Molecular and biochemical analysis of the diet of the black rhinoceros

A thesis submitted in fulfillment of the requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

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February 2009

ABSTRACT

The black rhinoceros, *Diceros bicornis*, is listed as critically endangered. The black rhinoceros population in the Great Fish River Reserve (GFRR) has increased steadily to a current estimate of one hundred animals since the re-introduction of four animals in 1986. In an effort to contribute to the effective conservation and management of this species, dietary composition was studied in the medium *Portulcaria* thicket vegetation of the GFRR. This study used a molecular approach to determine the diet of the black rhinoceros of the GFRR by sequencing the ribulose bisphosphate carboxylase large subunit (*rbc*L) gene in plants and dung.

Twenty-three plant species were collected from the reserve, and 802 bp of the *rbc*L gene were sequenced. These plant sequences were used as a reference database for the identification of plant sequences generated from black rhinoceros dung. Initial studies investigated the amplification, cloning and sequencing of DNA extracted from the dung samples which indicated the viability of the molecular approach. Thereafter, dung generated *rbc*L DNA was analyzed by GS FLX sequencing. Of the plant sequences identified by comparison to the GenBank database, *Carissa bispinosa* was the most prevalent.

The study further characterized the antioxidant activities and phenolic content of plants eaten by the black rhinoceros using four different assays. *Phyllanthus verrucosus, Putterlickia pyracantha, Maytenus capitata, Euclea undulata* and *Ozoroa mucrunata* consistently had high antioxidant activities when assayed against 2,2-azinobis (3-ethyl benzothiazolium-6-sulfonic acid) ($ABTS^{\cdot+}$), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), and ferric reducing antioxidant potentials (FRAP) and phenolic content when evaluated using the Folin-Ciocalteu assay. The majority of plants investigated showed low antioxidant potentials and low phenolic content. The extent to which antioxidants influenced the browse selection by the black rhinoceros remains inconclusive.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
(v/v)	Volume per volume
ABTS	2-azinobis (3-ethyl benzothiazolium-6-sulfonic acid)
AZA	Association of zoos and aquariums
BLAST	Basic local alignment search tool
bp	Base pair
CITES	Convention on international trade in endangered species of wild flora and fauna
CTAB	Cetyl trimethyl ammonium bromide
ddH_2O	Double deionized water
$dddH_2O$	Triple deionized water
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EDTA	Ethylenediamine tetraacetic acid
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalence (mg/l)
GFRR	Great Fish River Reserve
IUCN	International union of conservation of nature and natural resources
IPTG	Isopropyl-β-thiogalactosidase
LSC	Large single copy
LSU	Large subunit
LB	Luria broth
m/v	Mass per volume
MEGA	Molecular evolution genetics
MOPS	3-(N-morpholino) propanesulfonic acid
MPT	medium *Portulacaria* thicket
NCBI	National centre for biotechnology information
OD	Optical density
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PG	propylgallate
PVP	Polyvinyl pyrrolidone
rpm	Revolutions per minute
SD	standard deviation
SDS	Sodium dodecyl sulphate
SET	Short *Euphorbia* thicket
SOC	Super optimized culture
SSU	Small Subunit
TAE buffer	Tris-Acetate-EDTA buffer
TPTZ	2,4,6-Tri-2-pyridyl-*s*-triazine
UV	Ultra violet
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranosidase

DECLARATION

This work has originally being produced by Ananias Hodi Kgopa, submitted to Rhodes University in February 2009, for a Master of Science degree in Biochemistry.

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Co-supervisor: **Prof. John M. Brand**

ACKNOWLEDGEMENTS

I would like to thank the following for their contribution to this study:

The Almighty God of Zion, from my childhood till now. His presence, grace and guidance in good and difficult times of my research project, resulted in my academic achievement.

Dr Brendan Wilhelmi, for accepting me as his student and for introducing me into the area of multidisciplinary scientific research projects. Your guidance in research experimentations, science writing, presentations, the opportunity to attend conferences and the support you gave throughout my study program at Rhodes University made me realize your value in sharpening my future. I am now confident to stand forward and fear no challenges in the future.

My co-supervisor, Professor John Brand for his valuable input and constructive criticism in my research project. By guiding me how both the molecular and biochemical results of my research project should be critically analyzed, made me realize how data could be made meaningful and concrete.

Phetole, for his valuable critique, Dr Tandlich, Dr Adebyei, Dr Jiwaji, Dr Knox, Suzan, lab 412 honours students, Pholoshi, Caswell, Kwena and everyone who contributed to my research project.

My mother, Mamonyama, brother Maribe and Mankwana, sister Matsebe, son Tetelo and Nthabiseng for being supportive throughout my study programs. I wish my late brother Rakau and cousin Maribe (Jnr) could have been here to witness my progress in science.

The Andrew Mellon Foundation and National Research Fund for the financial support that helped me in fulfilling my dreams, and to Rhodes University staff in the Department

of Biochemistry, Microbiology and Biotechnology for the valuable support they gave throughout my studies.

CHAPTER ONE
LITERATURE REVIEW

1.1 Background

Dramatic land use changes have resulted in habitat loss that affects wildlife species (Bulte and Horan, 2003; Smith and Zollner, 2005). In addition to habitat loss, hunting has been a major cause of wildlife decline (Gavin, 2007). Because of the decline in certain animal populations, many programs have been initiated to protect wildlife populations (Amin *et al.*, 2003; Moehlman *et al.*, 1996). These programs rely on conserving and managing the remaining populations. This practice is applicable in developing countries such as South Africa and is considered an important method of protecting wildlife species (Kwapena, 1984; Schulz and Skonhoft, 1996; Gavin, 2007; Setsaas *et al.*, 2007).

The black rhinoceros has suffered a dramatic decline due to poaching for their horns, to a point of near extinction (Ashley *et al.*, 1990). As a result, conservation practices have been put in place to rescue the population (Flynn and Abdullah, 1984; Tivy, 1985). Areas preferred for conservation of wildlife populations are reserves and national parks (Setsaas *et al.*, 2007). Due to its conservation practices, South Africa has the highest number of the species, *Diceros bicornis* (O'Ryan *et al.*, 1994).

Although many disciplines are important for conservation, a thorough understanding of the species under conservation is a key for effective management (Hutchins and Kreger, 2006). Efforts to protect the black rhinoceros have focused on increasing security and creating suitable areas for the game to be introduced. The minimum habitat required to sustain a viable population of black rhinoceros is not known. However, for effective management, the availability and quality of food have been identified as major factors determining habitat suitability (Muya and Oguge, 2000). Further, secondary chemical compounds contained in foods should be considered as they play a role in animal health (Dierenfeld, 1997; Graffam *et al.*, 1997; Harley *et al.*, 2004). According to Velioglu *et al.* (1998), secondary chemicals include phenolic compounds, nitrogen compounds and carotenoids.

1.2 Rhinoceroses

Rhinoceroses are included in the family rhinocerotidae, in the order Perisodactyla, together with Tipiridae and Equidae (Tougard *et al.*, 2001). This family comprises of five living species: three in Asia and two in Africa (Emslie and Brooks, 1999). The two African species are the black rhinoceros (*Diceros bicornis*), which is a browser, and the white rhinoceros (*Ceratotherium simum*), which is a grazer. Both of these species are grey in colour, but are easily distinguished by their mouthparts (Emslie and Brooks, 1999; Tougard *et al.*, 2001). The black rhinoceros has a prehensile lip, which it uses to grasp stems, branches, twigs and leaves. It is sometimes referred to as the hook-lipped rhinoceros. The white rhinoceros can be identified by its "wide" mouth (Emslie and Brooks, 1999).

1.2.1 The status of the African black rhinoceroses

Black and white rhinoceros were formerly spread over most of the central part of southern Africa (Amin *et al.*, 2003). Black rhinoceros were the first population of large herbivores to be listed as a critically endangered species by the International Union of Conservation of Nature and Natural Resources (IUCN) 1996 *Red list of Threatened Animals* (Emslie and Brooks, 1999) and the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES, 1975) (Amin *et al.*, 2003).

In the 19th century, there were 100 000 black rhinoceros in Africa (Emslie and Brooks, 1999; Ausland and Sviepe, 2000). In 1970, the total population stood at around 60 000, then dropped steadily to 15 000 in 1980, 8 800 in 1984 and 3 800 in 1987 and to approximately 2 500 in 1995. Despite measures to protect black rhinoceros in Africa, the number dropped dramatically to 948 in 1998, a point of near extinction (Western, 1987; Emslie and Brooks, 1999).

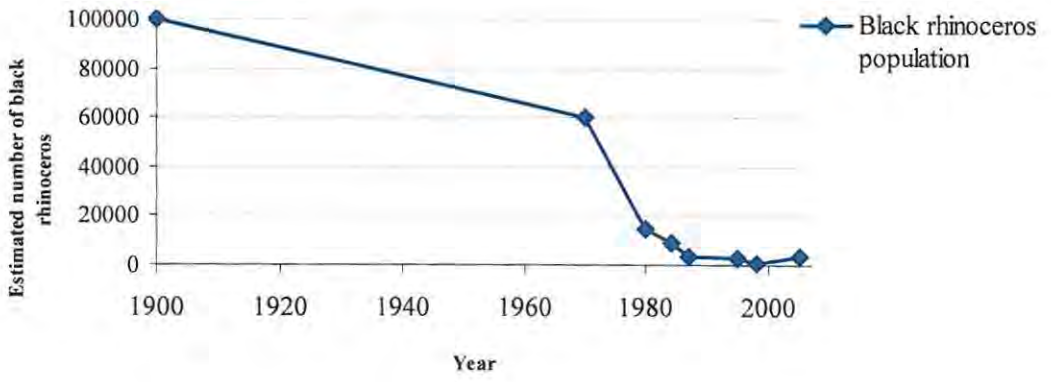

Figure 1.1: The decline of the black rhinoceros population in Africa (Emslie and Brooks, 1999).

