have only recently been used for snakes because the application to snakes is not always feasible. Nonetheless, snake predation research has been conducted typically in two ways. Following direct observations of predators (Diller 1990; Kupfer 2003) or indirect studies based on identifying prey from gut contents (Prestt 1971; Greene 1983; Slip & Shine 1988), faeces (Spellerberg & Phelps 1977; Slip & Shine 1988; Monney 1993; Luiselli et al. 1996), or regurgitates (Luiselli & Anibaldi 199l; Greene et al. 1994; Rugiero & Luiselli 1995; Luiselli et al. 1996).

Prey identification has been attained from gut contents from museum specimens, indigestible prey passing through the stomach (Mushinsky & Hebrard 1977; Burger et al. 1999) and from partially digested eggs (Broadley 1979). This approach provides direct information on prey consumption in the ecosystem, as animals remain undisturbed before collection (Sunderland 1988). Similarly, Daltry et al. (1998) found that faeces provide an alternative source of dietary information, as more prey items were identified from faecal contents than from stomach contents. Brown et al. (2014a) reported that reptiles may be more obliging to defecate when gently palpated than other vertebrate taxon. Moreover, Wallace and Diller (1990) proposed that faecal analyses from reptiles are more feasible than gut contents from museum specimens because the majority of the specimens have empty stomachs.

Importantly, we can answer different ecological questions with each method. For example, by palpating meals from snakes, we can collect data on prey size that can be used to answer whether snakes are size-selective predators, or whether larger snakes ingest a wider range of prey based on their body size compared to smaller snakes. Conversely, we can collect data from prey identified in faecal and stomach contents to answer questions related to the diversity of prey species in their diets. Each of these methods provide valuable insight into snake foraging ecology and dietary habits that can enhance our understanding of snakes and their role in the ecosystem.

While these methods have provided the basis for the global understanding of snake diets and foraging, they offer several drawbacks. Firstly, the examination of stomach contents of museum specimens is not always feasible in snakes as it frequently results in damage to the specimens. Furthermore, preserved specimens are often drawn from a large geographic distribution preventing detailed examination of local populations dietary trends. Moreover, prey is often recovered from live or preserved specimens at low frequency (Glaudas et al. 2017). As a consequence, this method might be biased toward detecting prey items that are less digestible or larger because these food items are digested more slowly (Mills et al. 2003; Jethva & Jhala 2004; Wachter et al. 2012; Glaudas et al. 2017). In addition, because snakes eat infrequent, large meals (Greene 1997), the palpation of meals from the stomachs of wild snakes might rob individual snakes of important meals that could influence energy acquisition rates for that feeding season and have knock-on effects for reproduction or activity patterns. Similarly, faecal analysis based on morphological identification of prey items may underestimate the occurrence and diversity of species (Brown et al. 2012) and requires expert knowledge to identify the prey (Brown et al. 2014a). In the instance of microscopic analysis, extensive taxonomic expertise is required because of the high rate of digestibility that could prevent identification of prey at a fine scale (Pompanon et al. 2012). Such studies offer useful data on prey selection and relative rates of predation but fail to produce absolute rates of predation (Greene 1983).

1.3.2. Non-invasive approaches

Detailed feeding records from a population can be obtained in a non-invasive manner using direct observations (Cezilly & Wallace 1988) such as social media observations (Maritz & Maritz 2020), camera traps (Hernández et al. 2005; Weckel et al. 2006) and videography (Lewis et al. 2004; Putman et al. 2015). The most direct approach uses field observations to identify prey from snake encounters with them (Sih et al. 1985; Shine et al. 1996). This method is particularly useful, as it allows for real-time observation of animal interactions (Stapp 2002; Meckstroth et al. 2007; Aguiar & Moro-Rios 2009; Maritz & Maritz 2020). However, direct observations often preclude working on elusive species such as snakes (Shine et al. 2004; Pompanon et al. 2012). This is because snake detection probabilities are often low, making snakes difficult to detect (Durso et al. 2011). Even if predator-prey interactions are observed, there is no information regarding whether the interaction is a common or rare event (Symondson 2002).

Camera traps have been used to study elusive terrestrial species that are otherwise difficult to study (Wemmer et al.1996; Burton et al. 2015). Although species abundance can be estimated relatively accurately when using camera traps (Chapman & Balme 2010), the probability of detection for those animals needs to be high (Maputla et al. 2013). Because many snakes have low detection probabilities (Greene 1997), capturing them on camera may prove challenging. Videography has been used for ambush predators like puff adders (Glaudas et al. 2017) and rattlesnakes (Clark 2006) but this is simply not feasible for active foraging snakes, unless a point resource (i.e., nests or hibernacula) is known. This method has principally been used in bird studies to identify nest predators (Brown et al. 1998) such as snakes (Robinson et al. 2005) and is known to record snake encounters (i.e., *Crotalus horridus*) with other prey (Clark 2006). The major limitation of videography is that it is likely biased towards detection of larger snakes while small snakes may not be easily detected. This may be dependent on the video resolution quality (Glaudas et al. 2017).

Molecular methods have long been used to study the diets of animals (reviewed in Symondson 2002). Earlier attempts to characterize carbon sources, organisms and food webs utilised stable isotope analyses (SIA) (Deniro & Epstein 1978; Peterson & Fry 1987). SIA has been used to determine long-term diet (Wallace et al. 2009). Although the diets of several snake species have been examined using stable isotopes (Rush et al. 2014; Durso & Mullin 2017), the major limitation to the method is that it seldomly provides information on species-specific identities (Symondson 2002; Wallace et al. 2009). Similar to SIA, protein-based studies including electrophoresis and antibody assays have been used to analyse the remains of predator guts (Murray 1989) and to determine prey at different taxonomic levels (Symondson et al. 1999).

DNA-based approaches are now widely used to study the diets of a diverse range of taxa (reviewed in Symondson 2002). In particular, the analysis of faecal DNA has become a popular method to investigate the feeding ecology in animal systems (Table 1.2). Previous studies that addressed dietary diversity followed methods such as DNA profiling and temperature or denaturing gradient gel electrophoresis (Deagle et al. 2005; Pompanon et al. 2012). These methods are useful when investigating dietary diversity, however they lack the ability to identify specific meal components (Felske et al. 1998). Furthermore, DNA profiling and temperature or denaturing gradient gel electrophoresis have proven difficult to visualise DNA bands clearly, and this can complicate the interpretation of results (Deagle et al. 2005).

DNA-metabarcoding is a technique used to identify prey using DNA-based methods and rapid DNA-sequencing. This method was implemented by Pereira et al. (2019) in diet studies of reptiles. Metabarcoding enables the identification of soft prey items which would otherwise be undetectable using faecal or stomach analysis (Esnaola et al. 2018; Moran et al. 2019). This technology can characterise the species compositions of mass samples of eukaryotes or of environmental DNA, however, it is limited mainly by its dependency on PCR and by the considerable investment needed to build comprehensive taxonomic reference libraries (Pompanon et al. 2012; Alberdi et al. 2018; Taberlet et al. 2021).

Common molecular strategies to identify fecal-DNA use PCR with a range of primers, which can be either taxon-specific or universal (Table 1.2). Taxon-specific primers are designed to target a limited number of species and provide an approach to detect the inclusion of specific prey types in the diet (King et al. 2008). Taxon-specific primers are thought to be better at making species-level identification than stable isotope analysis (Symondson 2002) because they are able to identify prey to species level. Faecal-DNA studies conducted on reptiles have made use of species-specific and group-specific primers to identify prey (Brown et al. 2012; Brown et al. 2014a; Kartzinel & Pringle 2015; Alenius 2016). However, the species-specific approach is a lengthy process that needs to be repeated over multiple occasions. Another limitation to using species-specific primers is the need to sequence and find primer sites prior to the application of a study. In order to obtain target DNA only, primers need to be tested against a wide range of non-target taxon whose DNA might also be present in the samples (Wallinger et al. 2012). *A priori* hypotheses are thus needed to identify which prey likely occur in their diets.

Universal primers are designed to amplify DNA from a taxonomically broad range of species. Several universal primers have been applied successfully to a range of animal taxon (Table 1.2). These studies demonstrated the viability of faecal by-products as non-invasive sampling source material. The advantage of using universal primers is that they can amplify a DNA region from species that remain to be studied (Jarman et al. 2004). Based on the literature review (Table 1.2), *COI* and *16S rRNA* are the two most commonly used gene regions in diet studies of reptiles. Currently, only one study has investigated snake diets using faecal DNA (Brown et al. 2014a). This study acquired detailed information on invertebrate and vertebrate prey.

Regardless of primer choice, quantifying snake diets remains challenging because snakes have low detection probabilities (Greene 1997; Durso et al. 2011) making samples difficult to acquire. Moreover, storage methods (Seutin et al. 1991; Wasser et al. 1997; Nsubuga et al. 2004; Roeder et al. 2004), poor quality and low quantity of DNA (Taberlet et al. 1999; Morin et al. 2001; Pompanon et al. 2012), preservation methods (Murphy et al. 2000; Murphy et al. 2002), extraction and amplification success (Goossens et al. 2000) and predator amplification (Deagle et al. 2006) are all factors that can influence the success of prey detection through PCR amplification. Although there are limitations to using universal primers these biases can be reduced or managed in a range of effective ways (Deagle et al. 2006; King et al. 2008).

Universal primers can be used in combination with cloning and sequencing (Höss et al. 1992) to overcome the constraints associated with prey and predator detection. DNA cloning methods are able to describe unknown DNA in samples such as faeces (Sheppard & Harwood 2005; Clare et al. 2009) and work better when targeting multiple prey species (Jarman et al. 2004). However, this is a labour-intensive approach, and requires sequencing of many clones. DNA contamination and secondary consumption can also be problematic when it comes to analysing the results (Sheppard & Harwood 2005). Despite these constraints, cloning methods have the potential to overcome many of the challenges described above.

More recently, Pompanon et al. (2012) assessed the developments of dietary ranges of predators and herbivores using Next-Generation Sequencing (NGS). NGS has widely been used to study the diets of several animal taxa (Table 1.2) and is more cost-effective compared to the commonly employed methods in DNA barcoding (Shendure & Ji 2008; Sonet et al. 2018). NGS is advantageous as it maximizes the resolution and detection of soft or small prey items and rare events (Pompanon et al. 2012). This method allows prey identification in reptile species that are difficult to observe. Furthermore, no *a priori* knowledge is required of the species involved. Even so, there are several limitations to using NGS. NGS cannot measure an organism's entire dietary breadth because of biases during DNA extraction, PCR and bioinformatics sorting (Pompanon et al. 2002), it requires sophisticated bioinformatics systems, large data storage capabilities (Alkan et al. 2011), the purchasing of expensive equipment and involves significant labour and time to analyse samples (Roh et al. 2010).

Regardless of all of these challenges, DNA-based approaches have the ability to circumvent the problems associated with traditional methods. Because many details concerning the roles

and interactions of snake species remain elusive, DNA-based studies could provide promising data to fill some of these gaps. These methods could be used to non-invasively analyse the range and diversity of prey consumed by generalist predators in complex ecosystems, which would allow molecular tracking of animal diets from sources such as faeces.

1.3.3. Overcoming challenges of faecal DNA-based approaches

Although there are several limitations to DNA-based approaches of dietary analyses—storage challenges, DNA detection, predator amplification (Deagle et al. 2006; Brown et al. 2014b) and method selection—prior studies (Seutin et al. 1991; Wasser et al. 1997; Nsubuga et al. 2004; Roeder et al 2004; Taberlet et al. 1999; Morin et al. 2001; Pompanon et al. 2012; Murphy et al. 2000; Murphy et al. 2002; Deagle et al. 2006) have shown that there is opportunity to modify existing methodologies that can be applied in the context of understanding snake diets. The above-mentioned studies have also shown that the success of DNA extractions from faecal samples vary in efficacy between different taxa.

Accurate identification of prey from snake predator faeces is critical for obtaining reliable data of their diets. As such, I conducted a thorough investigation of prior methodologies used to quantify animal diets (Table 1.2) and determined that prey identification from snake faeces will best be achieved using a two-step approach: (1) a species-specific approach and (2) a universal approach. The species-specific approach will be used to determine whether prey DNA is present and amplifiable in these two snake species faeces, while the universal approach will be used in combination with cloning and restriction enzymes to identify a broader range of prey, if present in the faeces, and to overcome challenges associated with predator detection.

Table 1.2 Review of DNA-based studies on faecal analysis in vertebrate systems. Amplicon sizes are represented in base pairs. Molecular approaches are simplified as "Metabarcoding" for DNA-metabarcoding, "Barcoding" for DNA-barcoding, "NGS" for Next Generation Sequencing, "SGS" for Second Generation Sequencing, "RFLP" for Restriction Fragment Length Polymorphism, "PCR-based (U)" for universal primers, "PCR-based (SS)" for species-specific, "PCR-based (GS)" for taxa-specific or group-specific. Organized by predator taxon.

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Bats	Insects	Automated glass fibre technique	PCR-based (U)	*COI*	648	Clare *et al.* 2009
Bats	Arthropods	Power Soil DNA Isolation Kit	PCR-based (U) + Metabarcoding	*16S rDNA, COI*	157, 20–500	Alberdi *et al.* 2020
Bats	Birds	QIAamp DNA Stool Mini Kit (QIAGEN) & Silica-method	PCR-based (U) + PCR-based (GS)	*CYTB, COI*	648, 758	Pastor-Beviá *et al.* 2014
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + NGS	*COI*	200	Vesterinen *et al.* 2013
Bats	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Metabarcoding	*COI*	157	Zeale *et al.* 2011
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	157–658	Bohmann *et al.* 2011
Bats	Birds & Pigs	CTAB method	PCR-based (U) + RFLP	*CYTB*	380	Bobrowiec *et al.* 2015
Bats	Arthropods	NucleoSpin DNA Stool Kit	PCR-based (GS) + Metabarcoding	*COI*	250	Vesterinen *et al.* 2018
Bats	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U)	*16S rDNA, COI*	112, 78	Sommer *et al.* 2019
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Metabarcoding	*COI*	157	Vesterinen *et al.* 2016
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	157	Gonsalves *et al.* 2013
Bats	Birds	QIAamp PowerFecal DNA Kit	PCR-based (GS)	*COI, CYTB*	648, 758	Heim *et al.* 2019
Bats	Domestic animals, Arthropods, Nematodes	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Metabarcoding	*16S rDNA, COI*	95, 313	Bohmann *et al.* 2018
Bats	Arthropods, Insects	Power Soil DNA Isolation Kit	PCR-based (GS) + Metabarcoding	*16S rDNA, COI*	157, 500	Aizpurua *et al.* 2018
Bats	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U)	*COI*	157-658	Krüger *et al.* 2014
Bats	Insects	Automated glass fibre technique	PCR-based (U)	*COI*	648	Clare *et al.* 2011

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + SGS	*CYTB*	405	Hope *et al*. 2014a
Bats	Plants	QIAamp DNA Stool Mini Kit (QIAGEN)	Metabarcoding + NGS	*ITS2*	225	Sobek & Walker 2020
Bats	Arthropods	QIAamp DNA Stool Mini Kit	Metabarcoding + Cloning	*COI*	157 & 180	Jusino *et al*. 2019
Bats	Invertebrates	QIAamp DNA Stool Mini Kit	PCR-based (U) + NGS	*COI*	177	Brown *et al*. 2014b
Bats	Frogs	PowerSoil Kit	PCR-based (U) + Metabarcoding	*16S rDNA, COI*	550, 650	Jones *et al*. 2020
Bats	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	157	Arrizabalaga-Escudero *et al*. 2015
Bats	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	157	Arrizabalaga-Escudero *et al*. 2019
Bats	Invertebrates	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Metabarcoding	*COI*	157	Andriollo *et al*. 2019
Bats	Insects	DNeasy Blood and Tissue Kit	PCR-based (SS)	*COI*	95, 114, 152, 185, 229	Schattanek *et al*. 2021
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	157	Hope *et al*. 2014b
Bats	Birds	GuSCN method	PCR-based (GS) + PCR-based (U) + Cloning	*COI, CYTB*	380, 160	Ibáñez *et al*. 2016
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI, CYTB*	648, 758	Clare 2014
Bats	Insects	QIAamp DNA Stool extraction Kit	PCR-based (GS)	*COI*	157	Razgour *et al*. 2011
Bats	Plants	CTAB based Lysis buffer	Barcoding	*rbnL*	550–600	Hayward *et al*. 2013
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Cloning	*COI*	157	Van Den Bussche *et al*. 2016
Bats	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + DNA-metabarcoding	*COI*	155, 159	Mata *et al*. 2019
Bats	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	157	Mata *et al*. 2016
Bats	Insects	DNeasy Power Soil Kit	PCR-based (GS) + Metabarcoding	*COI*	157, 133	Aldasoro *et al*. 2019

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Bats	Insects	Zymo Research Genomic DNA	PCR-based (GS) + Metabarcoding	*COI, CYTB*	157, 133	Browett *et al.* 2021
Rats	Birds, Invertebrates, Plants	QIAamp DNA Stool Mini Kit (QIAGEN), DNeasy *mericon* food Kit & CTAB method	PCR-based (GS)	*COX2*	350	Zarzoso-Lacoste *et al.* 2013
Rats, mouse, hedgehog	Frogs	Zymo D6010 Faecal DNA Kit	PCR-based (GS)	*12S rDNA*	130	Egeter *et al.* 2015
Mouse	Plants	Chloroform method	PCR-based (GS) + Metabarcoding	*trnL*	50–60	Sato *et al.* 2018
Shrew	Invertebrates	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U) + NGS	*COI*	177, 255	Brown *et al.* 2014b
Rodents	Plants, Molluscs, Arthropods, Invertebrates	DNeasy Blood Tissue Kit	PCR-based (GS) + Metabarcoding	*trnL, ITS1, 16S rDNA*	31–60, 69–75, 83–85, 37–38	Lopes *et al.* 2015
Rodents	Plants	DNeasy Tissue Kit	PCR-based (GS) + Metabarcoding	*trnL, COI*	146, 128	Soininen *et al.* 2015
Martens	Rodents, Birds, Fruit	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Barcoding	Micro-satellites	156–166, 128, 146-152, 176–200	Posłuszny *et al.* 2007
Martens	Birds, Rodents & Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Restriction enzymes	D-loop region	267	Balestrieri *et al.* 2011
Pyrenean desman	Mammals, Birds, Amphibians	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U) + NGS	*COI*	133	Gillet *et al.* 2015
Pyrenean desman & Shrew	Invertebrates	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + NGS	*COI*	133	Biffi *et al.* 2017
Pyrenean desman	Insects	QIAGEN Powerfecal DNA kit	PCR-based (GS) + Metabarcoding	*COI*	218, 133, 313, 130, 157	Esnaola *et al.* 2018
Monkey	Plants	QIAGEN DNeasy Blood and Tissue Kit	PCR-based (GS) + Metabarcoding	*trnL*	280–300	Srivathsan *et al.* 2015
Apes	Insects	QIAamp DNA Stool Mini Kit	PCR-based (U) + Cloning	*CYTB, COI*	157, 410	Hamad *et al.* 2014

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Monkey	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U)	*COI*	280	Mallott *et al*. 2015
Domestic cats	Rodents, Insects, Birds, Reptiles	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U) + Metabarcoding	*CYTB*	136	Forin-Wiart *et al*. 2018
Wolves & Coyotes	Deer, Moose, Rabbits	QIAGEN DNeasy 96 Blood & Tissue Kit	PCR-based (GS) + Metabarcoding	*CYTB, 12S rDNA*	170, 165, 208	Shi *et al*. 2021
Goats & Walia ibex	Plants	DNeasy Blood and Tissue Kit	PCR-based (GS) + Metabarcoding	*CYTB, trnL*	700, 426	Gebremedhin *et al*. 2016
Puma & Jaguar	Mammals, Lizards, Birds, Arthropods, Fish, Plants	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*CYTB*	102, 146	Farrell *et al*. 2000
Puma & Jaguar	Deer, Goat, Lizards, Raccoon, Opossum	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*CYTB*	102, 146	Rueda *et al*. 2013
Leopard cat	Mammals, Birds, Amphibians, Fish	DNeasy Blood and Tissue Kit	PCR-based (U) + NGS	*12S rDNA*	100	Shehzad *et al*. 2012
Wolves	Moose, Deer, Caribou, Beaver, Hare	DNeasy Tissue Kit	PCR-based (GS)	*CYTB*	117–200, 130–184, 78–150, 98–155	Shores *et al*. 2015
Felids	Rodents, Fowl, Ungulates, Birds, Fish, Lizards	2CTAB/PCI method	PCR-based (U) + NGS	*16S rDNA, 12S rDNA*	100, 350	Xiong *et al*. 2017
Lemur	Plant	2CTAB/PCI protocol	PCR-based (GS) + Metabarcoding	*trnL*	40, 456	Quéméré *et al*. 2013
Cow	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	Metabarcoding + NGS	*COI*	217	Sigsgaard *et al*. 2021
Otter, Polecat & Mink	Fish, Frogs, Birds	Phenol-chloroform, Chelex extraction, Guanidine thiocyanate method	PCR-based (SS) + Restriction enzymes	*CYTB*	189	Hansen & Jacobsen 1999
Otter	Fish	Real Pure Spin Food-Stool Kit	PCR-based (GS) + Metabarcoding	*CYTB, COI*	200	Martínez-Abraín *et al*. 2020
Otter	Fish	DNeasy Blood & Tissue Kit	PCR-based (U) + Barcoding	*12S rDNA*	77–123	Pertoldi *et al*. 2021
Wild boar	Birds	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*12S rDNA*	183	Oja *et al*. 2017