Poaching for rhinoceros horns used for medicinal and artistic purposes, as well as dagger handles, has been and still is the major reason for the decline of the black rhinoceros in the wild (http://www.kws.org/rhino.html; Western, 1987; Moehlman *et al.*, 1996; Emslie and Brooks, 1999; Walpole *et al.*, 2001; Amin *et al.*, 2003). The long-term solution for the protection of the remaining black rhinoceros is the establishment of breeding programs and reserves (Western, 1987; Moehlman *et al.*, 1996; Emslie and Brooks, 1999; Walpole *et al.*, 2001).

1.2.2 Captive conservation and breeding

Zoos have responded as the last option to prevent the extinction of many animals. However, attempts to breed animals in captivity is difficult, due to problems such as breeding only small numbers of animals due to limited space, resources and changes in the genetic diversity of animals. A further problem with captive breeding is that it gives the public the perception that as long as animals are kept in zoos, these species will not become extinct (Cohn, 1988).

Efforts to sustain viable global populations of black rhinoceroses through captive breeding programs have been hindered by health problems (Dierenfeld *et al.*, 1988; Harley *et al.*, 2004). Several diseases such as acute hemolytic anemia have been a major cause of deaths of black rhinoceroses in small captive areas (Harley *et al.*, 2004). Another prevalent disease causing deaths in captive black rhinoceros is a dermatologic and mucosal condition, characterized by recurrent plaques, vesicles and ulcers. The skin and mucosal diseases have not been identified in wild black

rhinoceroses, and it is not associated with *Stephanofilaria dinniki* infestations found associated with most ulcers in wild rhinoceros (Munson *et al.*, 1998).

A possible cause of these diseases in captivity may be due to inadequate nutrition. Alfalfa and timothy hay are often the main dietary components fed to black rhinoceros in zoos. Different types of *Acacia spp* and *Ficus spp.*, hoofstock and herbivores pellets are also fed to captive black rhinoceros in selected breeding areas (Munson *et al.*, 1998). For this reason, studies of the diet of wild black rhinoceroses may be of benefit to feeding captive animals.

1.2.3 Conservation of the black rhinoceroses in wild areas

Black rhinoceroses have been introduced into protected reserves, with the current idea for conservation being the translocation from areas of high density to reserves with low-density populations (Amin *et al.*, 2003; Dunn *et al.*, no date). Due to these conservation and breeding programs, there are currently approximately 3 725 black rhinoceros inhabiting protected areas.

South Africa is the strong-hold of the black rhinoceros, largely due to translocations of this species from areas approaching the ecological carrying capacity to new, suitable reserves (Hearne and Swart, 1991; Berger, 1994). The Great Fish River Reserve is one of the reserves in South Africa with an increasing number of the black rhinoceros subspecies *Diceros bicornis minor* (Fike, *pers. comm.* 2007).

1.2.4 Black rhinoceroses of the Great Fish River Reserve

The Great Fish River Reserve (GFRR) lies between Grahamstown and Fort Beaufort, 30 km north of Grahamstown in the Eastern Cape of South Africa (Mabinya *et al.*, 2002). The reserve comprises the Andries Vosloo Kudu Reserve, the Double Drift Nature Reserve and the Sam Knott Nature Reserve. These reserves are about 45,000 hectares in total and are divided by the Great Fish River. The primary purpose of this reserve is the conservation of the unique biodiversity, ecological processes, and the associated heritage features of the Eastern Cape Sub-Tropical Thicket. The reserve has a valley succulent bushveld with a variety of habitats which boast an abundance of game such as the black rhinoceros, buffalo, kudu, white rhinoceros, various antelopes and hippopotami (Mabinya *et al.*, 2002; http://www.ecparks.co.za).

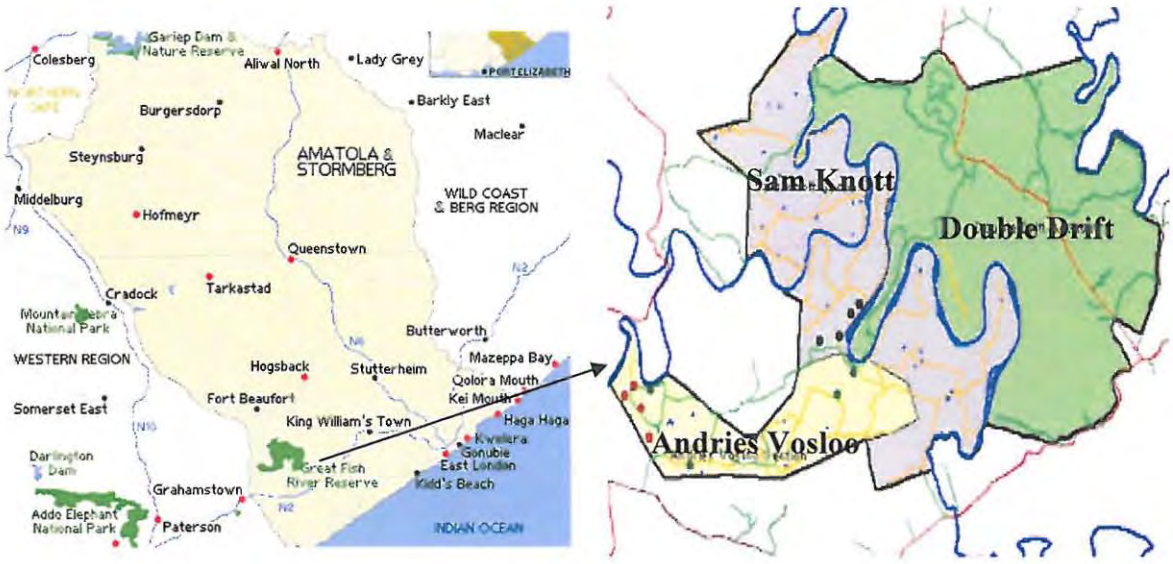

Figure 1.2: The three reserves that form the Great Fish River Reserve (Van Lieverloo and Schuiling, 2004).

The black rhinoceros population in the GFRR has increased steadily since the re-introduction in 1986 of four animals (Ndondo *et al.*, 2004; Fike, *pers. comm.* 2007). The current number is estimated at 100, with 30% of the adult females giving birth each year (Fike, *pers. comm.* 2007).

Figure 1.3: Black rhinoceroses of the Great Fish River Reserve (Picture by Morgan Brand).

Figure 1.4: *Euphorbia bothae*, a favoured food plant of the black rhinoceros of the Great Fish River Reserve (Picture by Morgan Brand).

Black rhinoceroses consume up to 200 different plants species, comprising 50 families, in a year. Eaten most are shrubs, herbs, succulents and woody plants (Graffam *et al.*, 1997; Fike, *pers. comm.* 2007). Success of the black rhinoceros in the GFRR is possibly due to the quality of habitat that comprises a variety of short and medium succulent plants (Brown *et al.*, 2003). The conservation plan is to achieve and maintain a high growth rate of the rhinoceros population without adversely affecting the vegetation, as this may have a negative impact on other herbivores, and can only be achieved if the reserve is not overstocked (Ausland and Sviepe, 2000). However, little is known about the minimum habitat that is suitable for a viable population of black rhinoceroses in a reserve (Muya and Oguge, 2000). For this reason, a better understanding of factors affecting herbivore populations is essential for their effective conservation and management, with diet being one of the most important factors in a successful conservation program.

1.3 Determination of the diet of herbivores

Wildlife viability is an important land management issue, and is a crucial component of healthy ecosystems (Bulte and Horan, 2003; Smith and Zollner, 2005). Specific problems encountered by range scientists are the accuracy in determining the botanical diet composition of herbivorous animals and the nutrient value offered by

the diet (Mofareh *et al.*, 1997). A thorough knowledge of the diet allows for the development of conservation and management strategies (Bradley *et al.*, 2007). Dietary information is a particularly important factor for the management of large free-ranging herbivores (Fitzgerald and Waddington, 1979; Mcinnis *et al.*, 1983; Mofareh *et al.*, 1997). This allows for the assessment of nutrient intake by the animal for evaluation of potential forage competition amongst herbivore species (Mcinnis *et al.*, 1983). For this reason, reliable methods for measuring plant species eaten are required (Fitzgerald and Waddington, 1979).

Several indirect methods have been proposed for analysis of the diet composition of herbivore species (Hansen *et al.*, 1973; Fitzgerald and Waddington, 1979; Kessler *et al.*, 1981; Mcinnis *et al.*, 1983; Mohammed *et al.*, 1995; Mofareh *et al.*, 1997; Henley *et al.*, 2001). Microscopic examination of plant residues recovered from oesophageal fistulae, stomach contents and faeces is a technique used to determine the food habits of herbivores. Direct observation is also a method used to determine the diet of foraging herbivores (Mcinnis *et al.*, 1983; Mclntire and Carey, 1989; Mohammed *et al.*, 1995; Mofareh *et al.*, 1997; Henley *et al.*, 2001).

Generally, microscopic techniques involve the identification of recognizable plant structures. Rumen or esophageal samples are usually analyzed using microanalytical methods whereby plant identification depends on the overall features of the ingested material (Kessler *et al.*, 1981). Microscopic techniques involve the study of structural components such as cuticle, epithelial cells, stomata, seeds and pollen to identify plants ingested (Mclntire, and Carey, 1989).

Each of these techniques is associated with a number of disadvantages and advantages (Mcinnis *et al.*, 1983; Fitzgerald and Waddington, 1979). The analysis of stomach contents may be biased toward the less digestible material in the diet (Mcinnis *et al.*, 1983). Both the esophageal fistula and rumen ingestion techniques require extensive training in the use of microscopic identification of plant fragments (Mohammed *et al.*, 1995). This has stimulated discussions as to which technique is most useful in interpreting food habits of large herbivores (Mcinnis *et al.*, 1983). For this reason, microhistological analysis of faecal material has become the technique several researchers have used (Fitzgerald and Waddington, 1979; Kessler *et al.*, 1981; Mcinnis *et al.*, 1983; Mohammed *et al.*, 1995). This technique is used to identify the

botanical composition of the diet selected by herbivores based on plant cuticular characteristics (Lee and MacGregor, 2004.).

1.3.1. Faecal microhistology

Analysis of herbivore faecal material involves the analysis of recognizable plant fragments in the faeces to determine its botanical composition (Hansen *et al.*, 1973; Mcinnis *et al.*, 1983; Mohammed *et al.*, 1995). This is simplified by the characteristic shape and arrangement of the epidermal cells of the leaves of each plant species. These features are molded on the overlying cuticle, which is indigestible and passes through the gut of herbivores (Fitzgerald and Waddington, 1979). Using a microscopic slide preparation technique, epidermal plant fragments are recognizable (Hansen *et al.*, 1973). Identification is possible with the aid of reference collections of representative leaf cuticles (Fitzgerald and Waddington, 1979).

An advantage of this method is the simplicity of collecting and storing faecal material (Fitzgerald and Waddington, 1979). Also, analysis of faecal material does not interfere with the normal feeding habits of the animal. This technique could be useful in distinguishing the dietary habits of two or more herbivores utilizing the same habitat (Mcinnis *et al.*, 1983; Dickman and Huang, 1988). A study on the feeding-habits of deer using faecal microhistology found similar dietary results when rumen and faecal material was compared (Kessler *et al.*, 1981).

Although faecal analysis is useful, its accuracy in providing an accurate evaluation of the diet of an animal has been questioned. A problem associated with feacal analysis is the differential digestion of epidermal tissues upon which species identification are dependent (Fitzgerald and Waddington, 1979; Kessler *et al.*, 1981). With this technique, most of the fragments of plant material ingested may decrease as the digestive processes proceed (Hansen *et al.*, 1973). Furthermore, even when the plants likely to have been eaten are known, preparation of reference plant material is time consuming and often impractical. As plant cuticles are often broken down completely through digestive processes, plant species may be difficult to identify. In addition, herbs, grasses and fragile-leaved tree species having long epidermal cells are often poorly defined post digestion and do not always produce a diagnostic cuticle (Fitzgerald and Waddington, 1979).

The value of microscopic faecal analysis for diet determination is unclear, because the proportion of various plant species present on the microscopic slide to the proportion of plants eaten, the digestive processes, and the effect of sample preparation are unknown (Fitzgerald and Waddington, 1979). Studies using microscopic faecal analysis suggest introducing correction factors for differential digestion in order to improve the accuracy of dietary representation (Kessler *et al.*, 1981). These factors would consider different digestion rates of the plant species, degradation of the cell wall material and the age of the faecal material sampled (Mofareh *et al.*, 1997).

Due to inherent problems encountered when working with ingested plant material, neither rumen, faecal nor esophageal microscopic analysis give consistent assessment of herbivores diet when compared (Kessler *et al.*, 1981; Mohammed *et al.*, 1995). For this reason, direct observation has been used as an alternative method for quantitative analysis of the botanical composition of herbivores diet (Henley *et al.*, 2001).

1.3.2 Direct observation

This technique focuses on the direct observation of plants being eaten by a particular animal at a specific time in its habitat (Oloo *et al.*, 1994; Mohammed *et al.*, 1995). It involves following feeding tracks of animals, identifying and recording plants consumed, and quantifying herbivory (Joubert and Eloff, 1971; Hall-Martin *et al.*, 1982; Oloo *et al.*, 1994; Brown *et al.*, 2003). The technique is considered to be easy and simple, requiring no equipment or surgery. However, there may be difficulties in identifying plant species, particularly when two or more plants are eaten at the same time (Mohammed *et al.*, 1995).

With this method, a bite on a plant species is regarded as the number of twigs which account for the dominating plant preferred (Brown *et al.*, 2003). Several studies have been conducted on the feeding habits of the black rhinoceros. Oloo *et al.* (1994) studied the feeding ecology of black rhinoceros in a dense bushland that comprised their preferred habitat by direct observation. More recently, Ausland and Sveipe (2000), Brown *et al.* (2003), Heilmann *et al.* (2006), Ganqa and Scogings (2007) and IJdema and de Boer (2008) studied the feeding ecology of black rhinoceroses of the GFRR using the direct observation technique.

1.3.3 Feeding ecology of black rhinoceroses of the GFRR

The GFRR was initially used for cattle farming after removal of wildlife. Due to difficult conditions, and with much of the land being unproductive, the area was slowly returned to natural bush and game reserves (http://www.adventurezone.co.za). The reserve is heterogeneous in terms of landscape, vegetation and land use. Rainfall is 430 mm/year with a coefficient of variation of 30%. The vegetation is semi-succulent thorny scrub, comprising dwarf shrub-land, succulent bush-clump savanna and grassland communities. The area comprises communal rangeland, commercial rangeland and nature conservation land, which have different dominant vegetation and degradation status. Degradation in this region has been accompanied by a decrease in edible grasses, succulents and herbaceous species to domestic stock, and an increase in less palatable dwarf shrubs, annual grasses and a reduction in total grass and woody biomass (Tanser and Palmer, 2000).

An increase in less palatable dwarf shrubs may, however, contribute to the increase in the number of herbivores in this reserve. Ausland *et al.* (2002) (cited from Brown *et al.*, 2003) initiated a study of the diet of the black rhinoceros of the GFRR particularly to contribute in their conservation. *Euphorbia bothae, Grewia robusta, Jatropha capensis, Plumabago auriculata* and *Azima tetracantha* were amongst the most frequently selected plants during the study period. Brown *et al.* (2003), further analyzed the diet of the black rhinoceros in two communities, which included the medium *Portulacaria* thicket (MPT) dominated by *Portulacaria afra*, and the short *Euphorbia* thicket (SET) dominated by *Euphorbia bothae*.

Plants species observed by Brown *et al.* (2003) to be the preferred diet of the black rhinoceros of the GFRR in the two communities are presented in the Fig 1.5 and 1.6. This study indicated that the diet varied markedly between the SET and MPT plant communities.

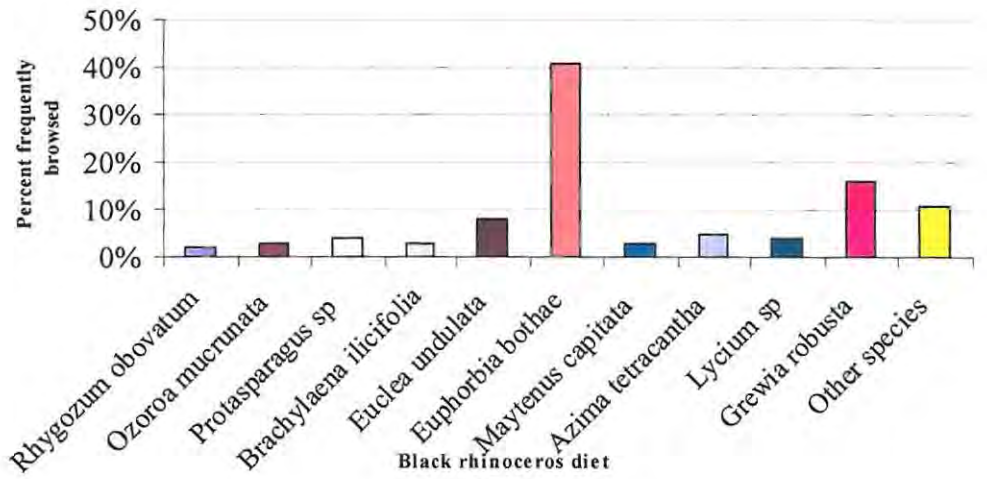

Figure 1.5: Tree and shrubs species most frequently browsed by black rhinoceros in the short *Euphorbia* thicket in the GFRR. The data is expressed as the percentages of bites recorded throughout the observation period (Brown *et al.*, 2003).

This SET study indicated *Euphorbia bothae* (41%) and *Grewia robusta* (16%) as the plant species preferred by the black rhinoceros. These plants contributed 57% of all bites (Brown *et al.*, 2003).