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Bears	Plants, Deer, Fish	QIAamp DNA Stool Mini Kit (QIAGEN), Silica pellet method	PCR-based (GS)	Micro-satellites	146, 200	Murphy *et al.* 2003
Bears	Plants	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	Micro-satellites	150, 180–200	Murphy *et al.* 2007
Bears	Plants, Vertebrates, Molluscs, Arthropods	DNeasy Blood and Tissue Kit	Metabarcoding + NGS	*12S rDNA, 16S rDNA, ITS1, ITS2, trnL*	51, 98, 36, 81, 69, 64, 82, 569, 407	De Barba *et al.* 2014
Seals	Fish	DNeasy Tissue Kit	PCR-based (GS)	*GH2*	112–124	Kvitrud *et al.* 2005
Seals	Fish, Squid	UltraClean Fecal DNA Isolation Kit	PCR-based (GS)	*16S rDNA, 28S rDNA*	160–237 100–180	Casper *et al.* 2007
Seals	Fish	DNAce Spin Stool extraction Kit	PCR-based (GS)	*16S rDNA, CYTB*	162, 327	Parsons *et al.* 2005
Seals	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA*	290–308, 282–300	Hui *et al.* 2017
Seals	Fish	Boiling in water, Boiling in Chelex-100, Proteinase K digestion & GuSCN method	PCR-based (GS)	Micro-Satellites	520	Reed *et al.* 1997
Seals	Fish	QIAamp DNA Stool Kit (QIAGEN)	PCR-based (GS)	*16S rDNA*	155	Thomas *et al.* 2014
Seals	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	Barcoding	*16S rDNA*	120	Deagle *et al.* 2013
Fur seals	Mammals, Fish, Birds, Invertebrates	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA, 12S rDNA*	70, 90, 150, 200, 230	Hardy *et al.* 2017
Fur seals	Fish, Cephalopods	QIAamp DNA Stool Mini Kit	PCR-based (GS) + NGS	*16S rDNA, 18S rDNA*	155, 280, 285	Deagle *et al.* 2009
Fur seals	Fish, Cephalopods	MO BIO Power Max Soil	PCR-based (GS)	*16S rDNA*	250	Emami-Khoyi *et al.* 2016
Sea lions	Fish, Squid, Shark	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA, COI*	100–250, 71	Peters *et al.* 2015a
Sea lions	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + restriction enzymes	*16S rDNA*	65–69	Deagle & Tollit 2007
Sea lions	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Cloning	*16S rDNA*	250	Deagle *et al.* 2005
Sea lions	Fish, Squid	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA, COI*	69–79, 77	Bowles *et al.* 2011

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Sea lions	Fish, Cephalopods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA*	100, 112	Peters *et al*. 2015
Walruses	Invertebrates	DNeasy stool Mini Kit	PCR-based (GS)	*16S rDNA*	183–280, 250	Bowles & Trites 2013
Dolphin	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U) + Cloning	*16S rDNA*	620	Dunshea 2009
Killer whale	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA*	330	Ford *et al*. 2016
Blue whale	Crustaceans	Reversible binding to silica method, Guanidine thiocyanate method	PCR-based (GS) + Cloning	*LSU rDNA*	286	Jarman *et al*. 2002b
Marine predators	Whales	Chelex rapid boiling method	PCR-based (GS)	*Valine tRNA*	370	Jarman *et al*. 2004
Birds	Plants	DNeasy Plant Mini Kit	Barcoding + NGS	*trnL*	577, 614, 389	Ando *et al*. 2013
Birds	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	211	Crisol-Martínez *et al*. 2016
Birds	Plants	DNeasy kit	Barcoding	*ITS2*	100–430	Volpe *et al*. 2021
Birds	Fish	Real Pure Spin Food-Stool Kit	PCR-based (GS) + Metabarcoding	*12S rDNA*	418–636, 221	Martínez-Abraín *et al*. 2020
Birds	Fish, Insects, Invertebrates	CTAB method	PCR-based (U) + NGS	*COI, 16S rDNA*	130–300	Gerwing *et al*. 2016
Birds	Invertebrates, Plants	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Metabarcoding	*COI, 16S rDNA, rbcL*	184–220	Shutt *et al*. 2020
Birds	Fish	BioSprint 96 DNA blood Kit	PCR-based (GS)	*COI*	82–94	Thalinger *et al*. 2017
Birds	Plants, Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Metabarcoding	*COI*	407, 658	Chung *et al*. 2021
Birds	Invertebrates	DNeasy Tissue Kits & QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U)	*COI*	710	King *et al*. 2015
Birds	Invertebrates	NucleoSpin® Soil Kit	PCR-based (U) + NGS	*COI*	286	Mansor *et al*. 2018
Birds	Invertebrates	CTAB protocol	PCR-based (U) + NGS	*16S rDNA, COI*	130–300	Bowser *et al*. 2013
Birds	Insects	DNeasy Kit	PCR-based (U) + Cloning	*COI*	658	Wong *et al*. 2015
Birds	Insects	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	185	Karp *et al*. 2013

22

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Birds	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U) + Cloning	*16S rDNA*	180–270	Deagle *et al*. 2007
Birds	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + NGS	*COI*	211	Crisol-Martínez *et al*. 2016
Birds	Arthropods	QIAamp DNA Stool Mini Kit & *Xpedition* Soil/Fecal DNA MiniPrep kits	PCR-based (U) + Cloning	*COI*	710	Jedlicka *et al*. 2013
Birds	Fish	QIAGEN BioSprint 96 instrument	PCR-based (GS)	*16S rDNA*, *COI*	77, 405	Oehm *et al*. 2017
Birds	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U)	*COI*	157	Trevelline *et al*. 2018
Birds	Invertebrates	DNeasy Tissue Kits & QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U) + Cloning	*COI*	282, 750, 751	King *et al*. 2015
Birds	Arthropods	CTAB method, UltraClean Fecal DNA Kit, Extract Master Faecal DNA Extraction Kit & QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	387, 128	Oehm *et al*. 2011
Birds	Fish & Lamprey	BioSprint 96 DNA blood Kit	PCR-based (GS)	*16S rDNA*, *COI*	77–405	Thalinger *et al*. 2016
Penguins	Cephalopods, Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA*	100–155, 250–310	Young *et al*. 2020
Penguins	Invertebrates, Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*16S rDNA*, *12S rDNA*	169, 300	Horswill *et al*. 2018
Penguins	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (SS) + Cloning	*16S rDNA*	180–270	Murray *et al*. 2011
Penguins	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U)	*18S rDNA*, *28S rDNA*, *16S rDNA*	245, 180, 300, 169	Xavier *et al*. 2018
Penguins	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Barcoding	*16S rDNA*	155, 260–310	Deagle *et al*. 2010
Penguins	Fish	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + Cloning	*16S rDNA*	180–270	Murray *et al*. 2011
Penguin	Fish, Copepods, Amphipods, Jellyfish	Tissue DNA Purification kit	PCR-based (U)	*SSU rDNA*	140–170	Jarman *et al*. 2013

Predator	Prey targeted	DNA extraction method	Molecular approach	Gene(s)	Amplicon sizes	Reference
Penguin	Crustaceans	Reversible binding to silica method, Guanidine thiocyanate method	PCR-based (GS) + Cloning	*LSU rDNA*	226, 224, 260–270, 440, 580	Jarman *et al*. 2002a
Fish	Fish, Amphibians, Arthropods, Molluscs	DNA-extracting reagent	PCR-based (GS)	*16S rDNA*	84, 80, 76	Taguchi *et al*. 2014
Turtle	Fish, Invertebrates	QIAGEN QIAamp PowerFecal DNA Kit	PCR-based (GS) + Metabarcoding	*COI, ITS, 16S rDNA*	168–1379, 546	Ducotterd *et al*. 2021
Frogs	Nematodes	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*18S rDNA*	402	Huggins *et al*. 2017
Frogs	Insects	Pure Link Genomic DNA Kit	PCR-based (GS) + Metabarcoding	*16S rDNA*	216, 156, 141	Pereira *et al*. 2021
Lizards	Invertebrates	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (U) + NGS	*COI*	177, 255	Brown *et al*. 2014b
Lizards	Invertebrates, Vertebrates, Plants	Stool DNA Isolation Kit	PCR-based (GS) + Metabarcoding	*COI, 12S rDNA, trnL*	40, 73–110, 110, 10–143, 146	Gil *et al*. 2020
Lizards	Arthropods	ZymoXpedition Soil & Fecal mini kit	PCR-based (GS)	*16S rDNA*	40–300	Kartzinel & Pringle 2015
Lizards	Arthropods	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*COI*	157	Alenius 2016
Lizards	Plants	QIAamp DNA Stool Mini Kit (QIAGEN) & Gentra Puregene method	PCR-based (GS)	Micro-satellites, *CYTB*	200	Pearson *et al*. 2015
Lizards	Invertebrates	Pure Link Genomic DNA Kit	PCR-based (U) + Metabarcoding	*16S rDNA, COI*	135–276, 658	Pereira *et al*. 2019
Lizards	Plants, Arthropods, Birds	Stool DNA Isolation Kit	PCR-based (U) + NGS	*16S rDNA, COI, trnL*	438, 324, 158, 710	Pinho *et al*. 2018
Lizards	Earthworms	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS) + PCR-based (U)	*12S rDNA, COI*	225--236, 287	Brown *et al*. 2012
Snakes	Shrews, Rodents, Frogs, Lizards, Invertebrates	QIAamp DNA Stool Mini Kit (QIAGEN)	PCR-based (GS)	*CYTB, 12S rDNA, COI*	176, 243, 216, 110, 206, 171, 108, 150, 250	Brown *et al*. 2014a

1.4.   Problem statement

The feeding ecology of snakes is poorly known in comparison to other vertebrate taxa, largely because of the unique challenges associated with quantifying snake diets at a fine-taxonomic scale. Snakes are generally secretive, usually difficult to observe while foraging in the wild and are able to digest prey at high rates, which makes obtaining feeding data difficult. Existing methods for identifying prey are currently invasive, limited to reveal only certain aspects of a species trophic ecology, and often biased in their approaches. Accordingly, investigations using traditional methods may result in an incomplete understanding of prey utilised by particular snakes. I plan to demonstrate an alternative non-invasive approach that uses faecal DNA to identify prey items utilised by snakes that could potentially provide a greater understanding of snake diets and allow us to answer questions at a finer geographic and temporal scale.

1.5.   Study aims and objectives

The aim of this study is to establish and utilise a molecular approach to investigate the diets of *P. crucifer* and *P. rhombeatus*, two sympatric South African snake species. The study objectives were:

1) To build a database of DNA sequences for known and potential prey from the study site, using tissue sequences from GenBank and sequences generated in this study.
2) To test whether faecal samples from captive-fed snakes have amplifiable prey DNA using prey-specific primers.
3) To establish a timeline of gut passage and retention of prey DNA using captive-fed snakes.
4) To develop a reduced-cost universal approach to identifying prey DNA using cloning and restriction enzyme digestion.
5) To conduct a DNA analysis on faecal samples from wild-caught snakes using a universal approach.

Chapter 2. Methods

2.1. Building a DNA sequence database of known and potential prey

2.1.1. Identifying known and potential prey

I compiled a comprehensive list representing the known small mammals, reptiles, amphibians, and birds at Koeberg Private Nature Reserve (KPNR) from previously collected biodiversity survey data. I also collected diet information of these snakes from the literature, Facebook, iNaturalist, Flickr, iSpot and YouTube. The list represents 71 known and potential prey species available at KPNR (Table 3.1). From this list, I built a database of potential prey for the two test snake species based on their known ecologies and apparent dietary preferences (Van Wyk 1988; Cottone & Bauer 2010; Bates et al. 2014). Additionally, the data from Cottone and Bauer (2010) was used as a guide to identify known prey species and species likely to be included in the diets of these snakes based on prey size (<11 g), taxonomic similarity to known prey items, and prey ecology. For example, Cottone and Bauer (2010) listed several species of *Pachydactylus* as prey of both *P. crucifer* and *P. rhombeatus*. I therefore included members of the same genus such as *P. geitje* and *P. maculatus* that overlap in distribution with these snakes. I used the same logic to identify potential prey species while also considering factors such as prey size and prey occurrence. For instance, I selected small mammals and juvenile birds that were taxonomically similar to known species and that occur at KPNR.

2.1.2. DNA sequences available on GenBank

I compiled available mitochondrial gene sequences, namely *CYTB* (cytochrome b), *COI* (cytochrome c oxidase subunit I) and *16S rRNA* (16S ribosomal RNA) which were derived from GenBank (NCBI) for several species of mammal, reptiles, birds, and amphibians for known and potential prey at KNPR based on the dietary review (Table 1.2). I selected gene regions based on their abundance in the literature (Table 1.2) and availability on GenBank (Table 3.1). In diet studies of vertebrates, *COI* and *16S* rRNA were the commonly used gene regions, however in reptile diets, *CYTB* has also been reported (Brown et al. 2012, 2014a; Kartzinel & Pringle 2015). Furthermore, on GenBank there were not many sequences for *COI* for the prey in which I was interested. As such, I excluded *COI* from subsequent analysis and focused exclusively on *CYTB* and *16S rRNA*.

2.1.3.   Collection of additional DNA material

DNA sequences were not available for all potential prey at KNPR. Therefore, I collected tissue samples of prey in the field and from previously collected specimens to be sequenced. Although some species that were sequenced were represented on GenBank, this was only for a certain gene region. I therefore sequenced a different region of the gene to the region available on GenBank. Tissue samples were collected from a range of available prey found at KPNR using both tail tips from live animals and material from previously collected and stored specimens. Sterile forceps, scissors and a scalpel were used to collect tissue samples from lizards and snakes in the field. Tissue samples were collected by taking tail tips which were stored in tubes containing 95% ethanol at -20°C until processing. Additionally, tissue samples were taken from deceased prey animals (i.e., small mammals) collected in the field as trap by-catch or from donated specimens. Frozen and stored tissue material was placed in a freezer for subsequent DNA analysis.

2.1.4.   DNA extraction, PCR amplification, and sequencing

The KAPA Express Extract Protocol (Kapa Biosystems) was used to extract DNA from tissue samples, following these conditions per 100 μl reaction: 88 μl PCR-grade water, 10 μl of 10X Kapa Express Extract Buffer, 2 μl Kapa Express Extract Enzyme, 2 mm^3 tissue samples. PCR was prepared for the prey items using KAPA2G Fast HotStart ReadyMix PCR Kit (Kapa Biosystems). The reactions were set up as follows: 9 μl of PCR-grade water, 12.5 μl HotStart ReadyMix, 1.25μl forward primer, 1.25 μl reverse primer, and 1 μl crude DNA template. All setups included a negative template control to test for contamination. PCR reactions were carried out using a Bio-Rad thermal cycler.

16S rRNA fragments were amplified using 16S-F01 (5'-CGC CTG TTT AHC AAA AAC ATM ACC-3') (based on 16Sar-wob) and 16S-R01 (5'-GTG ATG ATG AAT GGT AGG ATG AAG TG-3') (based on 16Sb-wob) (Elbrecht & Leese 2015) and 16S-F02 (5'-ACC GTG CAA AGG TAG CGT AAT-3') and 16S-R02 (5'-AYR GGG TCT TCT BGT CTT RT-3'). The following conditions were implemented: initial denaturation at 95°C for 3 min, 32 cycles of 95°C for 15 seconds, 50°C for 20 seconds and 72°C for 20 seconds, a final extension at 72°C for 5 minutes. *CYTB* sequences for *P. geitje*, *P. crucifer* and *P. rhombeatus* were amplified using species-specific primers: *gei-F1/gei-R1* (Table 2.1), *cruc-F4/cruc-R4* (Table 2.1) and *rhom-F6/rhom-R7* (Table 2.1), respectively. PCR conditions for *CYTB* followed: initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 15 s, 60°C for 20

seconds and 72°C for 20 seconds, and a final extension at 72°C for 5 minutes. Gel electrophoresis of the PCR products was implemented (5 µl DNA sample with 1 µl loading dye) to visualise these on a 1% gel made up of 0.4 g agarose and 40 ml 1X TBE buffer stained with 4 µl ethidium bromide. DNA samples and the base pair ladder (Sigma Direct load PCR 100 bp Low ladder) were loaded into wells and the gel ran for an hour at a voltage of 75 before viewing them within ENDURO software to visualise and compare DNA bands.

PCR products were sent to the Stellenbosch Sequencing Unit for post-PCR cleaning and Sanger sequencing using the respective forward primer. Newly sequenced samples appear in Table 3.1 as "This Study". DNA sequences were assessed using bioinformatics software BLAST to ensure that the sequences obtained corresponded to the known tissue source. DNA sequences from "This Study" and GenBank were analysed in ClustalX2 (Thompson et al. 1997) and BioEdit (Hall 1999). All positions containing gaps and missing data were eliminated. Aligned sequences were imported into MEGA7 (v.7.0.26) (Kumar et al. 2016) using the Neighbor-joining statistical method (Saitou & Nei 1987). The evolutionary distances were computed using the number of differences method (Nei & Kumar 2000).

2.2.   Utilisation of captive-fed snakes

2.2.1.   Source population and collection of study animals

Live snakes, *Psammophylax rhombeatus* (N = 4) and *Psammophis crucifer* (N = 2) (male or female, >220 mm SVL) were captured from KPNR (Cape Nature permit 19/7/1). Snakes were collected from under cover objects. Upon capture, snakes were placed into 1 L plastic containers containing air holes that were lined with paper towel or were temporarily stored in cloth bags and placed into a well-ventilated cardboard box for transport to the University of the Western Cape. Snakes were weighed, and their snout-vent length (SVL) and tail length (TL) were measured using a measuring tape. Snakes were transferred within a maximum of 4 hours to their housing.

Over the study course reptile prey (N =23) were collected for captive feeding trials (Cape Nature permit 19/7/1). Prey included *Pachydactylus geitje* (N = 18)*, Trachylepis capensis* (N = 4), and *Trachylepis homalocephala* (N = 1). Prey were captured by hand using gloves, weighed, and placed individually into plastic jars (350 ml volume; ventilation holes drilled into cap). Additionally, a small quantity of vegetation and substrate (i.e., grass, leaf matter, sand etc.) were placed into the container with the animal to provide cover. A bottle of 1 ml

water was sprayed into each container to ensure that the contents remain humid (but not wet) during transport. Prey animals were transported to the University of the Western Cape at the end of the day of fieldwork (maximum of 5 hours after initial capture; more typically 1–4 hours after initial capture).

2.2.2. Housing and maintenance of snakes

Captive snakes were individually housed at UWC in a plastic 360 x 210 x 160 mm enclosure (PT-2300, Exo Terra®) lined with paper towels. Animals were provided with fresh water and shelter within the enclosure. A 150 x 210 mm 9W-foil heating mat fitted to the base of the enclosure was used to warm half of the enclosure to 30°C. The enclosure was kept at air temperature and out of direct sunlight. The enclosure was large enough to allow the animals to select which temperature they would prefer. Daily checks were conducted to ensure captive animals had access to clean water and bedding. While enclosures were cleaned, snakes were temporarily housed in a 1 L plastic container. Animal well-being was monitored by collecting body mass and length measurements to ensure that mass did not change by more than 10%. A datasheet with these measurements was kept alongside each snake's cage to maintain a historical record of these data. Animals were held from October 2019 to March 2020 and then released at the point of capture as per permit conditions.

2.2.3. Handling and storage of prey items

After capture, prey items were transported from KPNR to UWC. Jars (including prey animals) were placed into a commercial refrigerator (ambient temperature = 5°C) for 60 minutes to ensure full body cooling to 5°C. Next, jars (with prey animals) were moved to a commercial freezer (ambient temperature -15°C) where they were left for at least 3 hours to ensure death. Frozen specimens were removed from jars and placed into labelled plastic sleeves (Ziplock bags) for storage until they were required for the feeding trials. The hypothermic euthanasia protocol (Shine et al. 2015; Lillywhite et al. 2017; Shine et al. 2019) was approved by the Animal Research Ethics Committee of the University of the Western Cape (CN44-31-11193) and Cape Nature permit (19/7/1).

2.2.4. Feeding of prey items to snakes

Snakes were not fed for the first two weeks after capture to allow their last meals to fully pass through their gastrointestinal system. This is not uncommon for snakes, as they can stay for much longer periods without food (Pope 1961; Greene 1997). Before feeding, prey animals

were removed from the freezer and allowed to defrost at room temperature. The mass of the prey was recorded before being offered to snakes. Using clean forceps, a prey item was placed in front of a snake (within 20 mm of the snout) to offer the snake the opportunity to feed. A single feeding trial started at the offering of a prey animal for voluntary consumption and ended with a snake successfully swallowing the animal (successful trial) or regurgitating a prey animal (failed trial). Following a successful trial, the snake was left to defecate, and was not offered another meal for approximately 10 days following defecation. Following a failed trial, the snake was left in its cage for a period of at least 2 days before a new feeding trial was attempted. Snakes were monitored by recording the time of ingestion and excretion of its prey (Appendix 1; Tables A2.1-A2.5). Mass, SVL, and tail length for each snake was recorded weekly. Snake cages were cleaned after feeding but prior to excretion.