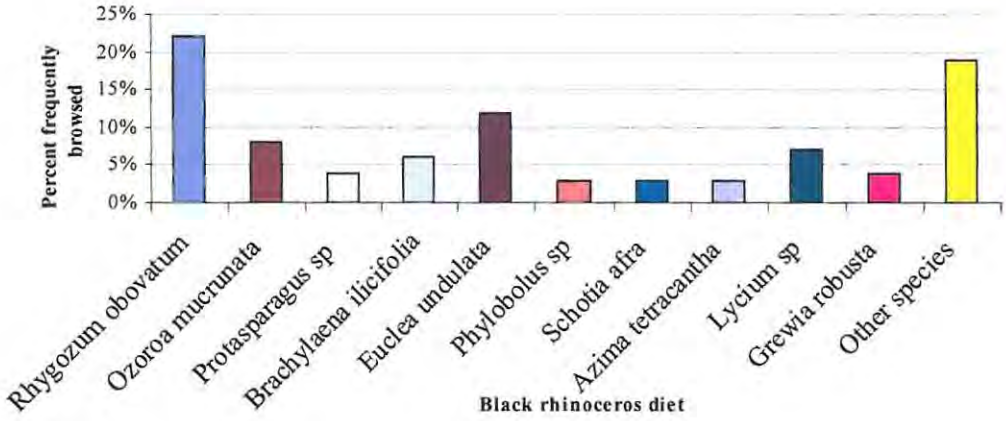

Figure 1.6: Tree and shrubs species most frequently browsed by the black rhinoceros in the medium *Portulacaria* thicket in the GFRR. The data is expressed as percentage of bites recorded throughout the observation period (Brown *et al.*, 2003).

In the MPT, the diet of black rhinoceroses was dominated by *Rhygozum obovatum* (22%), *Grewia robusta* (13%), *Euclea undulata* (12%), *Ozoroa mucrunata* (8%) and *Lycium spp* (7%). These plant species comprised 62% of all of the bites (Brown *et al.*

(2003). In a study conducted by Oloo *et al.* (1994) using backtracking to study the diet of black rhinoceroses in Kenya, the diversity of food plants was 15% greater during wet periods than during the dry period. They indicated that *Acacia spp*, *Phyllanthus spp*, *Carissa spp*, *Tinea aethiopia*, *Euclea spp*, were stable food plant species eaten during both wet and dry periods.

In a study conducted by Henley *et al.* (2001), the results of observation studies were different to faecal analysis and eosophageal extrusa. The observation of bites was affected by differences in bite sizes, possibly leading to incorrect estimation of feeding.

The observation technique has limitations, particularly when studying nocturnal animals and where habitat is not open. Human activity may also affect the normal feeding habitat of the animal. Further, there is an element of danger when studying herbivores such as the black rhinoceros. Therefore, directly observing animals feeding or checking the resulting browsed or grazed vegetation to assess the amount eaten does not always lead to accurate assessment of diet (Fitzgerald and Waddington, 1979).

Determination of diet of a herbivore is problematic in mixed plant habitats, particularly when plants are browsed by different animals (Duncan *et al.*, 2006). Obtaining dietary information through direct observation under these circumstances may not be practical such as when food items are difficult to distinguish. To overcome these problems, molecular methods are being investigated as a potential tool for the evaluation of the diet of herbivores (Bradley *et al.*, 2007).

1.4 Molecular analysis of plants

1.4.1 Characterization of plants by molecular analysis rather than morphology

Morphological characteristics remain the foundation of phylogenetic studies and are used to produce taxonomic identification of species (Kapli *et al.*, 2008). Currently, DNA sequences of various genomes have been used for taxonomic species identifications. These sequences are available to all scientists if deposited in databases such as GenBank (Zhang *et al.*, 2008). The genomic composition, organization and

evolution of higher plants is still incomplete, offering opportunities for plants to be characterized further (Terryn *et al.*, 1999; Martin *et al.*, 2005).

The challenge for molecular taxonomy is the identification of suitable DNA for comparison, which will give informative phylogenetic relationships between species, and will require large sequencing projects to be initiated (Savolainen and Chase, 2003; Martin *et al.*, 2005). Accurate identification of individual plant species requires sufficiently conserved DNA fragments, with specific variation to allow for differentiation (Taberlet *et al.*, 2006).

1.4.2 DNA barcoding

DNA barcoding is a diagnostic technique, which uses short fragments of DNA to distinguish species (Lahaye *et al.*, 2008). When comparing sequences, variations may reveal the evolutionary relationship within the group or between individual species (Hebert *et al.*, 2004; Fromme, 2005). This allows an efficient method for morphologically known species to be recognized and to accelerate the discovery of unknown species. A further intention of DNA barcoding is to use the information of one or a few genes to identify all living species, which will contribute to a wide range of ecological and conservation studies (Moritz and Cicero, 2004; Kress and Erickson, 2008).

Standardization of the DNA barcode is an important factor. The target gene should be highly informative to assign species easily to their taxonomic groups, and should consider order, family, genus and species level. For PCR amplification and sequencing, the target DNA region should have a highly conserved priming site. This is particularly important when a sample contains DNA from multiple species. Where the DNA is highly degraded, the DNA template should not be too long, as it may not amplify (Moritz and Cicero, 2004; Taberlet *et al.*, 2006; Lahaye *et al.*, 2008).

Molecular techniques have been successfully applied to animal taxonomy, but have been more problematic in plants studies, as plant genomes evolve differently (Hebert *et al.*, 2004; Stoeckle and Hebert, 2008). Both plant mitochondrial and chloroplast genomes evolve slowly, and provide limited variations. Researchers have used different plant genomic regions to barcode plants and attempt to find suitable genes

for plant identification. Genes that have been studied include *ITS*, *mat*K and *rbc*L (Kress *et al.*, 2005; Taberlet *et al.*, 2006; Lahaye *et al.*, 2008). In most studies, chloroplast specific genes have been used for plant taxonomy and identifications (Taberlet *et al.*, 2006). These genes have also been investigated for dietary determinations of herbivores (Höss *et al.*, 1992; Poinar *et al.*, 1998; Bradley *et al.*, 2007).

1.4.3 Structural arrangement of the chloroplast genome

The chloroplast genome is a major focus for studies in molecular evolution. The great majority of angiosperm chloroplast genomes studied consist of multiple copies of homogenous circular double-stranded DNA molecules, ranging in size from 135 to 160 kilo base pair (kb). Chloroplast DNA encodes a complete set of ribosomal RNAs, six tRNAs and many protein genes (Curtis and Clegg, 1984; Masood *et al.*, 2004).

The overall structure of the chloroplast genome is generally well conserved, with a number of mutations having been observed such as inversions, translocations and insertions/deletions as well as base substitutions (Curtis and Clegg, 1984). The manner in which the chloroplast genome is conserved with respect to size, structure and linear sequence of genes, suggests that any changes in structure, arrangement or content may have significant phylogenetic implications. Different portions of the genome evolve at different rates, with the non-coding regions evolving more rapidly than the coding regions (Masood *et al.*, 2004).

1.4.4 Coding sequence of the chloroplast genome and its function

There is considerable debate on which fragment of DNA is most suitable to infer phylogenetic relationships among plant species.

The plastid *trnH-psbA* intergenic spacer region has demonstrated a high percentage sequence divergence when compared to *rpl36-rpf8* and *trnL-F* regions. Universal priming sites of this region are known and its existing sequence database covers angiosperms, gymnosperms, ferns, mosses and liverworths and not nonflowering plants. Limitations to this gene are extensive length variations of 465 bp, with high numbers of insertions/deletions, making alignment difficult (Kress *et al.*, 2005).

Taberlet *et al.* (2006) used the chloroplast *trnL* intron for plant identification. Primer pairs used to amplify the entire region are well conserved in many plant species. A sufficiently extensive *trnL* sequence database is available for comparison studies. This region, along with the P6 loop could be suitable in amplifying highly degraded DNA from processed food samples, forensic samples, diet analysis from feaces and analyzing ancient DNA. The main drawback in using this region is that it does not allow plant identification to the species level when compared to other plant sequences in the GenBank database (Taberlet *et al.*, 2006).

Lahaye *et al.* (2008) used the *matK* gene to identify flowering plant species. Useful properties of this gene were reported in the study of Liang (1997), which includes resolution size of 1 500 bp, variable regions shown in the first and second codon positions, low transition and transversion ratio and the conserved 3' end region is useful in resolving phylogeny. The 3' region and the less conserved 5' region provide two characteristics that could be used at different taxonomic levels. This gene has only been tested on flowering plants.

A gene that has been extensively mapped within the chloroplast genome is the ribulose bisphosphate carboxylase large subunit (*rbc*L) (Curtis and Clegg, 1984). The plastid *rbc*L gene is located in the large single copy (LSC) region of the chloroplast genome and encodes for the large subunits of the multifunctional enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) (Curtis and Clegg, 1984; Gielly and Taberlet, 1994; Masood *et al.*, 2004). This is a key enzyme responsible for plant carbon dioxide fixation. The enzyme is comprised of eight large subunits (LSU) and eight small subunits (SSU) totalling approximately 500 kDa. The subunit contains the active site for the RUBISCO activity (Curtis and Clegg, 1984; Race *et al.*, 1999).