2.2.5. Faecal sample collection and storage

Faecal samples were collected from containers where snakes had defecated using forceps that had been sterilised with bleach and rinsed with water. Faecal samples were placed into a 2 ml centrifuge tube along with a solvent of 95% ethanol and stored at -20°C. A total of 83 faecal samples were collected from *P. crucifer* (N = 39) and *P. rhombeatus* (N = 44) individuals over the course of the study.

2.3. Genetic analysis of faeces from captive-fed snakes

2.3.1. DNA extraction and quantification

Faecal samples were centrifuged at 20 000 x *g* and the excess ethanol was discarded. DNA was extracted from the faecal samples following the QIAamp Fast DNA Stool Mini Kit (QIAGEN) as previously described (Deagle et al. 2005). The wet mass of most faecal samples weighed between 95 mg and 210 mg which was often less than the manufacturer's recommendation (180-220 mg per extraction). Samples were kept on ice until 1 ml Inhibitex buffer was added to each stool sample. Thereafter, samples were handled at room temperature. After adding the buffer, samples were vortexed for one minute. 25 μl of Proteinase K was pipetted into a new 1.5 ml microcentrifuge tube. 600 μl of supernatant was pipetted into a 1.5 ml microcentrifuge tube containing Proteinase K. 600 μl Buffer AL was added to the sample and was vortexed for 15 seconds. The sample was incubated at 70°C for 10 minutes. 600 μl of molecular-grade 100% ethanol was added to the lysate and was mixed by vortexing. 600 μl lysate was added to the QIAamp spin column that was centrifuged for

one minute and placed in a new 2 ml collection tube. The tube containing the filtrate was discarded. This step was repeated until all the lysate was loaded in the column. Thereafter, 500 µl Buffer AW was added to the QIAamp spin column and centrifuged for 1 minute. The QIAamp spin column was placed into a new 2 ml collection tube. Next, 500 µl Buffer AW2 was added to the QIAamp spin column and centrifuged for 3 minutes. The QIAamp spin column was placed in a new 2 ml collection tube that was centrifuged for 3 minutes. Finally, the QIAamp spin column was transferred into a new labelled 1.5 ml microcentrifuge tube and 200 µl Buffer ATE was pipetted directly onto the membrane. The microcentrifuge was incubated for one minute at room temperature and centrifuged for another minute to elute DNA.

The amount and quality of DNA present in each extraction were determined using a Nanodrop (2000) spectrophotometer. The sample reader was washed with molecular grade water and dried with a KimWipe. 2 µl of elusion water was used as a blank to initialise the system. 2 µl of DNA sample was loaded and measured. After the read was complete, the amount of DNA recovered (ng/µl) and A260/A280 and A260/A230 ratios were recorded. Thereafter, the sample reader was wiped clean with a dry KimWipe and the process was repeated for each extraction.

2.3.1.   Prey-specific approach

2.3.1.1.   Developing and testing prey-specific primers

Species-specific primers were designed using the following criteria: GC content was between 50-55%, secondary structures were low (i.e., hairpins), primer length ~15-20 bp and GC was found at the 5' end. Primers were tested for the above-mentioned criteria in NetPrimer (Premier Biosoft International). Using this criterion, multiple species-specific primers of different fragment lengths were designed (Table 2.1). This was based on the alignments of potential prey and predator species using AlleleID 7 (Premier Biosoft International, USA). Species-specific primers were designed to test for amplification of prey DNA in snake faeces, and primer specificity was tested on DNA extracted from tissue samples from prey (*P. geitje*) and predators (*P. crucifer* and *P. rhombeatus*). Estimated fragment lengths for sequences from *P. geitje* were 156 bp, 478 bp, and 573 bp, from *P. rhombeatus* were 149 bp, 316 bp and 458 bp and *P. crucifer* were 189 bp and 471 bp. Different primers were also used in combination with each other. For example, *gei-F3* was used with *gei-R1* and *gei-F1* was used

with gei-R3 to amplify DNA as the primers closely positioned one another and their base pairs overlap.

Table 2.1 Species-specific *CYTB* primer sequences. Intended species targets are represented as "gei" (*P. geitje*), "cruc" (*P. crucifer*) or "rhom" (*P. rhombeatus*). Annealing temperatures (T) and estimated PCR product lengths (base pairs) are provided.

Primer	Sequence	T (°C)	Product
gei-F1	5'-TAA TCT ACT GTC AGC CCT TCC ATA C-3'	55.8	156
gei-R1	5'-AAA GAG TAG GTG GAG TAA TAC GAC G-3'	55.8	
gei-F2	5'-GCC TAC TTA TTC AAA TCA CCA CGG-3'	55.8	478
gei-R2	5'-TAA CTA GGA CGA GAA GGA TTA CGC-3'	55.9	
gei-F3	5'-CCC AAC CGT CAA AAT TAT TAC CAC C-3'	56.6	251
gei-R3	5'-GTT CGT GTA GAA AGA GTA GGT GGA G-3'	56.4	
cruc-F4	5'-TAC CTG GGA ACG ATA ATA ACA ACC TG-3'	56.9	189
cruc-R4	5'-TTT TGT CTG TGT CTG AAT TAG TCC CC-3'	56.4	
cruc-F5	5'-GGA TCA ATA CTA TTA ACC TGC CTA GC-3'	55.5	554
cruc-R5	5'-GTG ATG ATG AAT GGT AGG ATG AAG TG-3'	55.0	
rhom-F6	5'-TTC AAC CTT CTC CCT GTC G-3'	54.3	458
rhom-R6	5'-ATT GAG AAT CCG CCT CAT AGT C-3'	54.3	
rhom-F7	5'-CAG TCG TAC ACA TTA CAC CAG A-3'	54.2	No product
rhom-R7	5'-ACG ACT GAT GAG AAT GCT AGG T-3'	55.5	

PCR reactions were set up using the KAPA2G Fast HotStart ReadyMix PCR Kit (Kapa Biosystems) as follows: 9 μl of PCR-grade water, 12.5 μl HotStart ReadyMix, 1.25 μl forward primer, 1.25 μl reverse primer, and 1 μl crude DNA template. PCR conditions for *CYTB* followed: initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 15 s, 50°C for 20 seconds and 72°C for 20 seconds, and a final extension at 72°C for 5 minutes. PCR reactions were carried out using a Bio-Rad thermal cycler. Gel electrophoresis of the PCR products was implemented (5 μl DNA sample with 1 μl loading dye) to visualise DNA on a 1% gel made up of 0.4 g agarose and 40 ml 1X TBE and were subjected to gel staining. The gel staining consisted of a working solution prepared as follows: One drop (50 μl) of 10 mg/ml ethidium bromide solution was added to 1000 ml deionised water. The gel was soaked for 15 minutes with gentle agitation and rinsed with deionised water and destained with fresh deionised water for 15 minutes with gentle agitation. DNA samples and the base pair ladder (Sigma Direct load PCR 100 bp Low ladder) were loaded into wells. Following this, the gel ran for an hour at a voltage of 75 before viewing them within ENDURO software to visualise

and compare DNA bands. A negative template control was included to test for contamination. Thereafter, the gels were ready for photographing.

To test species-specificity of primers, PCR was carried out using tissue DNA from either *P. crucifer*, *P. rhombeatus* or *P. geitje* with one of seven *CYTB* primer pairs (Table 2.1). Primer pairs were deemed specific if they only amplified DNA from the intended target (i.e., *gei-F1/ gei-R1*), such as only *P. geitje* and not *P. crucifer* or *P. rhombeatus*. The gels were examined by looking at which DNA amplified (prey or predator) from snake faeces using the primer sets. Visual inspection of the gels provided validation, as only PCR products of the intended target sequences (i.e., *P. geitje*) was amplified using a specific primer set (i.e., *gei-F1/gei-R1*). Where mtDNA amplification was successful (bands were evident for the target DNA), as determined by the presence of a band on an 1% agarose gel, amplification trials continued using the additionally developed species-specific primers (i.e., *gei-F2/gei-R1*, *gei-F3/ gei-F1*, *cruc-F5/cruc-R5*). Amplification success was visualised by inspecting the gels for specificity. Additionally, the PCR products that amplified for prey or predator DNA were sequenced to confirm the identity of the DNA sequences.

2.3.1.2. Analysis of faecal sample DNA

Using the newly tested species-specific *CYTB* primers, PCR reactions were prepared using faecal DNA from captive-fed *P. crucifer* and *P. rhombeatus*. Temperature gradient PCR was performed for the *CYTB* primers to determine the optimal annealing temperature at which the prey and predator species would amplify. PCR reactions followed the same protocol as described above but with a few modifications. The annealing temperature increased to 61.7°C and the DNA fragments were subsequently visualised on a 2% gel (0.8 g agarose) following the EtBr staining protocol (Section 2.3.2.1). Species-specific primers used include: *gei-F1/gei-R1*, *gei-F2/gei-F1*, *gei-F3/gei-R1* (*P. geitje*), *rhom-F6/rhom-R7* (*P. rhombeatus*) and *cruc-F4/cruc-R4*, *cruc-F5/cruc-R5* (*P. crucifer*). PCR products that showed positive DNA amplification for *P. geitje*, *P. rhombeatus* or *P. crucifer* DNA from the faecal samples of the predators were sent for Sanger sequencing using the respective forward primer. The sequences from Stellenbosch were aligned in ClustalX2 (Thompson et al. 1994) and edited in BioEdit (Hall 1999). Sequence similarity was compared using DNA from known tissue samples. Comparisons of the faecal DNA sequences of *P. geitje*, *P. rhombeatus* and *P. crucifer* returned a close match to tissue sequences in my database (Table 3.1). Faecal DNA

sequences were further assessed using bioinformatics by performing a local BLAST to identify prey and predator species from the faecal sample.

2.3.2. Universal approach

2.3.2.1. Developing and testing universal primers

DNA sequences of a broad range of taxa were obtained from GenBank and this study. DNA sequences were analysed in ClustalX2 (Thompson et al. 1994) and BioEdit (Hall 1999) to identify regions that were conserved and variable. This allowed me to design primers that could anneal to a wide range of species. Universal primers were designed for the *16S rRNA* gene region using PrimerDesign-M (Table 2.2) (Yoon & Leitner 2014; Brodin et al. 2013). *16S rRNA* primers (*16S-F01/16S-R01*) were designed based on 16Sar_wob and 16Sbr_wob in the following database:
https://v3.boldsystems.org/index.php/Public_Primer_PrimerSearch, following modifications (Elbrecht & Leese 2015). Primer pairs designed contained strongly conserved regions across taxa which were suitable to amplify a diverse range of species, alternated with less conserved short regions that exhibited species-specific differences.

To test the reliability of the primers, DNA from various known tissue samples of mammals and reptiles were tested with the newly developed universal primers (Table 3.1). Both mammal and reptile species were detected from tissue samples, indicating that the primers designed are universal (Figure 3.12–3.13). For the known species that I tested the primers against, the estimated fragment lengths varied between 595-630 bp (*16S-F01/16S-R01*) and 130-150 bp (*16S-F02/16S-R02*).

Table 2.2 *16S rRNA* universal primer sequences and their corresponding annealing temperature (T).

Primer	Sequence	T (°C)	Product (bp)
16S-F01	5'-CGC CTG TTT AHC AAA AAC ATM ACC-3'	54.8	595–630
16S-R01	5'-CGG TYT GAA CTC AGA TCA YGT-3'	54.7	
16S-F02	5'-ACC GTG CAA AGG TAG CGT AAT-3'	56.9	130–150
16S-R02	5'-AYR GGG TCT TCT BGT CTT RT-3'	53.3	

2.3.2.2. Conventional cloning

DNA extracts from faecal samples were amplified with both pairs of universal primers (*16S-F01/16S-R01* and *16S-F02/16S-R02*). PCR reactions were set up for *16S rRNA* following the above mentioned methodology (2.3.2) with these thermocycling conditions: initial denaturation at 95°C for 3 minutes, 32 cycles of 95°C for 15 seconds, 50°C for 20 seconds and 72°C for 20 seconds, a final extension at 72°C for 5 minutes (Figure 2.1).

LB agar plates were prepared by pouring 35 g of LB agar powder into a flask and adding 1000 mL dH_2O. Contents were mixed well and placed on a stirring hot plate for 1 minute. Contents were transferred to a 1 L Pyrex jar and labelled with autoclaved tape. The jar was autoclaved at liquid setting for 20 minutes in a basin with the top loosened and was left to cool to ~55°C.

Sterile petri dishes were removed from the plastic bag. 1000 µl of ampicillin was added to the cooled down LB agar solution. The solution was placed on a stirring hot plate for 1 minute. A thin layer (30mm) of LB agar was poured into each plate. Plates were swirled in a circular motion to distribute the agar on the bottom completely. Each plate was left to cool until it was solid (~20 minutes). Plates were inverted to avoid condensation on the agar. Plates were stored in plastic bags in the fridge with name, date and contents.

Ligation reactions were assembled by mixing 1 µl of linearized pMiniT Vector (25 ng/ µl) with 2 µl of DNA and 2 µl of dH_2O. 4 µl of the Cloning mastermix 1 (NEB®) and 1 µl of Cloning mastermix 2 (NEB®) was added to make up a total of 10 µl per ligation reaction. The reactions were incubated at room temperature (25°C) for 15 minutes and then placed on ice for 2 minutes. The reactions were transformed into NEB® 10-beta Competent *E. coli* (Figure 2.1).

A 50 µl tube of competent cells was thawed on ice for 10 minutes. 2 µl of ligation reaction was added to the competent cells and was mixed by flicking. The mixture was incubated on ice for 30 minutes and thereafter heat shocked at 42°C for 30 seconds. Following this, the mixture was placed on ice for another 5 minutes. 950 µl of LB media was added to the mixture. The mixture was placed at 37°C for 60 minutes in a rotation and shaking incubator at 250 rpm. Cells were thoroughly mixed by inversion and 50 µl of the 1 ml medium was

spread onto 37°C pre-warmed agar plates containing 100 μg/ml ampicillin. The plates were inverted and incubated overnight at 37°C (Figure 2.1).

A sterile toothpick was used to pick individual colonies. The colonies were placed into tubes containing 5 μl of PCR-grade water. The tubes were incubated at 95°C for 5 minutes to break open the cells and release DNA.
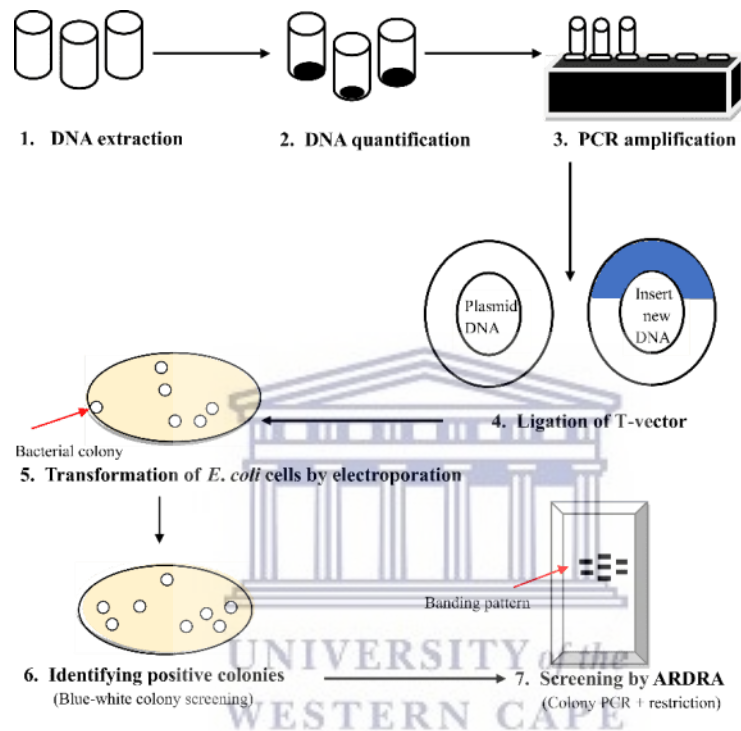
Figure 2.1 Diagram showing the molecular steps involved in cloning DNA from faecal samples of snakes.

2.3.2.3.   Restriction enzyme digest

All restriction enzyme sites were identified using NEBcutter (Vincze et al. 2003). Restriction sites for sequences of vertebrate species including potential prey were established using NEBcutter. Two restriction enzymes specific to the taxa under investigation were identified: RSAL and MseI.

2.3.2.4.   Post-PCR restriction enzyme digest

DNA was amplified in PCR with universal primers *16S-F02*/*16S-R02* (using conditions described in Section 2.3.3.2). Amplification success was established by gel electrophoresis stained with ethidium bromide. PCR products was digested by either RSAL and MseI using 1

µl of enzyme, 1 µl of buffer and 8 µl of colony PCR, in an incubation period of 4 hours at 37°C, followed by 20 minutes at 80°C for enzyme inactivation. PCR products were re-visualised by gel electrophoresis to determine whether the product had been digested (Figure 2.1).

2.4. Statistical analysis of faecal DNA retention

R version 3.4 (R Core Team 2017) was used for statistical analysis. To test which variable(s) correspond with the detection of prey DNA in faecal samples, a binomial regression analysis was performed using *faecal mass*, *days since last P. geitje meal*, *mass of last P. geitje meal*, *number of P. geitje meals fed*, *relative prey mass* and *snake identity* as predictor variables. This was tested following the function 'stats: glm'. The most parsimonious model was selected in the analyses.

2.5. Genetic analysis of faeces from wild-caught snakes

See above (2.3.1).

2.5.1. Collection and storage

Ten faecal samples were collected from wild-caught and captive snakes that defecated shortly after being captured at KPNR. Recovered wild faecal samples were stored in ethanol at -20°C as described above (2.2.5).

2.5.2. Universal approach

See above (2.3.3).

2.5.3. Identification of DNA sequences

Restriction enzymes (RSAL and MseI) were used with the intent to show differential banding for each species. However, differentiation was not possible for some of the species. Hence, subsamples of colonies from each faecal sample were sent for sequencing. Clone sequences were assessed using bioinformatics software BLAST to ensure that the sequences obtained corresponded to the known tissue source. Clone sequences were additionally analysed in ClustalX2 (Thompson et al. 1997) and BioEdit (Hall 1999). Aligned sequences were imported into MEGA7 (v.7.0.26) (Kumar et al. 2016) for further analysis.

Chapter 3. Results

3.1.   Database of prey DNA sequences

3.1.1.   Reference library construction

A total of 71 known and potential prey species of *Psammophis crucifer* and *Psammophylax rhombeatus* was identified from KPNR (Table 3.1). In total, the potential prey available to snakes at KPNR consists of 22 small mammal species, 31 reptile species, 10 anuran species and 8 bird species. Queries from GenBank produced a total of 136 DNA sequences representing potential prey species, 52 sequences of *16S rRNA*, 22 sequences of *COI* and 62 sequences of *CYTB* (Table 3.1). Overall, more sequences were available for *16S rRNA* and *CYTB* for the species of interest than for *COI*. Thus, I excluded *COI* sequences from all further analyses. The database was supplemented with an additional 11 sequences of *16S rRNA* and two sequences of *CYTB* which were generated during this study (Table 3.1; Table 3.2).

3.1.2.   Confirmation of newly sequenced prey DNA identities

BLAST searches were conducted on the newly obtained *16S rRNA* (N=11) and *CYTB* (N=2) sequences from known and potential prey. As anticipated, 76% of the new sequences were highly similar to sequences derived from intended prey targets that were available on GenBank. An exact match was not found for *Mus minutoides*, *Georychus capensis* and *Psammophis crucifer* (Table 3.2). For the above mentioned species, there were no DNA sequences available on GenBank for the gene or gene region that I amplified. However, there was sufficient sequence similarity to indicate that the sequences I obtained were similar to members within the same family or genus.

Table 3.1 Potential prey of *Psammophis crucifer* and *Psammophylax rhombeatus* available at KPNR. Prey species known to occur in the diet of either snake species (Table 1.1) are indicated with an "X". *16S rRNA*, *CYTB* and *COI* GenBank sequences are represented by an accession number. Taxonomic proxies are indicated where potential prey sequences are missing (indicated by an "accession number and species"). Sequences that I produced are marked "This study".