1.4.5 Molecular analysis of plants in animal faeces

The *rbc*L gene has been investigated in a number of studies to determine the botanical composition of animal faeces. Höss *et al.* (1992) analyzed a 356 bp DNA fragment to determine the plant composition of brown bear droppings. This technology was also applied by Poinar *et al.* (1998) in identifying the diet of the extinct ground sloth. Poinar *et al.* (2001) identified different plant species in 2 000 year old Native

American faeces found in a cave in Texas. Bradley *et al.* (2007) determined the diet of primates by molecular analysis of their faeces, sequencing the *rbc*L gene and *ITS*-2 region. This approached is discussed further in chapter three.

1.5 Dietary requirements of herbivores

Although availability of diet is important for herbivores, quality of food is also a requirement that could have a major impact on the survival and the reproduction of herbivores (Muya and Oguge, 2000; Oliver, 2007).

The feeding behaviour of African herbivores has been well researched. This has been done to set up suitable areas for conservation especially where competition between herbivores is high (Hall-Martin *et al.*, 1982; Muya and Oguge, 2000; de Garine-Wichatitsky *et al.*, 2004). These studies have indicated that herbivores favour specific plant species with respect to their grazing and browsing habits (Mabinya, 2002).

Natural abundances, morphological characteristics and nutritional factors determine diet preferences in most herbivores. Seasonal changes may affect the feeding habits of herbivores due to changes in the availability of different plants. Larger herbivores are less selective than small herbivores when feeding as they consume a greater amount of food to obtain sufficient nutrients required (Oliver, 2007). However, choice of foods is a limitation to herbivores in captivity (Cohn, 1988).

1.6 Health promoting factors in wildlife populations

Research indicates that rhinoceroses utilize food based on availability, season, quantity and quality. Availability of food sources to herbivores also depends on the extent to which animals can utilize them for their growth (Muya and Oguge, 2000; Oliver, 2007). Muya and Oguge, (2000), investigated the browse availability and quality of the diet of the black rhinoceros. They reported that black rhinoceroses feed on plants with low secondary constituents and high fiber contents.

Secondary plant compounds form part of the diversity of wildlife nutrition (Dierenfeld, 1997). Differences in the nutritional value of secondary compounds are hypothesized to be responsible for health and enhancing reproduction (Helary, 2007). Graffam *et al.* (1997) suggested that an understanding of black rhinoceroses nutrition, by assessing the chemical composition of the food they consume should be a priority.

Harley *et al.* (2004) demonstrated high uric acid, ATP and tyrosine levels in the red blood cells collected from the wild black and white rhinoceroses that were immobilized for translocation. Tyrosine and urate, both exhibit similar scavenging capacities of oxygen free radicals. Dierenfeld (1994) and Dierenfeld *et al.* (1988) demonstrated low vitamin E content in the browse of captive black rhinoceros compared to free ranging black rhinoceroses, which could be linked to many diseases observed in captive black rhinoceroses. These studies suggest a thorough investigation of health-promoting factors could be beneficial to both captive and wild black rhinoceroses, and may limit the number of deaths associated with feeding deficiencies (Dierenfeld *et al.*, 1988). In the present study, the antioxidant capacity of the browse of the black rhinoceros was investigated.

1.6.1 The principle of protection by the diversity of antioxidants

According to Halliwell (2005), "antioxidants may be defined as any chemical substances that when present at low concentrations compared with those of the targeted molecules, delays or prevent the oxidation of those substrates". This definition covers both enzymatic and nonenzymatic antioxidant substances (Sies, 1993).

The role of antioxidants is to guard against reactive free radical species that are generated in normal biological systems (Sies, 1993). Free radicals are chemical substances that contain one or more unpaired electrons in their outer orbital. They are continuously produced in cells during metabolic processes. Free radicals can be positively charged, negatively charged or neutral and have low molecular weight (Cheeseman and Slater, 1993; Nordberg and Arnér, 2001). They can either donate or remove an electron from a normal molecule (Cheeseman and Slater, 1993).

Mavi *et al.* (2004) noted that the most reactive radical species are those derived from oxygen and nitrogen. Oxygen derived free radicals have been well studied (Cheeseman and Slater, 1993; Martínez-Cayuela, 1995; Nordberg and Arnér, 2001). Cytoplasmic molecules, cytoplasmic proteins, membrane enzymes, peroxisomes and the mitochondrial electron transport system are sources of reactive oxygen species (Martínez-Cayuela, 1995). Superoxide and hydrogen peroxide are less reactive oxygen molecules, while hydroxyl radicals are highly reactive. It is difficult to monitor the involvement of reactive species in the development of disease, because the majority of these reactive oxygen species have different half-lives (Sies, 1993; Cheeseman and Slater, 1993). Nature has developed a variety of antioxidants by which free radicals can be scavenged in biological systems (Nordberg and Arnér, 2001).

Antioxidants protect the body from free radicals through prevention, interception and repair mechanisms (Sies, 1993). The most important defense mechanism is where both enzymatic and non-enzymatic reactions are active (Martínez-Cayuela, 1995; Pulido *et al.*, 2000). Non-enzymatic antioxidants include dietary antioxidants such as β-carotene, glutathione stimulating hormones, vitamin C, uric acid, albumin, bulirubin, and vitamin E (Martínez-Cayuela, 1995). These antioxidants are widely distributed in biological systems. Antioxidants molecules react with oxygen free radicals by donating a hydrogen ion or an electron (Martínez-Cayuela, 1995; Pulido *et al.*, 2000; Mosquera *et al.*, 2007). These antioxidants may transfer radicals away from a target area to a compartment in a cell where oxidative challenge is less damaging. This is achieved by transferring the oxidizing agent from a hydrophobic to an aqueous phase. Antioxidants are then capable of interacting with hydrophobic compounds for their own regeneration (Sies, 1993).

1.6.2 Antioxidants from plants

According to Antolovich *et al.* (2002), natural antioxidant mechanisms in mammalian systems may be inefficient. For this reason, dietary antioxidants are becoming popular as supplements. Their study indicated that the dietary intake of antioxidants is important in instances where the development of disease has been caused by dietary deficiencies.

Plants can be a good source of new anti-oxidant compounds with health-promoting properties (Silvia *et al.*, 2002; Mosquera *et al.*, 2007). The intake of plant antioxidants is related to reducing the risk of many degenerative diseases (Moure *et al.*, 2001). This has increased research on plant antioxidants (Sies, 1993; Moure *et al.*, 2001; Silvia *et al.*, 2002). As a result, many plants investigated were identified as having potential antioxidants activities (Katalinic *et al.*, 2006; Tawaha *et al.*, 2007; Mosquera *et al.*, 2007).

1.6.3 Structurally diverse secondary phenolic compounds

Plants synthesize thousands of different chemicals characterized by hydroxylated aromatic rings structures. The ability of plants to produce such an abundance of these compounds depends on the continuous evolution of genes by mutation and subsequent adaptation to specific functions and environmental changes. Phenolic compounds accumulate in plant tissues, enabling plants to adapt to changes in environmental conditions such as browsing herbivores, pathogens, UV radiation and pollution (Hutzler *et al.*, 1998; Boudet, 2007). Many of these plant compounds are highly toxic and are often stored in specific organs of the plants. Some may be reversibly degraded and fed into basic metabolism. Although generally common, certain of these compounds are restricted to closely related plant species (Stahl, 2003).

Phenolics from natural resources have received attention in many areas of plant research (Duan *et al.*, 2006; Katalinic *et al.*, 2006; Tawaha *et al.*, 2007). These compounds are derived from trans-cinnamic acid, formed by deamination of L-phenylalanine (L-Phe) by L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) (Boudet, 2007). These compounds are synthesized in plants via a common biosynthetic pathway and their precursors are derived from the shikimic-phenylpropanoid pathway (Thompson, 2004).

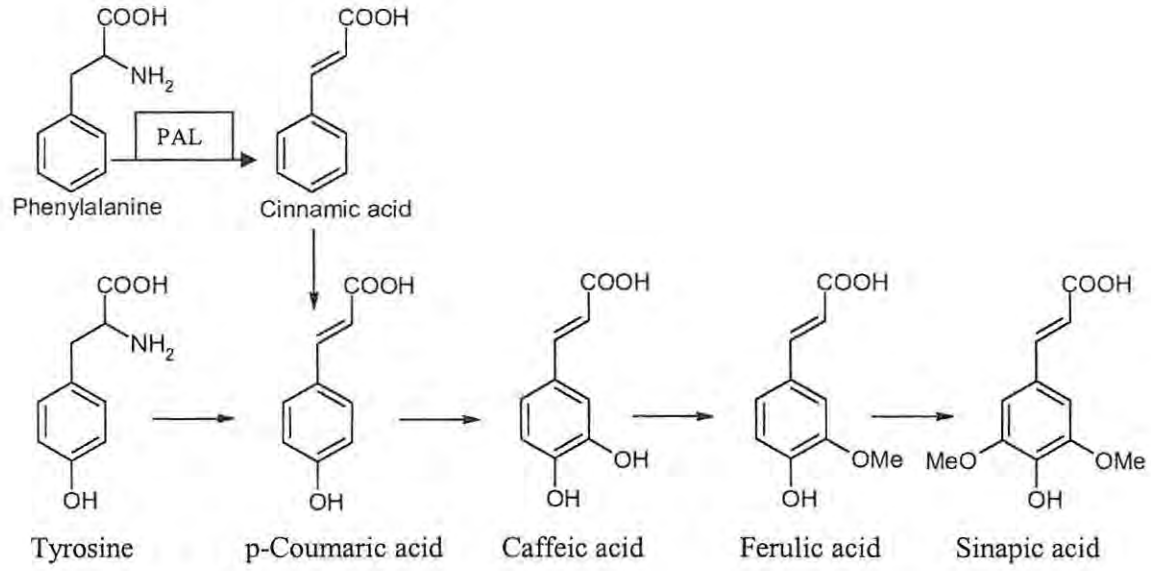

Figure 1.7: Biosynthesis of phenolic compounds via the shikimate pathway (Paixão *et al.*, 2007).