Potential Prey	Known Prey	*16S rRNA*	*COI*	*CYTB*
MAMMALS				
Cape mole-rat (*Georychus capensis*)	–	This study	This study	G496927.1
Cape dune mole-rat (*Bathyergus suillus*)	–	–	–	AY425912.1
African vlei rat (*Otomys irroratus*)	–	AF141253.1	–	KY754087.1
Cape gerbil (*Gerbilliscus afra*)	–	AM409232.1 This study	This study	AM409388.1
Egyptian rousette (*Rousettus aegyptiacus*)	–	AF134565.1	JF444443.1	AB085740.1
Cape horseshoe bat (*Rhinolophus capensis*)	–	–	–	KU531274.1
Geoffroy's horseshoe bat (*Rhinolophus clivosus*)	–	GU461872.1 *R. ferrumequinum*	–	EU436674.1
Egyptian slit faced bat (*Nycteris thebaica*)	–	AF044622.1	JF442546.1	HQ693723.1 *N. hispida*
Roberts's flat headed bat (*Sauromys petrophilus*)	–	AY495460.1	KF452684.1	HM802931.1
Egyptian free-tailed bat (*Tadarida aegyptiaca*)	–	KF059986.1	KF452715.1	HM802930.1
Natal long-fingered bat (*Miniopterus natalensis*)	–	–	JF442530.1	KF723608.1
Long-tailed serotine bat (*Eptesicus hottentotus*)	–	AY495466.1	MF038580.1	EU786823.1
Temminck's myotis (*Myotis tricolor*)	–	–	–	AJ504409.1
Cape serotine (*Neoromicia capensis*)	–	Q039231.1	KF452642.1	KJ756000.1
Forest Shrew (*Myosorex varius*)	–	KC505649.1	–	DQ630418.1 *M. cafer*
African pygmy mouse (*Mus minutoides*)	X	This study	–	LM994813.1
House mouse (*Mus musculus domesticus*)	X	LC062084.1	GQ905754.1	AY057807.1
Hairy-footed gerbil (*Gerbillurus paeba*)	–	–	–	KR089019.1
Gray African climbing mouse (*Dendromus melanotis*)	X	–	–	KF811235.1
Namaqua rock rat (*Aethomys namaquensis*)	X	AF141246.1	–	AF141215.1
Lesser gray-brown musk shrew (*Crocidura silacea*)	X	FJ486921.1	–	KF110763.1
Four-striped grass mouse (*Rhabdomys pumilio*)	X	AF141244.1 This study	MG773474.1 This study	AF533116.1

Potential Prey	Known Prey	*16S rRNA*	*COI*	*CYTB*
REPTILES				
Olive house snake (*Lycodonomorphus inornatus*)	–	AY611891.1 This study	This study	AY612073.1
Spotted house snake (*Lamprophis guttatus*)	–	AY611890.1	MF795180.1	AY612072.1
Cross-marked sand snake (*Psammophis crucifer*)	X	This study	This study	DQ486399.1 This study
Cape sand snake (*Psammophis leightoni*)	X	–	–	DQ486467.1 This study
Karoo sand snake (*Psammophis notostictus*)		–	–	DQ486463.1 This study
Spotted skaapsteker (*Psammophylax rhombeatus*)	X	FJ404215.1	–	FJ404312.1
Mole snake (*Pseudaspis cana*)		AY611898.1	–	AY612080.1
Common slug-eater (*Duberria lutrix*)	X	FJ404207.1	–	AF471061.1
Egg-eating snake (*Dasypeltis scabra*)	–	KX277241.1	–	AY612036.1
Spotted harlequin snake (*Homoroselaps lacteus*)	–	KX694686.1	–	AY612026.1
Herald snake (*Crotaphopeltis hotamboeia*)	–	AY611882.1	MH273689.1	AY612064.1
Delalande's beaked blind snake (*Rhinotyphlops lalandei*)	–	–	–	AY612022.1 *R. schlegelii mucuso*
Knox's desert lizard (*Meroles knoxii*)	X	LT745805.1	–	JX962928.1
Cape grass lizard (*Chamaesaura anguina*)	X	HQ167165.1	–	–
Cape dwarf chameleon (*Bradypodion pumilum*)	X	AY289856.1	AF448729.1	–
Austen's thick-toed gecko (*Pachydactylus austeni*)		AF449110.1	KY224250.1	AF449126.1
Ocellated thick-toed gecko (*Pachydactylus geitje*)	X	AF449116.1 This study	JN543887.1 This study	AF449132.1 This study
Spotted thick-toed gecko (*Pachydactylus maculatus*)	X	AF449111.1	KY224228.1	AF449127.1
Cape thick-toed gecko (*Pachydactylus capensis*)	X	AF449117.1	HQ165962.1	AF449133.1
Short-legged seps (*Tetradactylus seps*)	–	KF717417.1 This study	This study	AY167386.1
Silvery dwarf burrowing skink (*Scelotes bipes*)	–	JN561358.1	–	JN561411.1
Blouberg dwarf burrowing skink (*Scelotes montispectus*)	–	AY21798.1	–	AY217824.1
Cape skink (*Trachylepis capensis*)	X	AY028888.1 This study	DQ249079.1 This study	DQ239178.1
Red-Sided skink (*Trachylepis homalocephala*)	X	DQ238875.1	–	DQ239116.1
Variegated skink (*Trachylepis variegata*)	X	DQ238938.1 This study	–	DQ239179.1

Potential Prey	Known Prey	*16S rRNA*	*COI*	*CYTB*
REPTILES cont.				
Karoo sandveld lizard (*Nucras livida*)	X	HG005202.1	–	JX962944.1 *N. lalandii*
Namaqua sand lizard (*Pedioplanis namaquensis*)	X	DQ871102.1	AF206566.1	AF206546.1
Cape legless skink (*Acontias meleagris*)	X	JQ692571.1	AY028856.1	FJ972224.1
Yellow-throated plated lizard (*Gerrhosaurus flavigularis*)	X	KF717396.1	–	DQ090883.1
Karoo plated lizard (*Gerrhosaurus typicus*)	X	KF717403.1	–	AY167382.1
Cape girdled lizard (*Cordylus cordylus*)	–	KC700434.1	–	EU116507.1 *C. namaquensis*
FROGS				
Cape river frog (*Amietia fuscigula*)	X	EF136548.1	–	–
Cape sand toad (*Vandijkophrynus angusticeps*)	–	AF220899.1	KF665721.1	–
Cape sand frog (*Tomopterna delalandii*)	–	AY255086.1	–	JX564898.1 *T. cryptotis*
Clicking stream frog (*Strongylopus grayii*)	X	DQ022367.1 This study	–	–
Striped stream frog (*Strongylopus fasciatus*)	X	AF2215412.1	–	–
Banded stream frog (*Strongylopus bonaespei*)	X	DQ347345.1	–	–
Bronze dainty frog (*Cacosternum nanum*)	X	KJ461733.1	–	–
Senegal running frog (*Kassina senegalensis*)	X	FJ151067.1	KY177138.1	AF215495.1
Namaqua rain frog (*Breviceps namaquensis*)	X	JQ965933.1	–	–
Rose's rain frog (*Breviceps rosei*)	–	MH340426.1	–	FJ998375.1 *B. mossambicus*
BIRDS				
Karoo prinia (*Prinia maculosa*)	–	AF094647.1 *P. bairdi*	–	HQ608845.1 *P. atrogularis*
Cape white-eye (*Zosterops virens*)	–	–	–	LK056795.1
Bar-throated apalis (*Apalis thoracica*)	–	–	HQ998124.1 *A. goslingi*	KY273784.1
Cape spurfowl (*Pternistis capensis*)	–	–	–	AM236909.1
Cape canary (*Serinus canicollis*)	–	–	–	AY790891.1
Southern double-collared sunbird (*Cinnyris chalybeus*)	–	–	–	KJ456232.1 *C. asiaticus*
Karoo scrub robin (*Cercotrichas coryphaeus*)	–	–	–	KJ173615.1
Cape robin-chat (*Cossypha caffra*)	–	–	–	AY206954.1

Table 3.2 BLAST results for *16S rRNA* and *CYTB* sequences obtained from animal tissue samples. Top scoring results matched the species sequenced in all cases except those indicated by an '*'. "Query Cover" is described as the percent of the query length that is included in the aligned segments. "Score" gives an indication of how good the alignment is.

Taxon Sequenced	Most similar taxon (GenBank #)	Gene	Identities, %	Score	Query cover, %
Gerbilliscus afra	AM409232.1	*16S*	88.89	558	80
Georychus capensis	KT321364.1* *Fukomys damarensis*	*16S*	91.59	743	96
Mus minutoides	MN964117.1	*16S*	92.21	761	94
Rhabdomys pumilio	AF141244.1	*16S*	96.28	750	80
Strongylopus grayii	GU952077.1	*16S*	99.63	990	94
Trachylepis capensis	DQ238937.1	*16S*	98.42	896	95
Trachylepis variegate	MK792057.1	*16S*	90.35	675	96
Pachydactylus geitje	AF449116.1	*16S*	98.25	898	89
Tetradactylus seps	AY167369.1	*16S*	91	667	92
Lycodonomorphus inornatus	AY611891.1	*16S*	98.12	837	85
Psammophis crucifer	FJ4040220.1* *Psammophis* sp.	*16S*	93.17	706	91
Pachydactylus geitje	AF449132.1	*CYTB*	96.72	202	94
Psammophis crucifer	DQ486397.1	*CYTB*	86.67	94	38

3.2. Species-specific amplification of prey DNA from the faeces of captive snakes

3.2.1. Development of species-specific primers

To evaluate whether prey DNA was obtainable and amplifiable from snake predators, captive feeding experiments were conducted. As a proof of concept, both predator-specific (*P. crucifer* and *P. rhombeatus*) and prey-specific (*P. geitje*) primers targeting several regions within *CYTB* were developed (Table 2.1; Figure 3.1). From the alignments, I used a total of 478 bp for *P. geitje*, 458 bp for *P. rhombeatus* and 554 bp for *P. crucifer* to create the primers. 64.5% of the sites were identical across the taxa and 35.5% were variable. Variable regions were selected as species-specific primer binding sites. In total, seven primer pairs were developed to amplify products with varying fragment lengths to test whether larger DNA fragments would degrade more in faecal remains (*P. geitje*: 156 bp and 478 bp, *P. rhombeatus*: 149 bp, 316 bp and 458 bp and *P. crucifer*: 189 bp, 471 bp and 554 bp). Additional primer pair combinations that could work together include *gei-F2/gei-R1* (478 bp), *cruc-F5/cruc-R4* (554 bp), *rhom-F6/rhom-R7* (149 bp) (Figure 3.1).
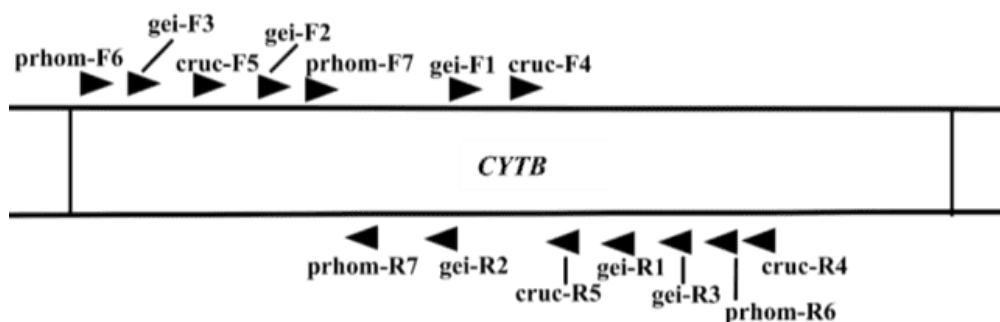
FORWARD PRIMERS

```
gei-F1           TAA TCT ACT GTC AGC CCT TCC ATA C
P. geitje        ... ... ... ... ... ... ... ... .
P. crucifer      C.. CC. ... AA. C.. .A. C.. ... G

gei-F2           GCC TAC TTA TTC AAA TCA CCA CGG
P. geitje        ... ... ... ... ... ... ... ...
P. crucifer      ... ... GAC .A. ... ... .A. .C.

gei-F3           CCC AAC CGT CAA AAT TAT TAC CAC C
P. geitje        ... ... ... ... ... ... ... ... .
P. crucifer      T.. ... .A. ..C .-- G.. ... T.A T

cruc-F4          TAC CTG GGA ACG ATA ATA ACA ACC TG
P. crucifer      ... ... ... ... ... ... ... ... ..
P. geitje        ... A.T ..C ..T .C. T.. GT. GTT TG

cruc-F5          GGA TCA ATA CTA TTA ACC TGC CTA GC
P. crucifer      ... ... ... ... ... ... ... ... ..
P. geitje        ..C ..C C.C ... GGC .TT ... ... CT

rhom-F6          TTC AAC CTT CTC CCT GTC G
P. rhombeatus    ... ... ... ... ... ...
P. geitje        C.. .TT GAC ... ..C AC. .

rhom-F7          CAG TCG TAC ACA TTA CAC GAG A
P. rhombeatus    ... ... ... ... ... ... ... .
P. geitje        .TA ..A C.. TA. ... GC. .T. .
```

REVERSE PRIMERS

```
gei-R1           AAA GAG TAG GTG GAG TAA TAC GAC G
P. geitje        ... ... ... ... ... ... ... ... .
P. crucifer      .TC ATA .TA CA. CTA AC. ACT ATA A

gei-R2           TAA CTA GGA CGA GAA GGA TTA CGC
P. geitje        ... ... ... ... ... ... ... ...
P. crucifer      AC. ... AT. AA. C.. TTG .C. AA.

gei-R3           GTT CGT GTA GAA AGA GTA GGT GGA G
P. geitje        ... ... ... ... ... ... ... ... .
P. crucifer      .C. AC. CC. C.. C.. A.. AC. CC. A

cruc-R4          TTT TGT CTG TGT CTG AAT TAG TCC CC
P. crucifer      ... ... ... ... ... ... ... ... ..
P. geitje        ... AT. G.. AC. A.. AC. AAT C.. GT

cruc-R5          GTG ATG ATG AAT GGT AGG ATG AAG TG
P. crucifer      ... ... ... ... ... ... ... ... ..
P. geitje        TGA CAT TAT TT. CC. TCT TCT TT. A.

rhom-R6          ATT GAG AAT CCG CCT CAT AGT C
P. rhombeatus    ... ... ... ... ... ... ... .
P. geitje        TGA CGC TTC GGT GGG GTG T.A G

rhom-R7          ACG ACT GAT GAG AAT GCT AGG T
P. rhombeatus    ... ... ... ... ... ... ... .
P. geitje        CAC TA. CTC C.T TT. CG. TCA C
```

Figure 3.1 Alignment of *CYTB* sequences for *Pachydactylus geitje*, *Psammophis crucifer* and *Psammophylax rhombeatus*. Dots represent sequence identity with the primer sequence. Differences are represented as nucleotides (A, C, G or T). Primers are all written in the 5' to 3' direction.

3.2.2. Evaluation of species-specific *CYTB* primers

To evaluate whether primers were specific to the target species, DNA analyses were conducted on tissue samples from *P. geitje, P. crucifer*, and *P. rhombeatus*. PCR products were screened visually to show specificity and non-specificity to target DNA (Figure 3.2; Appendix 2). In some instances, primer pairs were specific to the target species (Table 3.3). Two of the primer pairs designed (*gei-Fl/geiR1* and *geiF2/geiR1*) showed specificity to *P. geitje* and produced fragments sizes of 156 and 478 bp, respectively (Figure 3.2). The abovementioned primers amplified DNA of *P. geitje* and none amplified DNA of *P. rhombeatus* or *P. crucifer*. While four primer pairs were designed for *P. crucifer,* only two (*cruc-F4/crucR4* and *cruc-F5/cruc/R4*) showed specificity to *P. crucifer* (Figure 3.2).
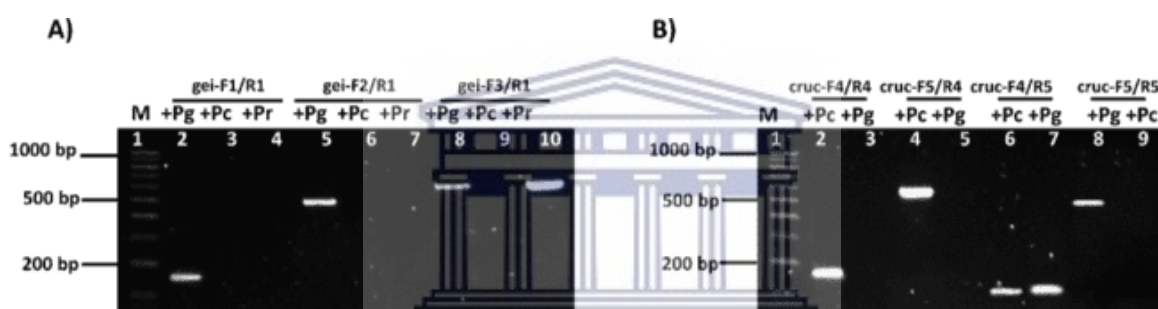
Figure 3.2 Specificity of *CYTB* PCR primers showing specificity to *P. geitje*, *P. crucifer* or *P. rhombeatus*. (**A**) Three *P. geitje* specific primer pair combinations. DNA template derived from either *P. geitje* (+Pg), *P. crucifer* (+Pc), or *P. rhombeatus* (+Pr). 100 bp DNA ladder (M). (**B**) Four *P. crucifer* specific primer pair combinations. DNA template derived from either *P. geitje* (+Pg) or *P. crucifer* (+Pc). 100 bp DNA ladder (M).

Table 3.3 Specificity of *CYTB* primers to *P. geitje*, *P. crucifer* and *P. rhombeatus*. Primer pairs showing specificity to intended target shown as PCR fragment lengths (base pairs) in bold. Primer pairs that are not specific to the target species (NS). Untested pairs (*) are represented by estimated fragment size. Incompatible primer pairs (–).

	gei-R1	gei-R2	gei-R3	cruc-R4	cruc-R5	prhom-R6	prhom-R7
gei-F1	**156**	71*	NS	–	–	–	–
gei-F2	**478**	NS	488*	–	–	–	–
gei-F3	NS	346*	NS	–	–		
cruc-F4	–	–	–	**189**	NS	–	–
cruc-F5	–	–	–	**554**	NS	–	–
prhom-F6	–	–	–	–	–	NS	**149**
prhom-F7	–	–	–	–	–	**316**	–

3.2.3.   Collection of faecal samples

To ascertain whether snake faecal samples have amplifiable prey DNA, faecal samples were collected from captive-fed snakes. A total of 83 faecal samples were collected and analysed from captive-fed *P. crucifer* (N=2) and *P. rhombeatus* (N=4), 39 and 44 respectively between October 2019 and March 2020 (Appendix 1). While in captivity, *P. rhombeatus* individuals produced on average 18 faecal samples (17.67 SD) and *P. crucifer* individuals produced on average 20 faecal samples (9.19 SD).

3.2.4.   Quantification of faecal DNA

To assess the efficacy of faecal sample extraction and the amount of DNA present in snake faeces, samples were quantified using spectrophotometry. On average, faecal samples contained 6.5 ng/nl (10.4 ng/nl SD) of DNA. Faecal sample mass was compared to the amount of DNA per sample to establish if initial faecal sample size influenced the extraction efficiency. On average, faecal samples weighed 0.63 g (0.56 g SD) (Figure 3.3).

Figure 3.3 Amount of DNA recovered from captive snake faeces in relation to the mass of faecal sample.

3.2.5.   Detection of prey and predator DNA in faecal samples

To investigate whether prey DNA could be detected in snake faeces and the amount of DNA degradation that occurred, faecal samples were tested from *P. crucifer* and *P. rhombeatus* using species-specific primers (Table 3.3). Visual size discrimination was used to check for prey and predator DNA amplification.

3.2.5.1.   Detection of prey DNA in relation to fragment size

Captive snakes were fed *P. geitje* individuals. Two sets of primers were used to detect *P. geitje* DNA in the faecal samples of both *P. crucifer* and *P. rhombeatus* —one set to target shorter DNA fragments and the other set to target larger DNA fragments. First, faecal sample DNA was evaluated using a prey-specific primer pair (*gei-F1/geiR1*) that targets short fragments (156 bp) of *P. geitje* DNA (Table 3.3). DNA amplification of *P. geitje* was evident in 75% of faecal samples analysed (Figures 3.4–3.5; Table 3.4; Appendix 3). More specifically, *P. geitje* DNA was exclusively detected in 15% of the faecal samples from *P. crucifer* and in 9% of the samples from *P. rhombeatus*. 8% of *P*. *crucifer*-derived samples and 25% of *P. rhombeatus*-derived samples contained only predator DNA. Of the 83 samples analysed, 7% yielded a low quantity or poor-quality DNA indicated by non-amplification of either taxon during PCR. Analysis of the remaining samples showed that 89% from *P. crucifer* (N = 35) and 95% from *P. rhombeatus* (N = 42) tested positive for the presence of either prey, predator or both species (Table 3.4). This shows that DNA can be detected in the faeces of these two snake species (Figure 3.6A).

Figure 3.4 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* and *cruc-F5/R5* shown in lanes 4 and 5, respectively. 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. crucifer*-derived faecal samples amplified with either *P. geitje*-specific primers (*gei-F1/R1*) or *P. crucifer*-specific primers (*cruc-F5/R5*). 100 bp DNA ladder (M).

Figure 3.5 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. rhombeatus* tissue (+Pr), or *P. rhombeatus*-derived faecal samples. Specificity of *gei-F1/R1* shown in lane 4. 100 bp DNA ladder (M). Negative template control (NTC). (**B**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. geitje*-specific primers (*gei-F1/R1*). 100 bp DNA ladder (M).
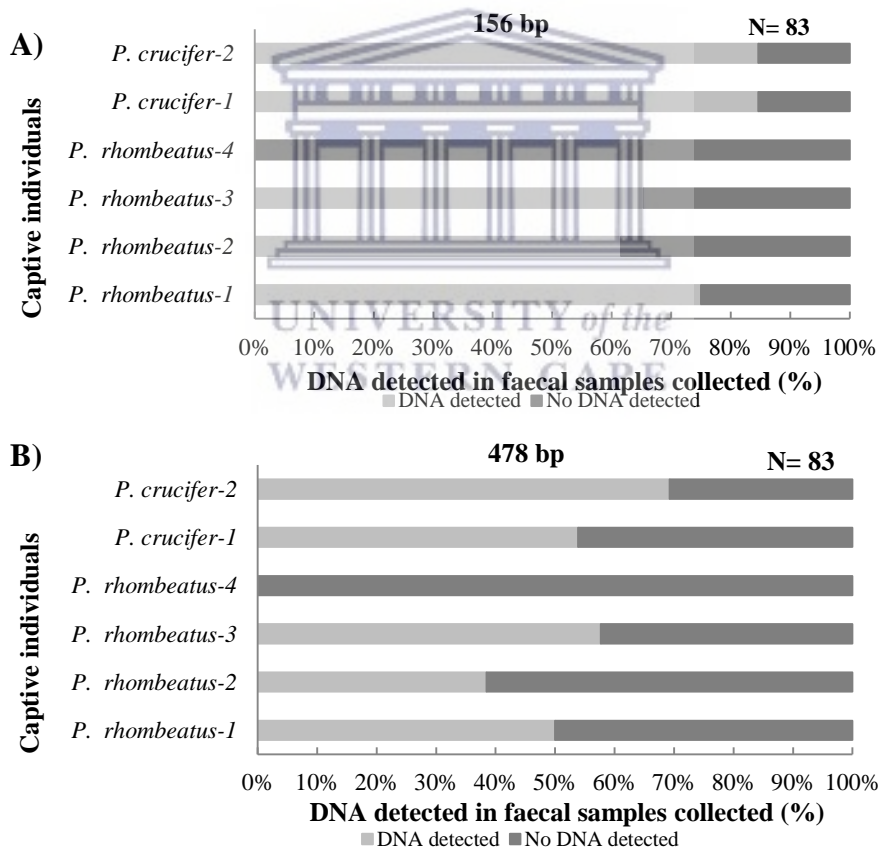
Figure 3.6 Proportion of faecal samples from captive snakes with amplifiable prey DNA. (**A**) Proportion of faecal samples with short, amplifiable fragments of prey DNA (156 bp). (**B**) Proportion of faecal samples with large, amplifiable fragments of prey DNA (478 bp).

Next, faecal samples were tested using the prey-specific primers (*gei-F2/geiR1*) that targeted a larger fragment (478 bp) of *P. geitje* DNA (Table 3.3). Overall, large fragments of prey DNA could be amplified from snake faeces (Figure 3.7–3.8; Appendix 4). Prey DNA was detected in 61% of faecal samples from *P. crucifer* and 59% of samples from *P. rhombeatus* (Figure 3.6B).
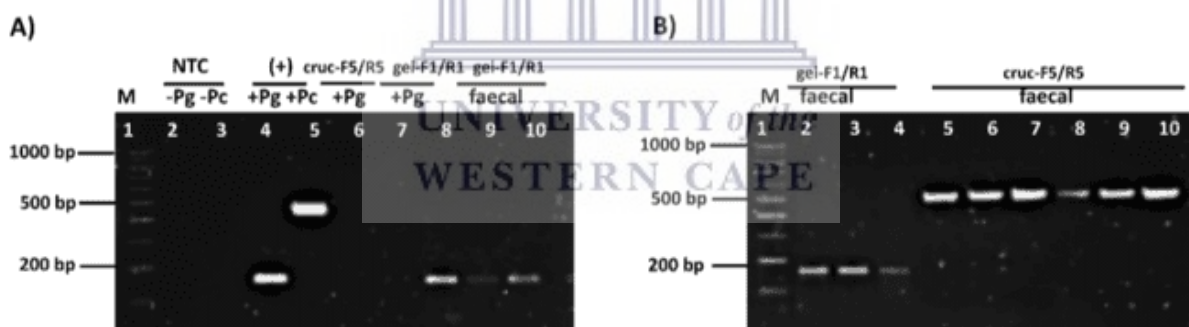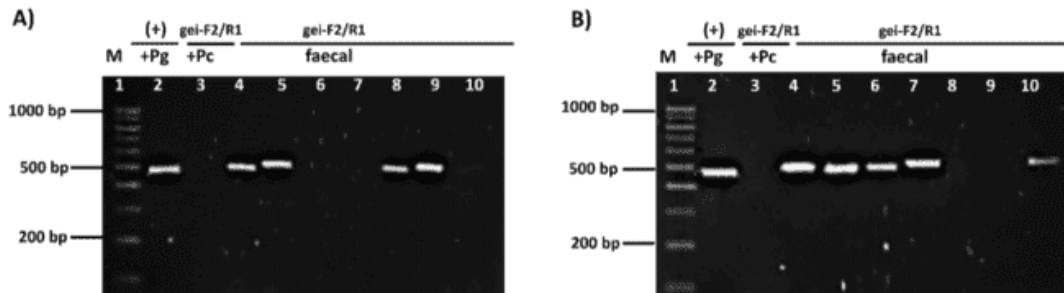

Figure 3.7 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F2/R1* (lane 2–3). 100 bp DNA ladder (M). (**B**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F2/R1* (lane 2–3). 100 bp DNA ladder (M).
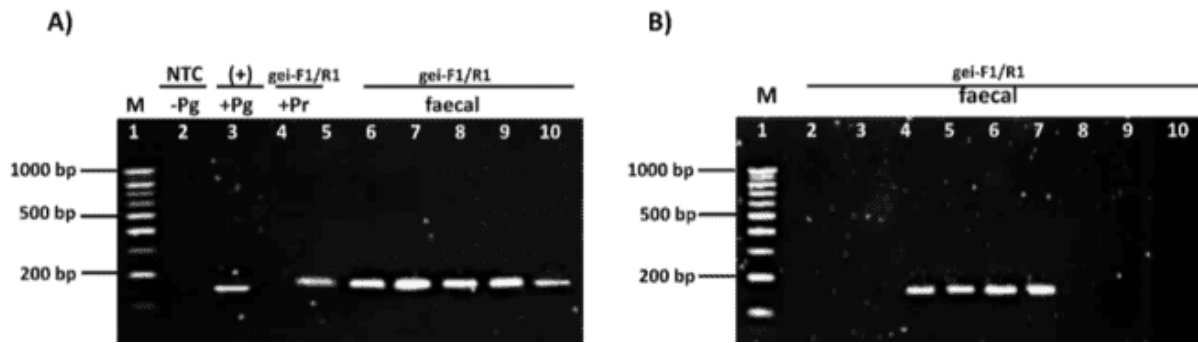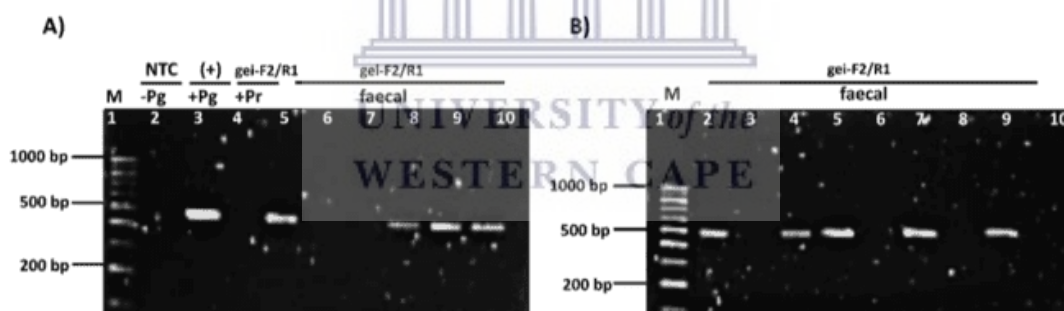

Figure 3.8 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. rhombeatus* tissue (+Pr), or *P. rhombeatus*-derived faecal samples. Specificity of *gei-F2/R1* (lane 3–4). Negative template control (NTC). 100 bp DNA ladder (M). (**B**) DNA template derived from *P. rhombeatus*-derived faecal samples. 100 bp DNA ladder (M).

DNA amplification produced large and small PCR fragments, that differed between 59% (*gei-F2/geiR1*) and 70% (*geiF1/geiR1*) for *P. rhombeatus* faecal samples, and 54% (*geiF2–geiR1*) and 82% (*geiF1/geiR1*) for *P. crucifer* faecal samples. Amplification of prey DNA from *P. crucifer* faecal samples was 28% higher when reactions targeted a 156 bp fragment of DNA (*geiF1/geiR1*) rather than 478 bp (*gei-F2/geiR1*). (Table 3.4; Appendix 4–5). Pearson's Chi-squared test showed that small and large DNA fragments were detected with similar efficiency from faecal samples of *P. crucifer* ($X^2 = 0.2$, $P = 0.6$) and *P. rhombeatus*

($X^2 = 0.04$, P = 0.8). The relationship between DNA detection and fragment size was not significant.

Table 3.4 Number of faecal samples testing positive for predator and/or prey DNA. Total number of samples analysed (N). Values represent the number of samples with DNA detected. "Prey only" is subcategorised into PCR product sizes (156 bp or 478 bp). At 156 bp the values for "Prey only" correspond to the values for "Predator only", "Both Predator & Prey" and "Neither Predator or Prey".

| | | | DNA detected from: | | | |
| | N | Predator only | Prey only | | Both Predator & Prey | Neither Predator or Prey |
			156 bp	478 bp		
P. rhombeatus	44	11	4	26	27	2
P. crucifer	39	3	6	24	26	4

3.2.5.2. Retention of prey DNA in gut-passage of snakes

Snake identity ($X^2 = 29.26$, P < 0.05) and days since last *P. geitje* meal ($X^2 = 8. 16$, P < 0.05) positively contributed to the detection of prey DNA (Table 3.5). No significant relationship was evident for faecal mass, mass of last *P. geitje* meal, number of *P. geitje* meals and relative prey size (Table 3.5). The general trend showed that the probability of detecting DNA from snake faeces decreased over time. Detection of prey was possible in fresh faeces collected within days, and in some cases, in older faeces collected after a month (Figure 3.10–3.11). The average day when prey detection decreased was after 30 days, however two individuals deviated from this. For these individuals, detection decreased shortly after prey was consumed or within a number of days following consumption (Figure 3.9–3.10). Overall, detection probabilities varied between individuals and species, with *P. crucifer* individuals showed greater detection than most *P. rhombeatus* individuals (Figure 3.11).

Table 3.5 Binomial regression results of the variables that predict DNA detection from *P. rhombeatus* and *P. crucifer* faeces. Variables that strongly correlate to a positive detection are indicated in bold.

Variable	AIC	P
Faecal mass	73.44	0.98
Snake identity	92.70	**2.055e-05**
Days since last *P. geitje* meal	79.60	**0.004**
Mass of last *P. geitje* meal fed	73.18	0.18
Number of *P. geitje* meals fed	74.60	0.07
Relative prey mass	72.82	0.23
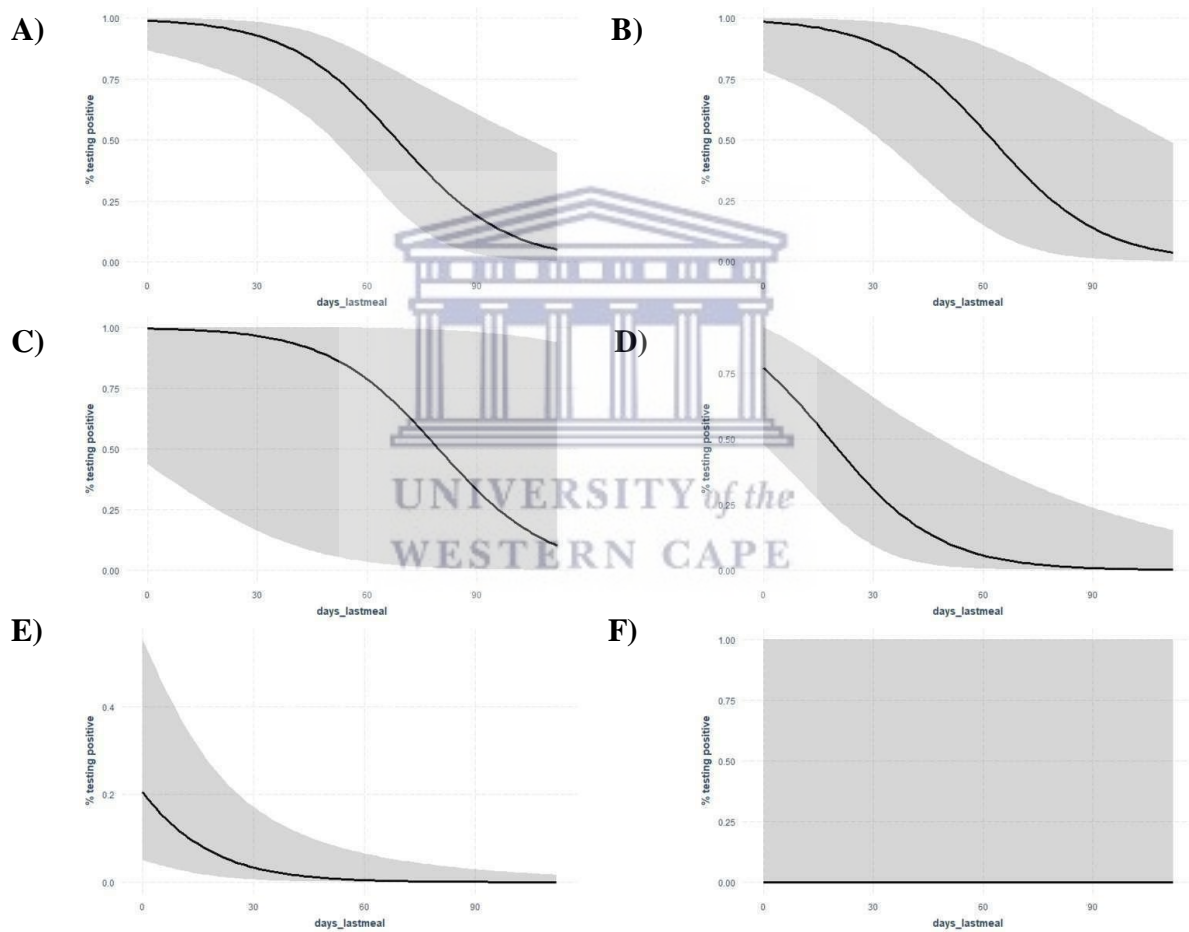
Figure 3.9 Probability of detecting prey DNA from faecal samples of captive snakes versus days since last eaten. Probability of detecting *P. geitje* DNA from faecal samples from individual (**A**) *P. crucifer*-1, (**B**) *P. crucifer*-2, (**C**) *P. rhombeatus*-1, (**D**) *P. rhombeatus*-2, (**E**) *P. rhombeatus*-3, (**F**) *P. rhombeatus*-4.
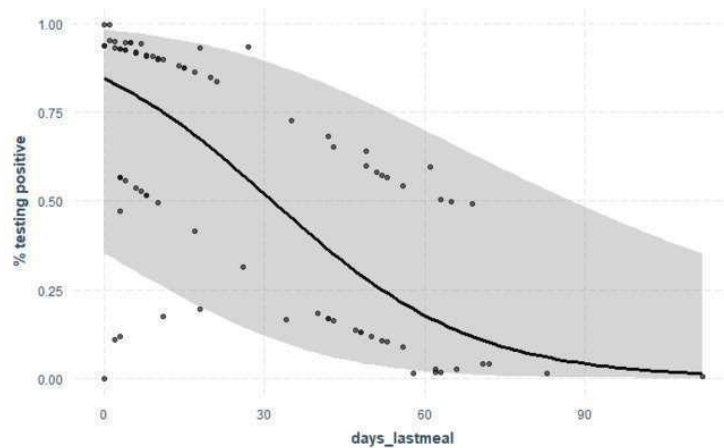
Figure 3.10 Probability of detecting DNA from faecal samples of 6 captive snakes based on the number of days since its last *P. geitje* meal.
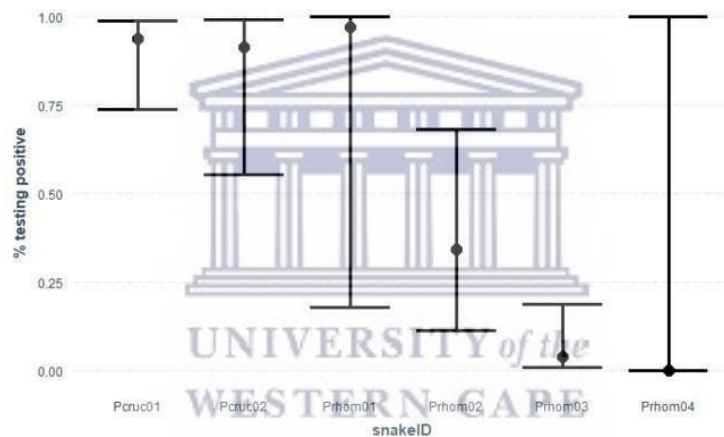
Figure 3.11 Probability of detecting DNA from captive snakes based on snake identity

3.3. Universal amplification of prey DNA from the faeces of captive snakes

3.3.1. Development of universal primers

Universal primers were designed to amplify multiple prey DNA from snake faecal samples. I designed primer pairs that would result in different PCR products that vary in size. The alignments of *16S rRNA* sequences contained highly conserved regions which were selected as best suited for making the primers (Figure 3.12). In total, two primer pairs were developed with varying fragment lengths (*16SF01/16SR0*1: 595–630 bp and *16SF02/16SR02*: 130–150 bp) (Table 2.2; Figure 3.12).

3.3.2.   Evaluation of universal primers

To evaluate whether universal primers amplify target species, DNA analyses were conducted on tissue samples from *Psammophis crucifer*, *Lycodonomorphus inornatus*, *Psammophylax rhombeatus*, *Tetradactylus seps*, *Trachylepis capensis*, *Trachylepis variegata*, *Pachydactylus geitje*, *Strongylopus grayii*, *Rhabdomys pumilio*, *Gerbiliscus afra*, *Georychus capensis*, and *M*us *minutoides*. DNA amplification was observed for each of the above species using primer pairs that produce short and long PCR products thus showing that they work (Figure 3.13–3.14).

Universal *16S rRNA* primer pairs were examined using the previously analysed faecal samples that tested positive for *P. geitje*, *P. crucifer* or *P. rhombeatus* (Section 3.2.5). The primer pair (*16SF01/16SR01*) was excluded from the analysis as the banding patterns did not match any of the expected PCR products. However, the primer pair (*16SF02/16SR02*) produced distinct DNA bands in 92% of the faecal samples analysed (N = 77/83) —77% from *P. crucifer* samples and 86% from *P. rhombeatus* samples (Figure 3.15; Appendix 5).

Figure 3.12 Alignment of *16S rRNA* sequences for a range of prey and predator species. Differences are represented as nucleotides (A, C, G or T). Primers are all written in the 5' to 3' direction.
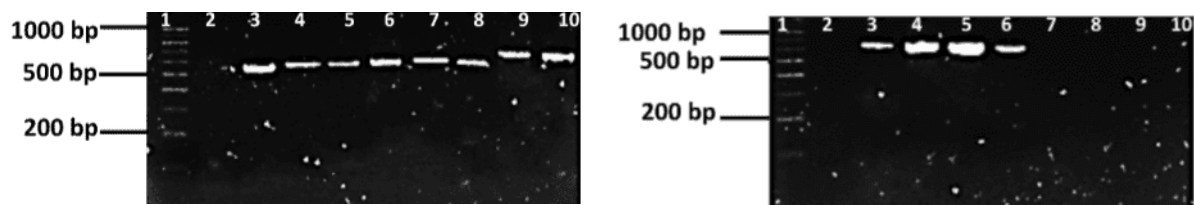
Figure 3.13 Amplification of *16S-rRNA* from tissue samples using universal primers (16SF01–16SR01). (**A**) 100 bp DNA ladder (1), NTC (2), *P. crucifer* (3), *L. inornatus* (4), P. *rhombeatus* (5), *T. seps* (6), *T. capensis* (7), T. *variegata* (8), *P. geitje* (9), *S. grayii* (10). (**B**) 100 bp DNA ladder (1), NTC (2), *R. pumilio* (3), *G. afra* (4), *G. capensis* (5), *M. minutoides* (6).
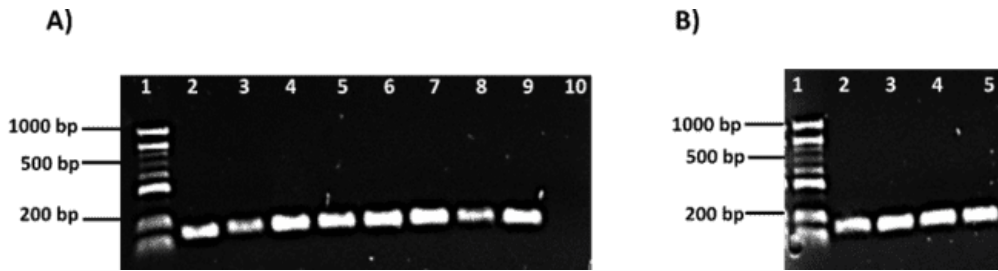
Figure 3.14 Amplification of *16S-rRNA* from tissue samples using universal primers (16SF02–16SR02). (**A**) 100 bp DNA ladder (1), *P. crucifer* (2), *S. grayii* (3), *R. pumilio* (4), *T. seps* (5), *G. afra* (6), *G. capensis* (7), *L. inornatus* (8), *M. minutoides* (9), NTC (10). (**B**) 100 bp DNA ladder (1). *T. capensis* (2), *T. variegata* (3), *P. geitje* (4), *P. rhombeatus* (5).
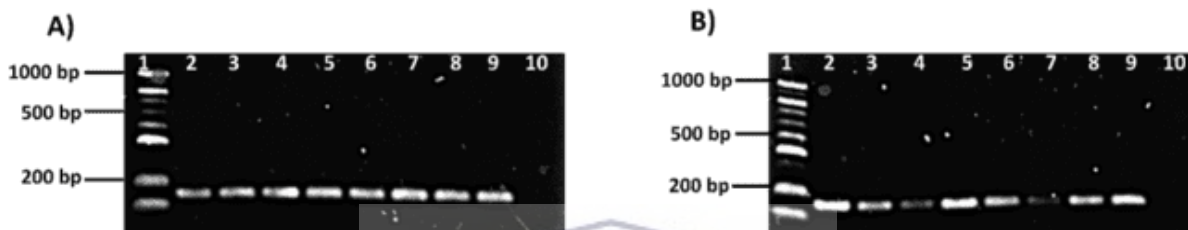
Figure 3.15 Amplification of *16S-rRNA* from *P. rhombeatus* and *P. crucifer* faecal samples using universal primers (16S-F02–16S-R02). (**A**) *P. rhombeatus* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10). (**B**) *P. crucifer* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10).