Within plant families, more than 8000 phenolic compounds, with distinct functional groups, have been identified and are categorized into 10 subclasses. Phenolics range from simple molecules (e.g. phenolic acids with a single ring structure) to biphenyls and flavonoids. Polyphenolics are another abundant group of compounds. Phenolic compounds are often esterified with sugars and other chemicals such as quinic acid to increase their solubility, and to prevent their enzymatic and chemical degradation (Thompson, 2004).

1.7 Objective of this research

Plants browsed by the black rhinoceros of the GFRR have been studied through observation methods. We suggest that the *rbc*L gene could be used as a marker to identify plants in the black rhinoceroses dung. A *rbc*L gene sequence database of GFRR plants was initially developed. These plants served as a reference collection against which unknown plants found in black rhinoceros dung could be identified by analyzing their *rbc*L gene sequences. For proof of concept, amplification, cloning and sequencing techniques were used for plant identification in a black rhinoceros dung sample. Further, the *rbc*L gene was amplified from four seasonal black rhinoceros dung samples and sequenced using the FLX genome sequencing technology.

Plant antioxidant capacities of black rhinoceros browse were studied. The free radical scavenging activities, ferric reducing antioxidant capacities and the total phenolic contents of the plants were assessed.

CHAPTER TWO
*rbc*L GENE SEQUENCES OF PLANTS FROM THE GREAT FISH RIVER RESERVE

2.1 Introduction

The chloroplast *rbc*L gene was used as the target DNA to identify plants from the GFRR. Plants most likely to form part of the black rhinoceros diet were collected, and the partial *rbc*L sequences were used as a reference database for comparison with sequences generated from analysis of black rhinoceros dung.

The *rbc*L gene is abundant due to the high number of chloroplasts in plants. The gene is present as a single copy per chloroplast genome (Gutteridge and Gatenby, 1995). The size of this gene is variable, ranging between 1428 and 1433 bp, with variable regions found towards the 3' end. The *rbc*L gene is highly conserved and for this reason has been used for phylogenetic studies. The gene allows for the identification of plant families, but not always to a genus or species level (Gielly and Taberlet, 1994; Poinar *et al.*, 1998; Taberlet *et al.*, 2006).

A reason the *rbc*L gene was chosen for plant identification was that an extensive database exists for its sequences (Anderson and Buckland, 2008). Although certain of the plants to be sequenced in this study are not in the GenBank, these sequences will be deposited in due course and add to the existing database. The *rbc*L gene has no introns in higher plants, which allows for sequence alignment (Curtis and Clegg, 1984). Further, as this gene is plant specific, it can be used to differentiate DNA from a complex origin. This approach has been used by a number of researchers to establish diet of herbivores (Höss *et al.*, 1992; Poinar *et al.*, 1998; Hofreiter *et al.*, 2003; Bradley *et al.*, 2007).

2.2 Material and methods

2.2.1 Reagents

DNeasy Plant Mini and QIAprep Spin Miniprep Kits were purchased from Southern Cross Biotechnology (South Africa). Liquid nitrogen was obtained from Afrox (South Africa). M13 forward and reverse primers were synthesized by Inqaba Biotechnologies (South Africa). O'GeneRuler 1kb DNA Ladder Plus, *E. coli* FastMedia LB agar IPTG/X-Gal, *E. coli* FastMedia LB Liquid Amp, 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal), isopropyl β-D-1-thiogalactopyranoside (IPTG) and Topvision LE GQ agarose were purchased from Inqaba biotechnologies (South Africa). pGem-T Easy Vector System II and GoTag® PCR Core System II were purchased from Whitehead Scientific group (South Africa). 3-(N-morpholino) propanesulfonic acid (MOPS) and ampicillin were purchased from Roche (South Africa). Silica gel, Bacto®-tryptone, Bacto®-yeast extract, agar bacteriological, D-glucose monohydrate, sodium chloride and potassium chloride, hydrochloric acid, N'N dimethyl formamide, propan-2-ol, chloroform, isoamyl alcohol, ethanol, glycerol, sodium dodecyl sulfate were purchased from Merck (South Africa). Potassium acetate, trizma® base, calcium chloride, glacial acetic acid, ethylenediaminetetraacetic acid, manganese chloride, cetyl trimethyl ammonium bromide, magnesium sulfate and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Germany).

2.2.2 Collection and preservation of plant material

Twenty-three plant samples (Table 2.1), were collected from the Great Fish River Reserve complex in the Andries Vosloo Kudu reserve. Plant samples collected were partly based on the observation study of Brown *et al.* (2003) of the diet of the black rhinoceros in the medium *Portulacaria* thicket. Collected leaves were placed separately in ziploc plastic bags (10 x 25 cm) with silica gel distributed between the layers of the leaves and stored at –20°C. Plant samples were identified at the Selmer Schonland Herbarium in Grahamstown. Plants names were also confirmed using the International Plant Names Index (http://www.ipni.org/ipni/plantnamesearchpage.do).

Table 2.1: Inventory of plant species collected from the GFRR for partial *rbc*L gene sequencing for the purpose of generating a GFRR specific *rbc*L gene sequence database.

Number	Plant family	Genus	Species
1	**Anacardiaceae**	*Ozoroa*	*mucrunata*
2	**Anacardiaceae**	*Rhus*	*pterota*
3	**Apocynaceae**	*Carissa*	*haematocarpa*
4	**Apocynaceae**	*Carissa*	*bispinosa*
5	**Asparagaceae**	*Protasparagus*	*suaveolens*
6	**Asparagaceae**	*Protasparagus*	*crassicladus*
7	**Asparagaceae**	*Protasparagus*	*africanus*
8	**Asteraceae**	*Brachylaena*	*ilicifolia*
9	**Celastraceae**	*Gymnosporia*	*capitata*
10	**Celastraceae**	*Maytenus*	*capitata*
11	**Celastraceae**	*Maytenus*	*nemorosa*
12	**Celastraceae**	*Putterlickia*	*pyracantha*
13	**Ebenaceae**	*Euclea*	*undulata*
14	**Euphorbiaceae**	*Euphorbia*	*bothae*
15	**Euphorbiaceae**	*Jatropha*	*capensis*
16	**Euphorbiaceae**	*Phyllanthus*	*verrucosus*
17	**Plumbaginaceae**	*Plumbago*	*auriculata*
18	**Portulacaceae**	*Portulacaria*	*afra*
19	**Rhamnaceae**	*Scutia*	*myrtina*
20	**Rubiaceae**	*Codia*	*rudis*
21	**Salvadoraceae**	*Azima*	*tetracantha*
22	**Solanaceae**	*Lycium*	*ferocissimum*
23	**Stoculaceae**	*Grewia*	*robusta*

2.2.3 DNA isolation and analysis

DNA from fresh silica-gel dried leaves (0.1 g) was extracted using a DNeasy Plant Mini Kit as per the manufacturer's instructions (Appendix A). The plant samples analyzed are presented in Table 2.1. The CTAB extraction procedure, as used by Doyle and Doyle, (1987) (cited from Bulani (2007), was used in extracting genomic DNA from *Phyllanthus verrucosus*. Extracted DNA was quantified using a Biowave S2100 Diode Array Spectrophometer (Biochrom) at 260/280 nm. DNA was electrophoresed at 12 V/cm for 30 min in 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in 1 X Tris-Acetate-EDTA (TAE) buffer. Extracted DNA was compared to a 0.75 – 20 kb DNA ladder. Electrophoresed gels were visualized under translumination radiation using a gel documentation system (UviproChemi, United Kingdom). Extracted DNA samples were stored at -20°C.

2.2.4 Polymerase chain reaction (PCR)

2.2.4.1 Primer design for the amplification of the partial rbcL gene

Known *rbc*L gene sequences were used to design the reverse primer. The forward primer used for the amplification of *rbc*L gene was originally designed by Zurawski (DNAX Research Institute). This primer is composed of the first 20 bases of the *rbc*L gene. The primers are listed in Table 2.2.

Table 2.2: The pair of primers used for the amplification of the *rbc*L gene (802 bp).

Name	5' → Primer 3'	Nucleotides
1For	ATGTCACCACAAACAGAGAC	20
*rbc*L rev 802	CATGCATTACGATAGGAACTC	21

2.2.4.2 Amplification of the partial chloroplast rbcL gene

The *rbc*L gene (802 bp) was amplified from all the plant samples listed in Table 2.1 using GoTag® PCR Core System II. PCR was performed in a total reaction mixture of 15 µl, consisting of 1 X Go*Taq*® Flexi Buffer, 0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, 200 ng of DNA template and 0.8 µM of each primer. BSA was added to the PCR if required as shown in Appendix D. Go*Taq*® Flexi DNA polymerase of 1.0 U was added per reaction mix prior to initiation. Plasmid DNA of 323 bp provided with the Go*Taq*® PCR Core System II kit, was used as a positive control. The negative control contained no DNA template. The PCR reaction mixture is further described in Appendix C.

PCR was performed in a Multigene II thermal cycler (Labnet International, Inc.) with the following profile: initial denaturation of 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 1 min, DNA polymerization at 72°C for 1 min, and a final extension step at 72°C for 5 min. The final hold temperature was 4°C. The amplified products were visualized using agarose gel electrophoresis to verify the presence and the size of amplified products in comparison to a standard DNA molecular weight marker. The amplicons were electrophoresed at 12 V/cm for 25 min using 2% agarose gel stained with 0.4 µg/ml

ethidium bromide in 1 X TAE buffer. The gels were visualized under gel documentation system (UviproChemi, UK). Amplicons were stored at –20°C.

2.2.5 Cloning and screening of the partial 802 bp *rbc*L gene

2.2.5.1 Ligation reactions

The 802 bp *rbc*L gene amplification products were cloned directly into a TA cloning vector using pGem-T Easy Vector System II, following the manufacturer's protocol. A 542 bp plasmid insert positive control (supplied by the manufacturer), negative and background controls were performed. Ligation reactions were carried out using 10 µl reaction volumes containing 5 µl of 2 X rapid ligation buffer, 2 µl of 5 ng/ml of the pGem-T Easy Vector, 2 µl of the PCR product and 1 µl of T4 DNA ligase. Ligation reactions were incubated at 4°C for 12 h.

2.2.5.2 Transformation of the E. coli competent cells

The JM109 *E. coli* strain cells, made chemically competent by the procedure described in Appendix E, were transformed by the vector constructs, following the procedure described in Appendix G. The transformants were grown at 37°C for 12 h using the X-gal/IPTG LB agar plates prepared as described in Appendix F2. White colonies containing plasmid inserts were selected.

2.2.5.3 Plasmid preparation and purification

Recombinant colonies were cultured separately in 1 ml LB broth in the presence of 100 µg/ml ampicillin. Cultures were grown by incubation at 37°C with continuous shaking at 180 rpm for 12 h. Easy prep (Bergman and Auer, 1993), was used for the screening of the plasmid constructs from all cultures as described in Appendix H. Plasmid DNA fragments were then electrophoresed at 12 V/cm for 25 min using 2% agarose gels stained with a 0.4 µg/ml ethidium bromide in 1 X TAE buffer. The correct size of the plasmid DNA was confirmed by comparison to a 0.75 – 20 kb DNA ladder. Plasmid DNA was then purified by an alkaline lysis method using a QIAprep Spin Miniprep kit, following the manufacturer's protocol (Appendix I). Plasmid DNA was electrophoresed at 12 V/cm for 25 min using 2% agarose gels stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer to confirm the correct size. DNA was compared to 0.75 – 20 kb O'Gene Ruler DNA Ladder plus.

Electrophoresed gels were visualized using a gel documentation system (UviproChemi, UK). The purified plasmid DNA from all samples was stored at –20°C.

2.2.5.4 PCR amplification of the plasmid DNA

Plasmid PCR was carried out following the protocol as described in Appendix J. A pair of M13 primers (Table 2.3) flanking the region of the insert and complementary to the plasmid DNA was used for the amplification of the *rbc*L gene. The thermal cycling parameters and the screening of the *rbc*L gene inserts were analyzed as described in section 2.2.4.2.

Table 2.3: M13 primers used for the screening of partial 802 bp *rbc*L gene.

Name	5' Primer 3'	Nucleotides
Forward M13	GTTTTCCCAGTCACG	15
Reverse M13	CAGGAAACAGCTATGACCATGA	22

2.2.6 DNA Sequencing

Double stranded plasmid DNA was sequenced in both directions at Inqaba Biotechnologies (Hatfield, South Africa) using a pair of pGem-T Easy vector primers, SP6 and T7 using a 3130 XL Genetic Analyzer (Applied Biosystems, Hitachi).

2.2.7 Analyses of sequences

Forward and reverse sequences obtained from each plant were aligned and edited using Bioedit software. The plant *rbc*L sequences were compared to sequences at GenBank, provided by the National Center for Biotechnology Information, using the BLASTn server program (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was done using the ClustalW algorithm from NCBI. Tree phylogeny of all plant sequences was constructed using the MEGA program.

Protein translation of sequences was done using Microsoft Word Template Software (MBCS 1.2 dot).

2.3. Results

2.3.1 Genomic DNA of the plants collected from the GFRR

Genomic DNA from seven of twenty-three plants species are shown in Fig 2.1 below.

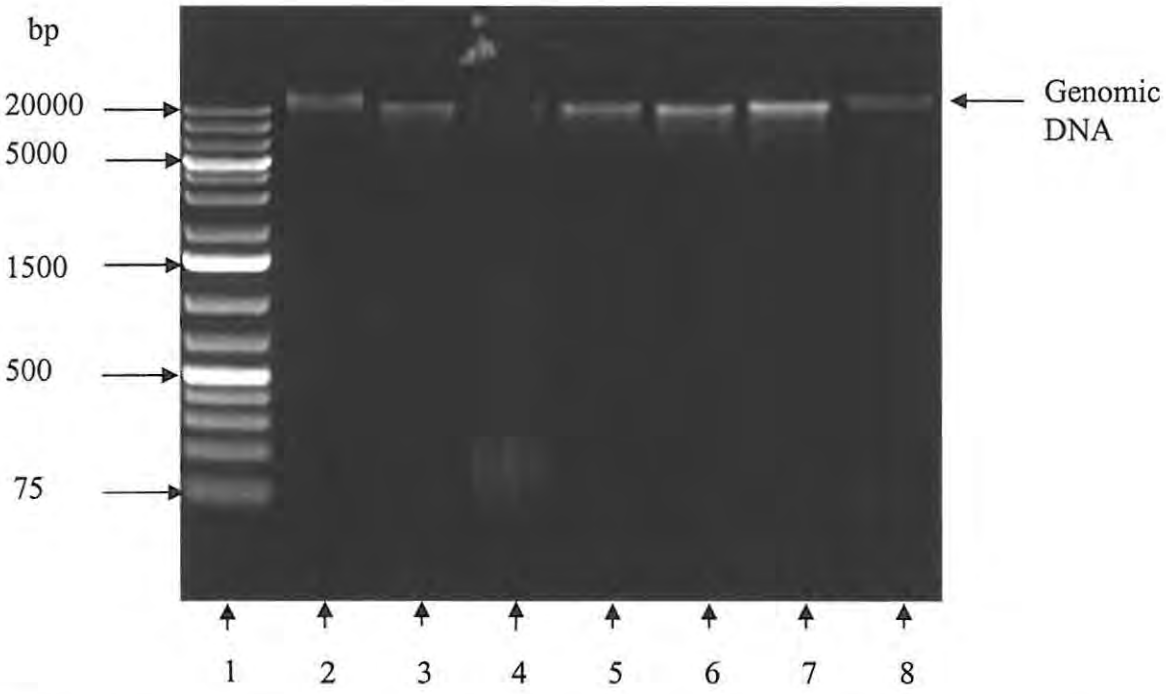

Figure 2.1: The genomic DNA extracts of selected plant species observed to be part of the diet of black rhinoceros of the GFRR. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: *Azima tetracantha*, Lane 3: *Plumbago auriculata*, Lane 4: *Coddia rudis*, Lane 5: *Protasparagus crassicadus*, Lane 6: *Protasparagus suaveolens*, Lane 7: *Protasparagus africanus* and Lane 8: *Phyllanthus verrucosus*.

The size of this DNA is greater than 20 kb, with the size of chloroplast genomic DNA expected in the range between 135 and 160 kb.

2.3.2 Amplification of the partial *rbc*L gene from plants

Partial amplification (802 bp) of the *rbc*L gene from the twenty-three plant samples was performed and the selected PCR products are shown in Fig. 2.2.