3.3.3. Restriction endonuclease digest patterns

Tissue DNA of *P. crucifer*, *P. rhombeatus* and *T. capensis* were cut by MseI and tissue DNA of *P. geitje* was cut by RSAL (Figure 3.16). Well resolved patterns of two bands were observed on a 2% agarose gel from the digested products of *P. crucifer* and *P. rhombeatus*. In contrast, there were no clear differences between the digested products of *P. geitje* and *T. capensis* as they remained the original length and did not cut (Figure 3.16).
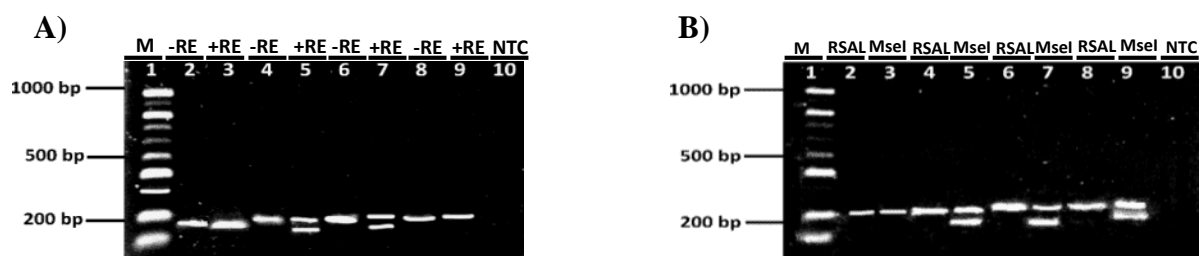
Figure 3.16 Undigested (-RE) and digested (+RE) PCR products using the restriction enzymes, RSAL and MseI. (**A**) Amplified tissue-derived DNA from: *P. geitje* (2), *P. geitje* (3, RSAL), *P. crucifer* (4), *P. crucifer* (5, MseI), *P. rhombeatus* (6), *P. rhombeatus* (7, MseI), *T. capensis* (8), *T. capensis* (9, MseI). Negative template control (NTC). 100 bp DNA ladder M (1). (**B**) Digestion of amplified faecal samples known to contain either *P. crucifer* (5, 7)*, P. rhombeatus* (9), or *P. geitje* (lanes 2–4, 6, 8), DNA from prior analyses. 100 bp DNA ladder M (1). Negative template control (NTC).

3.3.4.   Examination of cloning success

On average, four colonies were sampled for each faecal sample (N= 11). 58% of colonies showed predator DNA. For a given faecal sample, 2 out of 4 sampled colonies were positive for prey DNA (Table 3.6). Overall, the sequencing of cloned products confirmed that both prey and predator DNA can be derived from snake faeces. Although the restriction banding results were unable to discriminate between the prey species (Figure 3.16), the cloned sequencing results showed that prey could be identified from faecal remains of snakes (Table 3.6).

Table 3.6 Local BLAST results of cloned faecal samples from captive-fed animals using sequences produced in this study (*P. geitje, T. capensis, P. crucifer, P. rhombeatus*). All samples were amplified using universal primers 16S-F02/16S-R02, targeting 16S-rRNA. "Query Cover" is described as the percent of the query length that is included in the aligned segments. "Score" gives an indication of how good the alignment is.

Clone ID	Snake ID	Faecal sample	Species similarity	Identities, %	Score	Query cover, %
C1	Pcruc01	Pcruc1-015	*Psammophis crucifer*	91.00	155	86
C2	Pcruc01	Pcruc1-015	*Psammophis crucifer*	93.02	177	92
C3	Pcruc01	Pcruc1-015	*Trachylepis capensis*	92.86	172	98
C4	Pcruc01	Pcruc1-015	*Trachylepis capensis*	90.06	117	82
C5	Pcruc01	Pcruc1-004	*Pachydactylus geitje*	91.00	138	88
C6	Pcruc01	Pcruc1-004	*Psammophis crucifer*	96.15	178	98
C7	Pcruc01	Pcruc1-004	*Psammophis crucifer*	93.00	173	86
C8	Pcruc01	Pcruc1-004	*Psammophis crucifer*	97.35	188	99
C9	Pcruc01	Pcruc1-012	*Psammophis crucifer*	95.02	188	94

Clone ID	Snake ID	Faecal sample	Species similarity	Identities, %	Score	Query cover, %
C10	Pcruc01	Pcruc1-012	*Psammophis crucifer*	94.02	176	92
C11	Pcruc01	Pcruc1-012	*Psammophis crucifer*	94.02	176	92
C12	Pcruc01	Pcruc1-012	*Trachylepis homalocephala*	76.85	99.6	100
C13	Pcruc01	Pcruc1-012	*Psammophis crucifer*	90.01	166	88
C14	Pcruc02	Pcruc2-002	*Psammophis crucifer*	96.49	191	99
C15	Pcruc02	Pcruc2-002	*Psammophis crucifer*	94.23	188	98
C16	Pcruc02	Pcruc2-002	*Pachydactylus geitje*	98.00	189	93
C17	Pcruc02	Pcruc2-002	*Pachydactylus geitje*	95.37	173	90
C18	Pcruc02	Pcruc2-002	*Pachydactylus geitje*	96.26	176	97
C19	Pcruc02	Pcruc2-008	*Psammophis crucifer*	97.12	179	99
C20	Pcruc02	Pcruc2-008	*Psammophis crucifer*	96.43	183	97
C21	Pcruc02	Pcruc2-008	*Psammophis crucifer*	95.65	168	99
C22	Pcruc02	Pcruc2-008	*Trachylepis capensis*	95.47	171	92
C23	Prhom01	Prhom1-004	*Psammophylax rhombeatus*	94.45	184	89
C24	Prhom01	Prhom1-004	*Psammophylax rhombeatus*	95.31	190	92
C25	Prhom01	Prhom1-004	*Pachydactylus geitje*	96.33	188	97
C26	Prhom01	Prhom1-004	*Pachydactylus geitje*	96.30	178	97
C27	Prhom01	Prhom1-004	*Trachylepis capensis*	92.96	169	98
C28	Prhom02	Prhom2-002	*Psammophylax rhombeatus*	95.58	180	98
C29	Prhom02	Prhom2-002	*Pachydactylus geitje*	96.30	178	97
C30	Prhom02	Prhom2-002	*Pachydactylus geitje*	96.33	188	93
C31	Prhom02	Prhom2-002	*Pachydactylus geitje*	96.30	178	96
C32	Prhom02	Prhom2-005	*Pachydactylus geitje*	92.31	171	95
C33	Prhom02	Prhom2-005	*Psammophylax rhombeatus*	95.54	178	96
C34	Prhom02	Prhom2-005	*Psammophylax rhombeatus*	94.78	178	99
C35	Prhom02	Prhom2-005	*Pachydactylus geitje*	92.31	171	95
C36	Prhom02	Prhom2-012	*Pachydactylus geitje*	91.00	140	87
C37	Prhom02	Prhom2-012	*Psammophylax rhombeatus*	96.00	193	96
C38	Prhom02	Prhom2-012	*Pachydactylus geitje*	90.00	122	93
C39	Prhom02	Prhom2-012	*Pachydactylus geitje*	92.01	140	89
C40	Prhom03	Prhom3-019	*Trachylepis capensis*	97.22	186	94
C41	Prhom03	Prhom3-019	*Pachydactylus geitje*	94.02	176	94
C42	Prhom03	Prhom3-019	*Pachydactylus geitje*	96.30	178	92
C43	Prhom03	Prhom3-019	*Pachydactylus geitje*	96.33	178	90
C44	Prhom03	Prhom3-019	*Pachydactylus geitje*	96.33	178	91
C45	Prhom04	Prhom4-01	*Pachydactylus geitje*	93.05	153	85
C46	Prhom04	Prhom4-01	*Pachydactylus geitje*	91.03	139	89
C47	Prhom04	Prhom4-01	*Pachydactylus geitje*	93.00	169	90
C48	Prhom04	Prhom4-01	*Pachydactylus geitje*	97.16	201	98

3.4. Analysis of faecal samples from wild-caught animals

In total, 17 colonies were sampled from four faecal samples of wild-caught *P. rhombeatus* individuals. 50% of the colonies contained prey DNA. Predator DNA was evident in at least 2 out of 4 colonies (Table 3.7). Overall, the sequences of cloned products confirmed that both prey and predator DNA can be derived from faeces of wild-caught specimens. In three cases, there was evidence of wild-caught specimens feeding on *Trachylepis homalocephala* and *Pachydactylus geitje* and *Tetradactylus seps*. *T. seps* is a new prey species for *P. rhombeatus*. snakes Human contamination was evident in three colonies.

Table 3.7 BLAST results of cloned faecal sample DNA from wild-caught animals. All samples were amplified using universal primers 16S-F02/16S-R02, targeting *16S-rRNA*. "Query Cover" is described as the percent of the query length that is included in the aligned segments. "Score" gives an indication of how good the alignment is.

Clone ID	Snake ID	Faecal sample	Species similarity	Identities, %	Score	Query cover, %
C1	Prhom-W1	Prhom-W01	*Tetradactylus seps*	94.39	169	92
C2	Prhom-W1	Prhom-W01	*Tetradactylus seps*	95.00	173	94
C3	Prhom-W1	Prhom-W01	*Tetradactylus seps*	95.00	172	93
C4	Prhom-W1	Prhom-W01	*Psammophylax rhombeatus*	94.62	186	88
C5	Prhom-W1	Prhom-W01	*Tetradactylus seps*	93.58	174	93
C6	Prhom02-W	Prhom2-W02	*Trachylepis homalocephala*	77.78	107	100
C7	Prhom02-W	Prhom2-W02	*Trachylepis homalocephala*	79.00	109	96
C8	Prhom02-W	Prhom2-W02	*Psammophylax rhombeatus*	79.89	104	72
C9	Prhom02-W	Prhom2-W02	*Psammophylax rhombeatus*	78.08	101	70
C10	Prhom-W2	Prhom-W04	*Homo sapiens*	78.57	99.6	76
C11	Prhom-W2	Prhom-W04	*Homo sapiens*	75.78	489	80
C12	Prhom-W2	Prhom-W04	*Homo sapiens*	80.00	102	71
C13	Prhom-W2	Prhom-W04	*Psammophylax rhombeatus*	79.01	103	71
C14	Prhom04-W	Prhom4-W01	*Psammophylax rhombeatus*	96.46	187	98
C15	Prhom04-W	Prhom4-W01	*Psammophylax rhombeatus*	90.05	188	89
C16	Prhom04-W	Prhom4-W01	*Psammophylax rhombeatus*	92.95	178	89
C17	Prhom04-W	Prhom4-W01	*Pachydactylus geitje*	97.00	182	97

Chapter 4. Discussion

The results of this study demonstrate the utility of DNA analysis of faecal samples to assess the diets of co-occurring snake species in southern Africa. In this study, I demonstrated that it is possible to obtain diet information from snake faeces using non-invasive genetic approaches, but the efficiency of detecting prey decreased over time. Targeting shorter fragments of prey DNA produced better, but not statistically better results. The probability of species-identification was highest for prey items consumed one day prior to faeces collection. Detection success and gut retention time varied among snake species and individuals. I was able to scale the prey-specific approach to universal via cloning, which resulted in the detection of a new prey type. These approaches confirm the utility of genetic approaches to identify prey species in snake faeces, and highlight the need to account for bias in possible faecal degradation, especially in wild-collected faeces.

4.1.   Molecular tools as key identifiers

Extensive dietary studies have focused on defining trophic links of mammals using faecal-DNA approaches (Table 1.2), while relatively few studies have attempted to extract prey DNA from reptile faeces (Brown et al. 2012, 2014a; Kartzinel and Pringle 2015; Pearson et al. 2015; Pinho et al. 2018; Pereira et al. 2019; Gil et al. 2020). Even though snake faeces have previously been used to identify morphological remains of prey items (Weatherhead et al. 2003), the feasibility of molecular analysis as a non-invasive approach has been heavily underutilised. To my knowledge, only Brown et al. (2014a) examined the diet of smooth snakes (*Coronella austriaca*) and grass snakes (*Natrix natrix*) using sequencing of reptiles, mammals and invertebrate DNA from faecal contents. In this paper, the authors showed that the molecular approach provides a relatively unbiased picture of prey utilisation by these snakes. Congruently, the PCR-based methods developed in this study to identify *P. geitje* DNA in the faeces of *P. crucifer* and *P. rhombeatus* were surprisingly efficient. In >70% (N= 63/83) of the predator's faeces, prey DNA could be amplified.

Previous research has shown an increase in prey detection success when targeting <300 bp fragments of prey DNA (Symondson 2002; De Barba et al. 2014; Brown et al. 2014a). This is mainly because of the assumption that in degraded samples, short fragments are present in larger amounts (Deagle et al. 2006). By targeting a short fragment (156 bp), I was able to amplify prey DNA from faeces that were tested. By contrast, I was able to amplify larger

DNA fragments (478 bp) from the faeces of both snake species with similar efficacy. These observations suggest that prey DNA may not always be highly degraded and therefore enable the amplification of larger fragments. Interestingly, amplification success showed no advantage of short fragments over large fragments, despite several studies indicating that prey DNA in faecal samples is often degraded into smaller fragments (Zaidi et al. 1999; Chen et al. 2000; Agusti et al 2003; Symondson 2002; von Berg et al. 2008). Moreover, of the 83 faecal samples examined, PCR amplification failed for only six samples, which could be due to low DNA concentration or quality, as these samples were collected long after the prey was consumed.

4.2.   Gut-passage times

Although relatively long gut passage times have been reported in reptiles (Bjorndal 1987), the greatest variation in passage times have been measured in snakes (Lillywhite et al. 2002). Lillywhite et al. (2002) found that terrestrial viperids can retain their faeces from multiple feeding events until they reach a storage threshold. In contrast, the authors found that for arboreal snake species no variations in passage times were observed as snakes defecated shortly after every meal. In the current study, I found that gut passage times varied between both snake species and individuals. Over multiple occasions after snakes consumed prey, numerous faecal samples were produced, with excretion events of the same meal occurring for multiple days. In some cases, however, excretion events continued for months after prey was consumed. These results suggest that prey DNA detection greatly decreased over time despite snakes producing faeces. For these snakes, gut passage time appears to be within days after feeding and up to a month, meaning that a sample collected in the wild could potentially provide information on the snakes' diet over the past few days. However, a lack of detection of a potential prey species may not necessarily mean its absence, but possibly a failure to sample within the detection window (Weaver 1993; Klare et al. 2011). A stringent protocol for collecting faeces may be required.

The results showed that prey species are detected soon after feeding, with some prey possibly detected after 3-4 days. These findings were supported by the prey detection model that showed the probability of detection depends on the days since the last meal fed and snake species. In comparison, Brown (2010) found that mouse DNA could be detected in *Thamnophis marcianus* faeces by at least the fifth day post-feeding. Moreover, mouse DNA could be detected up to sixteen days post-feeding (Brown et al. 2010). Contrary to the short

detection time reported in the previous study, our results indicate that prey detection is possible in snake faeces for up to 30 days post-feeding. Higher prey detection rates were generally observed in *Psammophis crucifer* individuals compared to *Psammophylax rhombeatus* individuals. This is likely because these snakes digested the majority of the prey within the first few days, and so faeces excreted after multiple prior excretions would contain very little remnant prey DNA (Thomas & Pough 1979; Secor & Diamond 1995, 1998; Jones et al. 2009; Lillywhite et al. 2002). However, this long post-feeding detection time is important to consider when interpreting prey DNA detection in field-collected faeces, as feeding occurence and consequently predation impact could be overestimated (McMillan et al. 2007). For these snakes, the number of days post-feeding directly influenced the quality of the faecal samples in terms of the quantity produced and the ability to adequately obtain DNA for amplification from the PCR products. These results suggest that prey may remain detectable for a much longer period in psammophiid snakes compared to garter snakes.

4.3.   Importance of primer selection and design

Two species-specific *CYTB* primers were developed successfully for the detection of prey. Both primer pairs (*geiF1/geiR1* and *geiF2/geiR1*) were specific to *Pachydactylus geitje*, which proved to be useful for obtaining species-level discrimination. The use of species-specific primers aided, in this case, in a better understanding of the passage times of prey DNA in the gut of snakes (King et al. 2008; Falk & Reed 2015), its degradation or lack thereof (Symondson 2002; Brown et al. 2014b), and dynamics of prey retention (Lillywhite et al. 2002). In other cases, as Brown et al. (2012) showed, it can be highly useful when a taxon is a specialist or is known to eat only a few prey types. Otherwise, the number of primer pairs required is too large and taxa-specific or universal primers would be recommended due to handling time. Similarly, this study showed that prey-specific primers are appropriate for the analysis of the diet of skaapstekers and sand snakes.

The species-specific results allowed the opportunity to test whether the universal primers and cloning approach could be utilised. Known prey, *Pachydactylus geitje*, were successfully detected from faecal DNA extracts of captive-fed snakes using a universal *16S rRNA* primer pair (*16SF02* and *16SR0*2). These findings correspond to the prey detection data above. The prey species fed to the snakes during the feeding experiment were detected and therefore show the potential utility of this approach in field studies where prior information on diet of snakes is not known.

A frequently encountered problem with the use of cloning and sequencing with universal primers, however, is the detection of predator DNA in faeces (Siers et al. 2018). In Deagle et al. (2005) a large majority of clones from faecal extracts contained sequences belonging to the predator. One commonly employed method for the sequencing of prey PCR products is a blocking primer (Pompanon et al. 2012). Vestheim & Jarman (2008) designed blocking that provides a practical route to preferentially amplify the DNA of interest. In the current study, however, predator-specific blocking primers were not incorporated. Yet, I was still able to verify the presence of prey DNA. However, rare prey may be harder to detect without the presence of these blocking primers (Vestheim & Jarman 2008). Restriction enzymes proved ineffectual to distinguish between banding patterns of prey, however for predators, distinct species-specific banding patterns were observed. Sequencing of the clones following PCR with universal primers resulted in a sufficient yield of prey DNA in 40% of the sequences. This differs from prior studies that found all cloned sequences belonged to the predator (Jarman et al 2004; Deagle et al. 2005; Brown et al. 2012).

Analysis of the faecal content of a wild-caught specimen revealed a one case of *P. rhombeatus* feeding on *Tetradactylus seps*. Based on previously published records from preserved museum specimens of skaapstekers, this is the only *Tetradactylus* dietary record for *P. rhombeatus* (Broadley 1977, 1983; Branch & Bauer 1995; Jacobsen 2005; Cottone & Bauer 2008, 2010). This opportunistic foraging behaviour has previously been documented in this species (Cottone & Bauer 2008). These findings reveal that the cloning approach is effective to identify unknown prey in snake diets, which may have gone unnoticed using the species-specific approach.

4.4.   Considerations for future research

There are strengths and weaknesses to the approaches employed in this study. Firstly, a study like this is limited by the sequence availability for a specific gene region, which is particularly limiting if species-specific identities are needed. This can easily be resolved if genus-level or family-level is sufficient for the purposes of the study. Proxies can be used to narrow the gap of missing sequence data and where applicable, DNA can be extracted and sequenced for species that are not represented in the database. Furthermore, primer tests can be performed for closely and distantly related organisms to elaborate on the reference sequences available in public databases. Alternatively, you can select a gene region for which more sequences exist.

Secondly, while many studies have employed species-specific primers to analyse predator diets (reviewed in Pompanon et al. 2012), this approach relies on *a priori* knowledge of the possible prey in a faecal sample before DNA analysis is performed. In a captive feeding experiment, these primers would be ideal, as primers can be developed specifically for known prey. However, in situations where prey is consumed in the wild, they can likely go undetected using this approach. Prey-specific primers are of limited utility for generalist predators as the analysis has to be repeated for a large number of potential and often unknown prey (King et al. 2008; Piñol et al. 2014). For example, in a study on penguin diets, Deagle et al. (2007) found that species-specific PCR tests carried out on faecal DNA led to an underrepresentation of prey, as only a limited range of prey for which the primers were designed could be detected. The analysis of prey without any prior knowledge can be achieved with universal primers, followed by prey identification through cloning and sequencing. However, this approach may not be appropriate where qualitative data is required (Deagle et al. 2009).

Thirdly, while DNA analysis provides good taxonomic resolution, identifying prey by extracting DNA from faeces may be subject to biases (Tollit et al. 1997). For example, the digestion of prey DNA is variable between species and, as a result, may only reflect prey whose DNA remained sufficiently intact after digestion to be amplified. One of the advantages of PCR-based analysis is its ability to provide taxonomic resolution to prey that may be consumed but not defined using traditional analysis.

Lastly, although universal primers, restriction enzymes and cloning enabled amplification of non-target prey, it involved sequencing of many clones (Sutherland 2000; Deagle et al. 2007). This study was limited in scale because of the costs and effort required to sequence clones (Pegard et al. 2009). An alternative approach using Next Generation Sequencing technologies provide the opportunity to sequence all prey species present in a predator's faeces simultaneously (Shendure & Ji 2008; Valentini et al. 2009; Deagle et al. 2009). However, it is limited by problems such as amplification efficiency and PCR errors (Deagle et al. 2013). The predator species in this study consume a wide range of prey, making them appropriate targets for both cloning and NGS. However, in my opinion, snakes are better suited to cloning given that one prey item moves through the gut at a time.

Chapter 5. Conclusion

To conclude, the ability to identify prey items that snakes have been eating from their faeces is necessary, because it precludes the need for invasive sampling which can seldom differentiate species from digested remains. The results of the approaches tested in this study demonstrate that the two methods are capable of assessing snake diets by providing species-level identification of prey. The species-specific approach proved particularly useful where *a prior* knowledge of prey was available, while the universal approach enabled the identification of an unknown prey type.

The faecal-DNA analysis in this study provides a new avenue to study trophic interactions of snakes and the ecology of prey species. Dietary studies like these support research examining resource partitioning (Kruger et al. 2014; Sedlock et al. 2014) and competition (Brown et al. 2014a), particularly between co-occurring species. It is also a powerful way to provide broader insights into ecosystem composition and health. Faecal-DNA analysis may ultimately provide a functional context for the ecology of African psammophiines. In a broader context, I can conclude that analysis of prey DNA in snake faeces is a useful method to obtain accurate information on diet. When combined with cloning, such analyses can provide valuable ecological information about the diets of generalist predators. However, there are confounding factors that should be considered when implementing such a study. Although prey DNA can be detected from snake faeces, many sequences emanated from the predator and this could potentially reduce prey information. I therefore recommend the use of prey-specific primers where few prey is consumed, however, universal primers used with cloning or NGS are more applicable for highly generalist predators, as it can potentially reduce biases associated with prey information and predator amplification.

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Appendices

Appendix 1: Feeding and excretion in captive *Psammophylax rhombeatus* and *Psammophis crucifer*

Table A2.1. Feeding and excretion timeline for P. rhombeatus-1. Animal was checked daily for consumption and excretion events. (Initial mass: 69.6 g; final mass: 70.1 g)

Date	Time	Prey fed	Eaten	Faecal sample (g)
16.11.19	11:48 am	*T. capensis* (4.7 g)	Not consumed	-
18.11.19	09:15 am	*P. geitje* (0.3 g)	-	-
18.11.19	11:48 am	-	Consumed	-
24.11.19	13:30 pm	P. geitje (0.6 g)	-	-
25.11.19	10:12 am	-	Consumed	-
04.12.19	14:33 pm	-	-	1.27
12.12.19	07:20 am	*T. capensis* (6.2 g)	-	-
12.12.19	10:10 am	-	Consumed	-
15.12.19	13:20 pm	-	-	2.22
06.01.20	10:20 am	-	-	2.1
16.03.20	09:55 am	-	-	0.1

Table A2.2. Feeding and excretion timeline for P. rhombeatus-2. Animal was checked daily for consumption and excretion events. (Initial mass: 7.5g; final mass: 7.9 g; shed skin: 0.2 g)

Date	Time	Prey fed	Eaten	Faecal sample (g)
24.10.19		T. capensis (10.2 g)	Not consumed	-
05.11.19	09:40 am	*P. geitje* (1.9 g)	-	-
05.11.19	15:15 pm	-	Consumed	-
11.11.19	08:44 am	-	-	-
20.11.19	08:47 am	*P. geitje* (0.9 g)	-	-
21.11.19	12:08 pm	-	Consumed	-
21.11.19	15:03 pm	-	-	0.98
21.11.19	15:03 pm	-	-	0.98

Table A2.2. continued

Date	Time	Prey fed	Eaten	Faecal sample (g)
22.11.19	11:41 am	-	-	1.01
24.11.19	12:59 pm	-	-	1.00
27.11.19	08:57 am	-	-	0.97
01.12.19	08:50 am	-	-	1.3
08.12.19	21:10 pm	-	-	1.06
10.12.19	07:49 am	*P. geitje* (0.9 g)	-	-
12.12.19	10:10 am	-	Consumed	1.02
06.01.20	10:21 am	-	-	1.04
13.01.20	08:50 am	-	-	0.97
14.01.20	10:25 am	T. capensis (4.7 g)	Not consumed	-
20.01.20	11:40 am	-	-	-
23.01.20	09:25 am	*P. geitje* (1.7 g)	Consumed immediately	-
27.01.20	09:10 am	-	-	0.04
28.01.20	14:40 pm	-	-	0.39 + uric acid
29.01.20	08:42 am	-	-	0.18
11.02.20	09:13 am	-	-	0.03

Table A2.3. Feeding and excretion timeline for *P. rhombeatus*-3. Animal was checked daily for consumption and excretion events. (Initial mass: 7.9 g; final mass: 8.3 g)

Date	Time	Prey fed	Eaten	Faecal sample (g)
21.10.19	-	-	-	1.04 wild sample
24.10.19	-	-	-	1.03 wild sample
11.11.19	12:34 pm	*P. geitje* (1.3 g)	Not consumed	Uric acid
14.11.19	08:30 am	*P. geitje* (1.4 g)		-
14.11.19	10:30 am	-	Consumed	Uric acid
17.11.19	-	-	-	Uric acid
18.11.19	08:49 am	-	-	-
21.11.19	11:25 am	*P. geitje* (0.3 g)	-	0.08
22.11.19	11:34 am		Consumed	-
24.11.19	12:43 pm	*P. geitje* (0.1 g)	Consumed immediately	0.98

Table A2.3. continued

Date	Time	Prey fed	Eaten	Faecal sample (g)
25.11.19	10:07 am	-	-	1.05
26.11.19	11:04 am	-	-	0.97
28.11.19	11:30 am	-	-	1.05
29.11.19	10:33 am	-	-	1.4
02.12.19	13:34 pm	-	-	1.2
04.12.19	10:39 am	*P. geitje* (0.9 g)	Consumed immediately	-
12.12.19	11:02 am	-	-	0.97
30.12.19	13:59 pm	-	-	1.08
07.01.20	12:05 pm	-	-	Uric acid
13.01.20	08:54 pm	-	-	0.4
15.01.20	11:14 am	-	-	0.2
16.01.20	10:10 am	-	-	0.01
16.01.20	12:40 am	*T. capensis* (3.7 g)	Consumed immediately	-
17.01.20	08:49 am	-	-	0.05
20.01.20	11:43 am	-	-	0.23
21.01.20	18:33 pm	-	-	0.15
22.01.20	10:12 am	-	-	0.31
24.01.20	07:40 am	-	-	0.25
25.01.20	15:43 pm	-	-	0.27
27.01.20	09:15 am	-	-	0.13
30.01.20	08:37 am	-	-	0.17
10.02.20	08:33 am	-	-	uric acid
14.02.20	08:33 am	-	-	0.06+ uric acid
15.02.20	08:51 am	-	-	0.1
21.02.20	09:00 am	*P. geitje* (0.9 g)	Consumed immediately	-
28.02.20	08:52 am	-	-	0.07
02.03.20	09:18 am	-	-	0.97
09.03.20	09:33 am	-	-	0.07

Table A2.4. Feeding and excretion timeline for *P. rhombeatus*-4. Animal was checked daily for consumption and excretion events. (Initial mass: 48.6 g; final mass: 49.5 g)

Date	Time	Prey fed	Eaten	Faecal sample (g)
25.01.20	15:41 pm	-	-	0.58-wild sample
03.02.20	09:00 am	-	-	0.29-wild sample
21.02.20	09:10 am	*T. capensis* (22.2 g)	Not consumed	-
21.02.20	14:05 pm	*P. geitje* (1.4 g)	Not consumed	-
24.02.20	10:30 am	*P. geitje* (1.3 g)	-	-
24.02.20	11:46 am	-	Consumed	-
28.02.20	08:43 am	-	-	0.18

Table A2.5. Feeding and excretion timeline for *P. crucifer*-1. Animal was checked daily for consumption and excretion events. (Initial mass: 18.4 g; final mass: 19.5 g, shed skin: ~0.45 g)

Date	Time	Prey fed	Eaten	Faecal sample (g)
14.11.19	13:30 pm	-	-	1.53-wild sample
18.11.19	11:43 am	*T. capensis* (4.41 g)	Not consumed	Uric acid
22.11.19	11:45 am	-	-	-
24.11.19	13:05 pm	*P. geitje* (1.7 g)	Consumed immediately	1
25.11.19	10:15 am	-	-	1.01
27.11.19	09:00 am	-	-	1.03
01.12.19	12:36 pm	-	-	1.03
03.12.19	09:33 am	-	-	1.03
04.12.19	10:48 am	*T. homalocephala* (8.8 g)	Consumed immediately	-
05.12.19	08:05 am	-	-	1.12
08.12.19	21:05 pm	-	-	1.92
08.12.19	21:05 pm	-	-	-
10.12.19	07:05 am	-	-	-
10.12.19	08:40 am	-	-	1.64
10.12.19	11:00 am	-	-	1.11
12.12.19	07:10 am	-	-	1.08
15.12.19	13:11 pm	-	-	1.01

Table A2.5. continued

Date	Time	Prey fed	Eaten	Faecal sample (g)
19.12.19	11:25 am	*T. capensis* (3.8 g)	Not consumed	-
30.12.19	-	-	-	1.04
07.01.20	11:54 am	-	-	0.96
13.01.20	08:34 am	-	-	0.03
14.01.20	08:45 am	*T. capensis* (3.7 g)	Not consumed	Uric acid
15.01.20	11:08 am	-	-	0.02
15.01. 20	15:00 pm	-	-	1
17.01.20	08:50 am	-	-	0.06
20.01.20	11:36 am	-	-	0.25
21.01.20	18:30 pm	-	-	0.03
23.01.20	09:00 am	*T. capensis* (1.1 g)	Consumed immediately	-
25.01.20	15:42 pm	-	-	0.2
27.01.20	08:48 am	-	-	0.12
27.01.20	12:50 pm	-	-	0.1
28.01.20	14:30 pm	-	-	0.09 + uric acid
02.02.20	12:30 pm	-	-	0.05 + uric acid
16.02.20	08:50 am	-	-	0.07 + uric acid
21.02.20	09:35 am	*T. capensis* (2.2 g)	Consumed immediately	-

Table A2.6. Feeding and excretion timeline for *P. crucifer*-2. Animal was checked daily for consumption and excretion events. (Initial mass: 4.8 g; final mass: 5.2 g)

Date	Time	Prey fed	Eaten	Faecal sample (g)
18.11.19	11:00 am	-	-	1.06-wild sample
19.11.19	10:20 am	*P. geitje* (1.5 g)	-	-
19.11.19	13:21 pm	-	Consumed	-
21.11.19	14: 24 pm	*P. geitje* (1.56 g)	Not consumed	-
28.11.19	11:29 am	*P. geitje* (1.2 g)	Consumed immediately	1.03
01.12.19	12:22 pm	-	-	1.04
02.12.19	08:48 am	-	-	1.06
04.12.19	08:26 am	*T. capensis* (3.8 g)	Not consumed	-

Table A2.6. continued

Date	Time	Prey fed	Eaten	Faecal sample (g)
04.12.19	10:31 am	*T. capensis* (7 g)	Not consumed	-
12.12.19	09:25 am	*T. capensis* (3.7 g)	Not consumed	-
17.01.20	08:45 am	-	-	0.05
23.01.20	09:40 am	*T. capensis* (1.1 g)	Not consumed	-
28.01.20	14:35 pm	-	-	1.04
30.01.20	09:55 am	*T. capensis* (1.22 g)	Consumed	0.07
02.02.20	12:15 pm	-	-	0.07 + uric acid
03.02.20	08:45 am	*P. geitje* (1.1 g)	Consumed	-
03.02.20	11:05 am	-	-	0.1 + uric acid
07.02.20	15:45 pm	-	-	0.11 + uric acid
10.02.20	08:28 am	*P. geitje* (0.6 g)	Not consumed	0.04 + uric acid
11.02.20	09:00 am	-	-	0.03
21.02.20	09:00 am	*P. geitje* (0.6 g)	Consumed	-
26.02.20	08:28 am	-	-	0.06
03.03.20	09:14 am	-	-	0.04

Appendix 2: Evaluation of species-specific *CYTB* primers
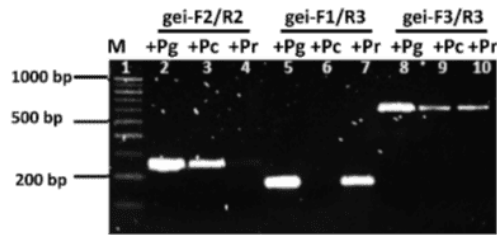
Figure A2.1 Specificity of *CYTB* PCR primers showing specificity to *P. geitje*, *P. crucifer* or *P. rhombeatus*. Three *P. geitje* specific primer pair combinations. DNA template derived from either *P. geitje* (+Pg), *P. crucifer* (+Pc), or *P. rhombeatus* (+Pr). 100 bp DNA ladder (M).
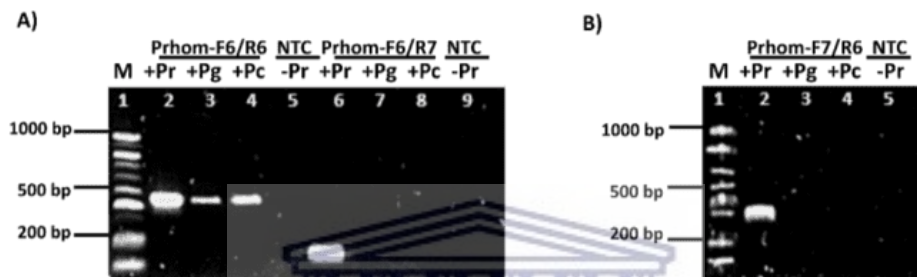
Figure A2.2 Specificity of *CYTB* PCR primers showing specificity to *P. geitje*, *P. crucifer* or *P. rhombeatus*. **(A)** Two *P. rhombeatus* specific primer pair combinations. DNA template derived from either *P. geitje* (+Pg), *P. crucifer* (+Pc), or *P. rhombeatus* (+Pr). 100 bp DNA ladder (M). **(B)** One *P. rhombeatus* specific primer pair combination. DNA template derived from either A template derived from either *P. geitje* (+Pg), *P. crucifer* (+Pc), or *P. rhombeatus* (+Pr). 100 bp DNA ladder (M).

Appendix 3: Faecal DNA analysis targeting a short fragment
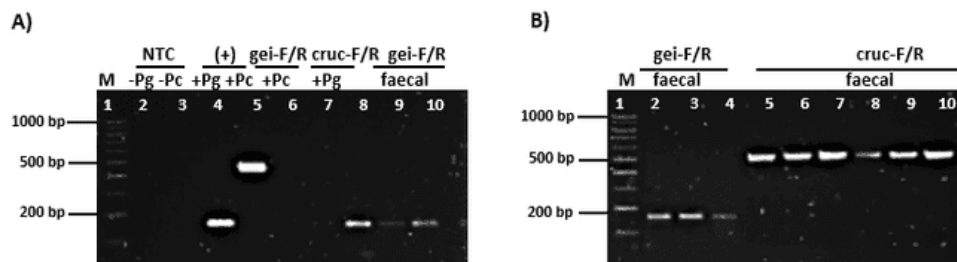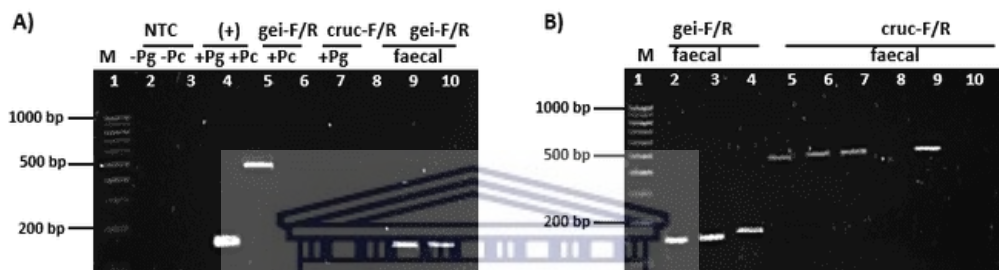

Figure A3.1 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* and *cruc-F5/R5* shown in lanes 4 and 5, respectively. 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. crucifer*-derived faecal samples amplified with either *P. geitje*-specific primers (*gei-F1/R1*) or *P. crucifer*-specific primers (*cruc-F5/R5*). 100 bp DNA ladder (M).
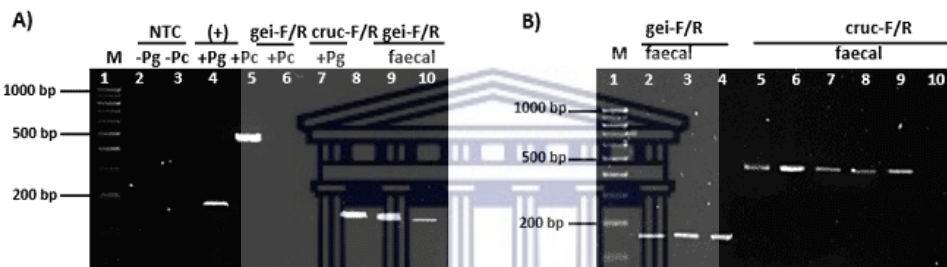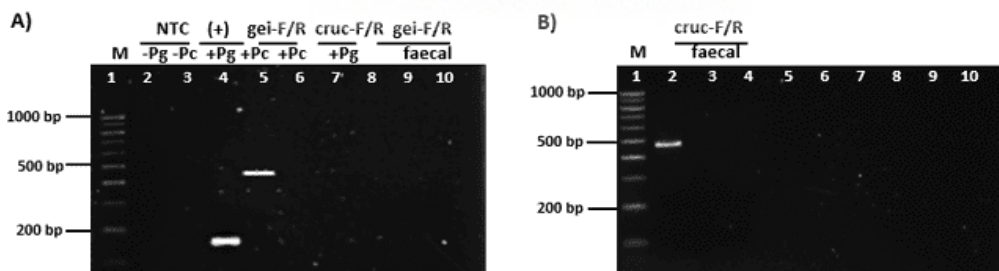

Figure A3.2 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* and *cruc-F5/R5* shown in lanes 4 and 5, respectively. 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. crucifer*-derived faecal samples amplified with either *P. geitje*-specific primers (*gei-F1/R1*) or *P. crucifer*-specific primers (*cruc-F5/R5*). 100 bp DNA ladder (M).
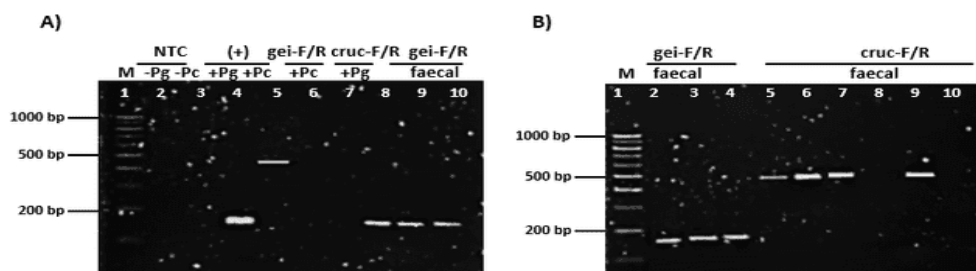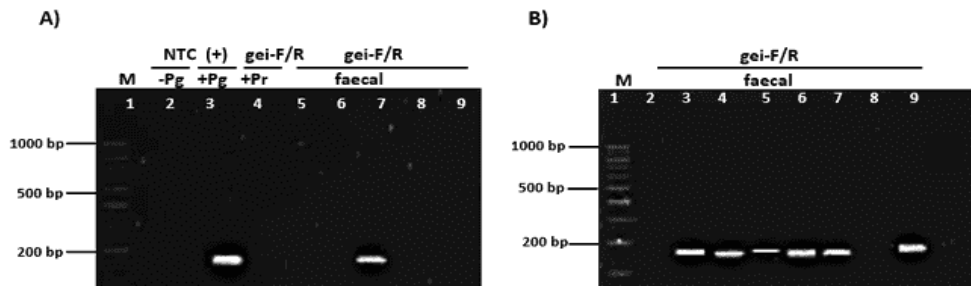

Figure A3.3 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* and *cruc-F5/R5* shown in lanes 4 and 5, respectively. 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. crucifer*-derived faecal samples amplified with either *P. geitje*-specific primers (*gei-F1/R1*) or *P. crucifer*-specific primers (*cruc-F5/R5*). 100 bp DNA ladder (M).
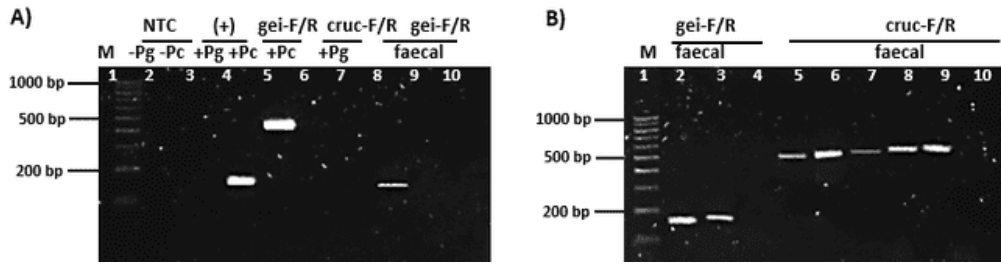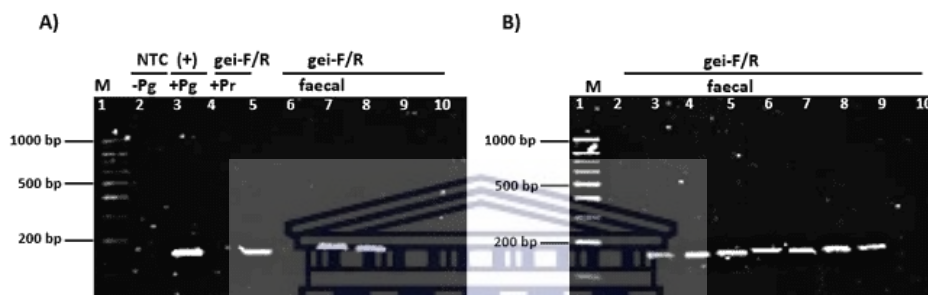
Figure A3.4 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. **(A)** DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* and *cruc-F5/R5* shown in lanes 4 and 5, respectively. 100 bp DNA ladder (M). Negative template controls (NTC). **(B)** DNA from *P. crucifer*-derived faecal samples amplified with either *P. geitje*-specific primers (*gei-F1/R1*) or *P. crucifer*-specific primers (*cruc-F5/R5*). 100 bp DNA ladder (M).

Figure A3.5 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. **(A)** DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* and *cruc-F5/R5* shown in lanes 4 and 5, respectively. 100 bp DNA ladder (M). Negative template controls (NTC). **(B)** DNA from *P. crucifer*-derived faecal samples amplified with either *P. geitje*-specific primers (*gei-F1/R1*) or *P. crucifer*-specific primers (*cruc-F5/R5*). 100 bp DNA ladder (M).

Figure A3.6 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. **(A)** DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* and *cruc-F5/R5* shown in lanes 4 and 5, respectively. 100 bp DNA ladder (M). Negative template controls (NTC). **(B)** DNA from *P. crucifer*-derived faecal samples amplified with either *P. geitje*-specific primers (*gei-F1/R1*) or *P. crucifer*-specific primers (*cruc-F5/R5*). 100 bp DNA ladder (M).

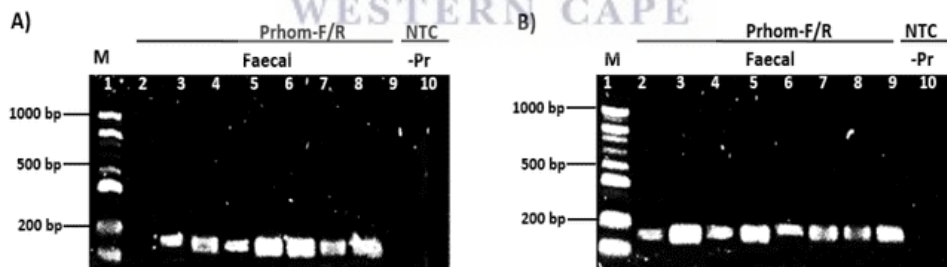**Figure A3.7** Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. rhombeatus* tissue (+Pr), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* shown in lane 3. 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. geitje*-specific primers (*gei-F1/R1*). 100 bp DNA ladder (M).
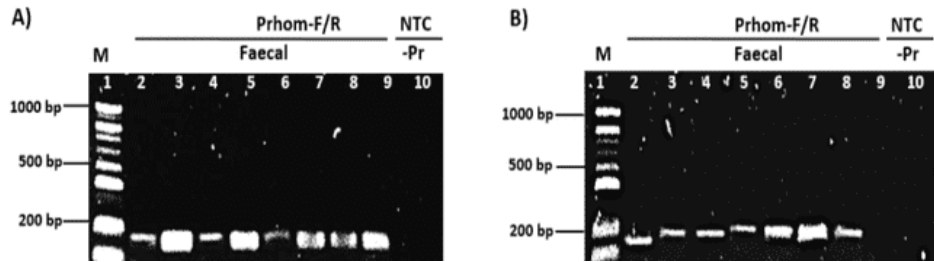

Figure A3.8 Amplification of a 156 bp fragment of prey DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. rhombeatus* tissue (+Pr), or *P. crucifer*-derived faecal samples. Specificity of *gei-F1/R1* shown in lane 3. 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. geitje*-specific primers (*gei-F1/R1*). 100 bp DNA ladder (M).

Figure A3.9 Amplification of a 156 bp fragment of predator DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. rhombeatus*-specific primers (*prhom-F6/R9*). 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. rhombeatus*-specific primers (*gei-F6/R7*). 100 bp DNA ladder (M).
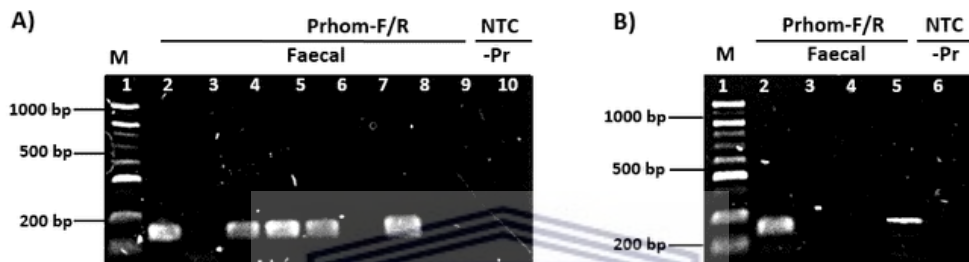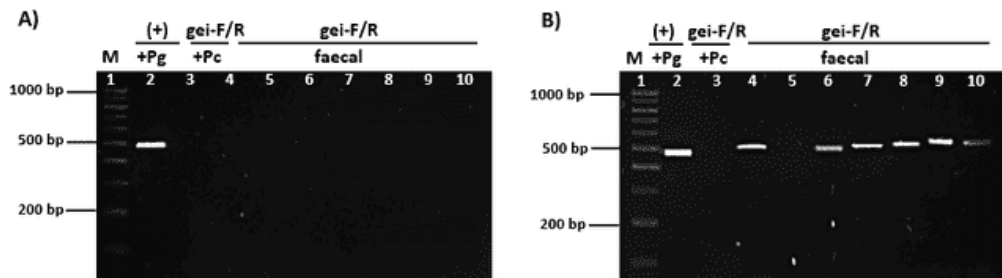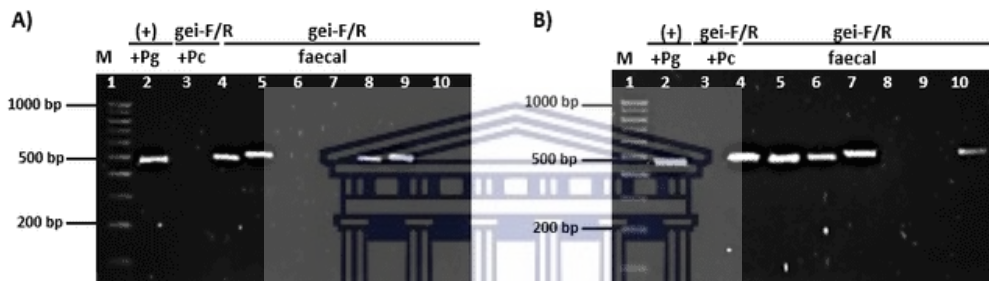
Figure A3.10 Amplification of a 156 bp fragment of predator DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. rhombeatus*-specific primers (*prhom-F6/R7*). 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. rhombeatus*-specific primers (*gei-F6/R7*). 100 bp DNA ladder (M).

Figure A3.11 Amplification of a 156 bp fragment of predator DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. rhombeatus*-specific primers (*prhom-F6/R9*). 100 bp DNA ladder (M). Negative template controls (NTC). (**B**) DNA from *P. rhombeatus*-derived faecal samples amplified with *P. rhombeatus*-specific primers (*gei-F6/R7*). 100 bp DNA ladder (M).

Appendix 4: Faecal DNA analysis targeting a large fragment

Figure A4.1 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specifici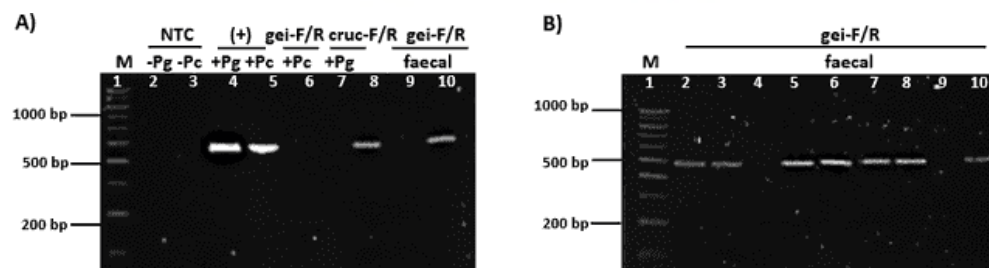ty of *gei-F2/R1* (lane 2–3). 100 bp DNA ladder (M). (**B**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F2/R1* (lane 2–3). 100 bp DNA ladder (M).

Figure A4.2 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F2/R1* (lane 2–3). 100 bp DNA ladder (M). (**B**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F2/R1* (lane 2–3). 100 bp DNA ladder (M).

Figure A4.3 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. crucifer* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. crucifer* tissue (+Pc), or *P. crucifer*-derived faecal samples. Specificity of *gei-F2/R1* (lane 6–7). 100 bp DNA ladder (M). (**B**) DNA template derived from *P. crucifer*-derived faecal samples. 100 bp DNA ladder (M).
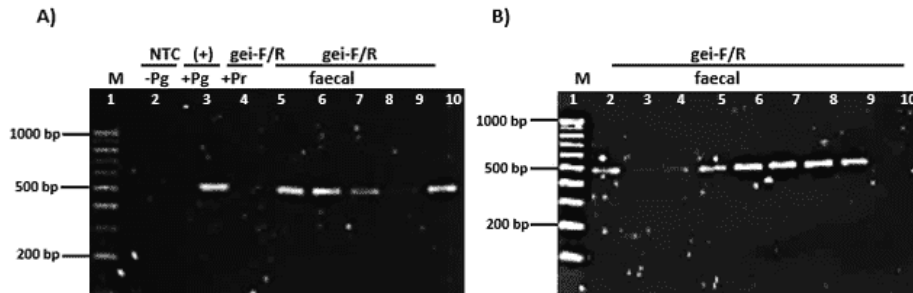
Figure A4.4 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. rhombeatus* tissue (+Pr), or *P. rhombeatusr*-derived faecal samples. Specificity of *gei-F2/R1* (lane 3-4). 100 bp DNA ladder (M). (**B**) DNA template derived from *P. rhombeatus*-derived faecal samples. 100 bp DNA ladder (M).
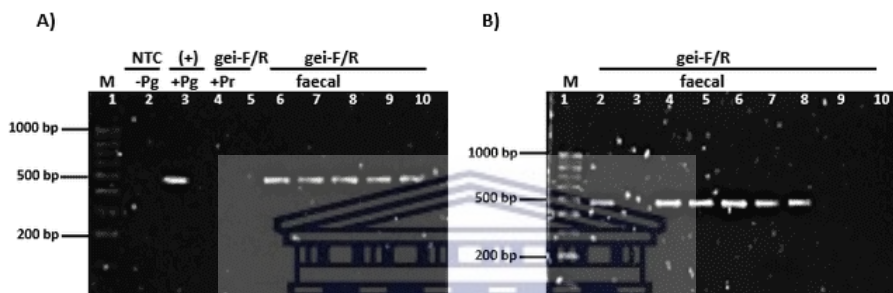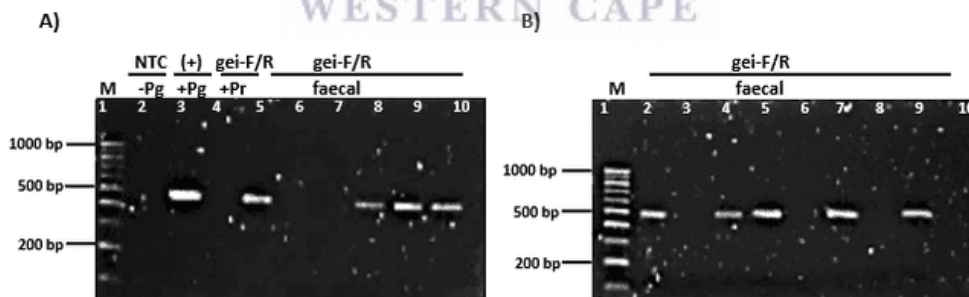

Figure A4.5 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. rhombeatus* tissue (+Pr), or *P. rhombeatusr*-derived faecal samples. Specificity of *gei-F2/R1* (lane 3-4). 100 bp DNA ladder (M). (**B**) DNA template derived from *P. rhombeatus*-derived faecal samples. 100 bp DNA ladder (M).

Figure A4.6 Amplification of a 478 bp fragment of prey DNA (*CYTB*) from *P. rhombeatus* faecal samples. (**A**) DNA template derived from either *P. geitje* tissue (+Pg), *P. rhombeatus* tissue (+Pr), or *P. rhombeatusr*-derived faecal samples. Specificity of *gei-F2/R1* (lane 3-4). 100 bp DNA ladder (M). (**B**) DNA template derived from *P. rhombeatus*-derived faecal samples. 100 bp DNA ladder (M).

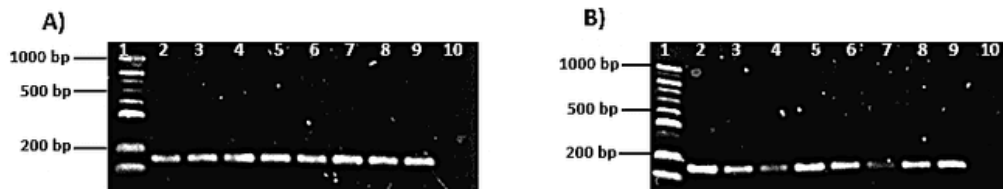**Appendix 5: Faecal DNA analysis using 16S universal primers**

Figure A5.1 Amplification of *16S-rRNA* from *P. rhombeatus* and *P. crucifer* faecal samples using universal primers (16S-F02–16S-R02). (**A**) *P. rhombeatus* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10). (**B**) *P. crucifer* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10).
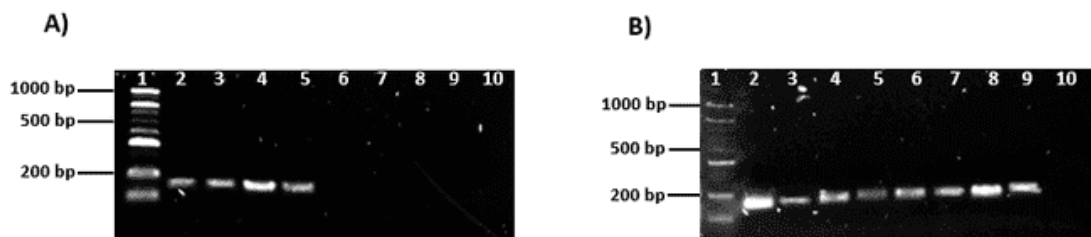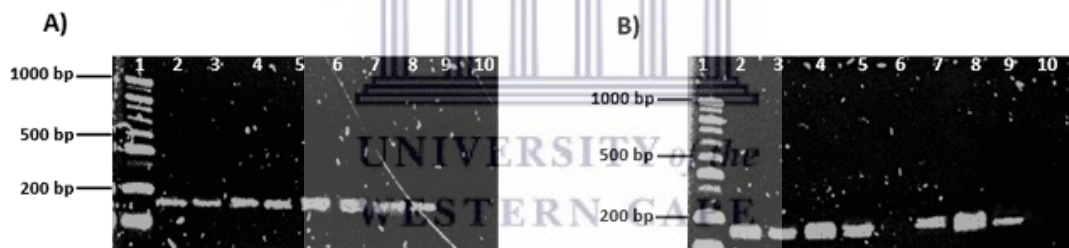

Figure A5.2 Amplification of *16S-rRNA* from *P. rhombeatus* and *P. crucifer* faecal samples using universal primers (16S-F02–16S-R02). (**A**) *P. rhombeatus* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10). (**B**) *P. crucifer* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10).
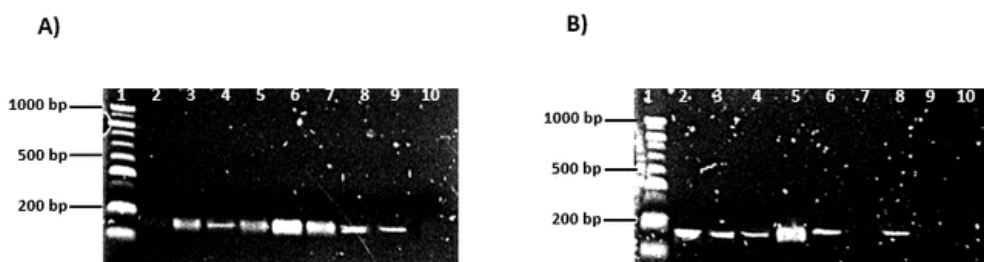

Figure A5.3 Amplification of *16S-rRNA* from *P. rhombeatus* and *P. crucifer* faecal samples using universal primers (16S-F02–16S-R02). (**A**) *P. rhombeatus* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10). (**B**) *P. crucifer* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10).

Figure A5.4 Amplification of *16S-rRNA* from *P. rhombeatus* and *P. crucifer* faecal samples using universal primers (16S-F02–16S-R02). (**A**) *P. rhombeatus* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10). (**B**) *P. crucifer* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10).
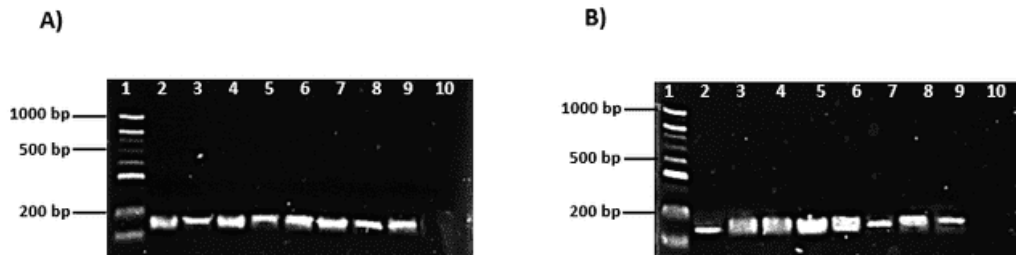
Figure A5.5 Amplification of *16S-rRNA* from *P. rhombeatus* and *P. crucifer* faecal samples using universal primers (16S-F02–16S-R02). (**A**) *P. rhombeatus* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10). (**B**) *P. crucifer* faecal samples (2–9), 100 bp DNA ladder (1), NTC (10).
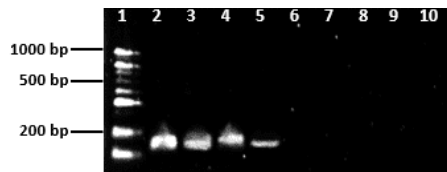

Figure A5.6 Amplification of *16S-rRNA* from *P. rhombeatus* and *P. crucifer* faecal samples using universal primers (16S-F02–16S-R02). *P. rhombeatus* faecal samples (2–5), 100 bp DNA ladder (1), NTC (6).