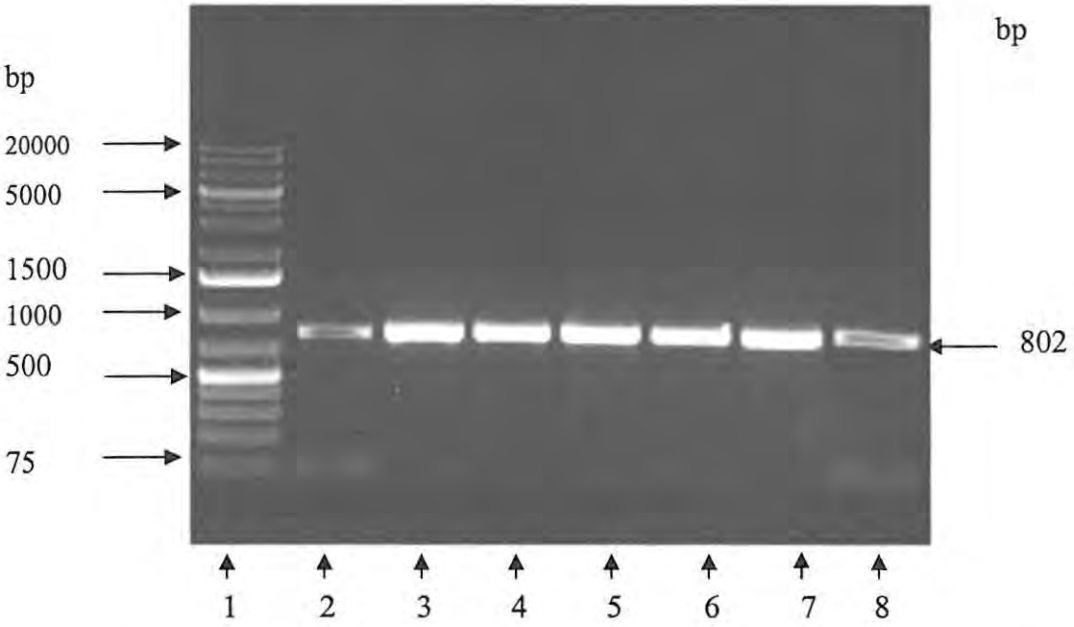

Figure 2.2: Amplification of the 802 bp *rbc*L gene from plants observed to be part of the diet of the black rhinoceros of the GFRR. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer. Lane1: 0.75 - 20 kb DNA ladder, Lane 2: *Azima tetracantha*, Lane 3: *Plumbago auriculata*, Lane 4: *Coddia rudis*, Lane 5: *Protasparagus crassicadus*, Lane 6: *Protasparagus suaveolens*, Lane 7: *Protasparagus africanus* and Lane 8: *Phyllanthus verrucosus*.

The PCR products of the seven plants are shown.

2.3.3 Plasmid PCR for screening of the partial *rbc*L gene

Selected clones from each plant sample were amplified for the 802 bp *rbc*L gene using M13 primers as shown in Fig. 2.3.

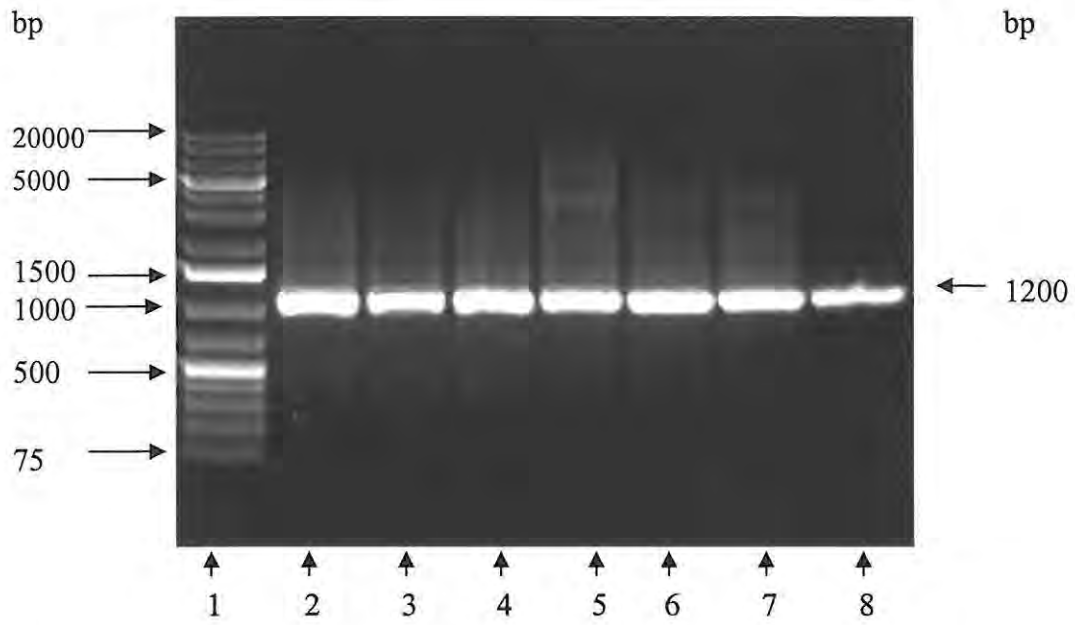

Figure 2.3: Amplification of the 802 bp *rbc*L gene insert cloned into the pGem-T Easy vector using M13 primers. The DNA was electrophoresed at 12V/cm for 25 min using 1% agarose gel stained with 0.4 µg/ml ethidium bromide in 1X TAE buffer. Lanel: 0.75 - 20 kb DNA ladder, Lane 2: *Azima tetracantha*, Lane 3: *Plumbago auriculata*, Lane 4: *Coddia rudis*, Lane 5: *Protasparagus crassicadus*, Lane 6: *Protasparagus suaveolens*, Lane 7: *Protasparagus africanus* and Lane 8: *Phyllanthus verrucosus.*

The PCR products of the seven plants shown are of high intensity. The size of the plasmid PCR product is 1.2 kb due to additional sequence from the pGem-T Easy vector.

2.3.4 Comparison of *rbc*L sequences to the GenBank sequences database

A total of twenty-three *rbc*L gene sequences (802 bp) from individual plant samples from the GFRR were aligned and edited using Bioedit software program. Partial *rbc*L gene (802 bp) sequences were compared to the plant *rbc*L sequences from the GenBank database using BLASTn program as shown in Table 2.4.

Table 2.4: Comparison of the partial *rbc*L gene sequences of plants from GFRR with sequences in the GenBank database.

Plants from the GFRR		**Best GenBank blast search results**			
Plant family	**Genus and Species**	**Plant family**	**Accession no.**	**Genus and species**	**% Match**
Anacardiaceae	*O. mucrunata*	**Anacardiaceae**	AY510148 AY510146	*C. coggygria* *B. javanica*	99
Anacardiaceae	*R. pterota*	**Anacardiaceae**	AM23484811	*R. lucida*	99
Apocynaceae	*C. bispinosa*	**Apocynaceae**	X919738	*C. bispinosa*	99
Apocynaceae	*C. haematocarpa*	**Apocynaceae**	AJ419738	*C. bispinosa*	99
Celastraceae	*M. nemorosa*	**Apocynaceae**	AJ419738	*C. bispinosa*	99
Euphorbiaceae	*P. verrucosus*	**Apocynaceae**	AJ419738	*C. bispinosa*	99
Asparagaceae	*P. crassicladus*	**Asparagaceae**	AM234843	*A. capensis*	99
Asparagaceae	*P. suaveolens*	**Asparagaceae**	AM234843	*A. capensis*	97
Asparagaceae	*P. africanus*	**Asparagaceae**	AM234843	*A. capensis*	99
Asteraceae	*B. ilicifolia*	**Asteraceae**	EU385023	*T. camphoratus*	100
Celastraceae	*M. capitata*	**Celastraceae**	AY380352	*M. arbutifolia*	99
Celastraceae	*P. pyracantha*	**Celastraceae**	AM234959	*P. pyracantha*	98
Celastraceae	*G. capitata*	**Celastraceae**	AM234955	*G. buxifolia*	99
Ebenaceae	*E. undulata*	**Ebenaceae**	Z80186	*E. natelansis*	99
Euphorbiaceae	*E. bothae*	**Euphorbiaceae**	AY794824	*E. abyssinica*	99
Euphorbiaceae	*J. capensis*	**Euphorbiaceae**	AM234978	*J. capensis*	100
Plumbaginaceae	*P. auriculata*	**Plumbaginaceae** **Polygonaceae**	EU002283 Y16906 M77702	*P. auriculata,* *P. capensis* *R. x cultorum,*	100
Portulacaceae	*P. afra*	**Portulacaceae**	AM235080	*P. afra*	100
Rubiaceae	*C. rudis*	**Rhamnaceae**	AJ390070	*Rhamnus lycioides*	98
Rhamnaceae	*S. myrtina*	**Rubiaceae**	AJ286695	*C. rudis*	99
Salvadoraceae	*A. tetracantha*	**Salvadoraceae**	U36782	*A. tetracantha*	99
Solanaceae	*L. ferocissimum*	**Solanaceae**	AM235152	*L. ferocissimum*	99
Tiliaceae	*G. robusta*	**Tiliaceae**	AJ233152	*G. occidentales*	99

Three plants were identified correctly with a 100% match. The *B. ilicifolia* sequence gave a 100% match to *T. camphoratus*, which was incorrect. Comparisons of known plant sequences based on correct taxonomic identification were assessed and are summarized in Table 2.5.

Table 2.5: Comparison of GFRR plant *rbc*L sequences with the GenBank sequence database for the assessment of full taxonomic identification.

Plant taxonomic identifications				
Correctly identified to family, genus and to species level	Identified to family and genus level	Identified to family level	Incorrectly identified	Total plants correctly identified to family level
7	9	3	4	19

Seven plant sequences gave full taxonomic identification to family, genus and species level. Nine plant sequences gave the correct family and genus but incorrect species. Three plant sequences were identified to family level. Four sequences of the twenty-three plants were incorrectly identified, at family, genus and species level. Nineteen plant sequences were correctly identified to family level, and sixteen plants were correctly identified at the genus level.

2.3.5 Genetic diversity between the collected plants from the GFRR

The genetic diversity of the plants collected from the GFRR was assessed by constructing a phylogenetic tree, based on the *rbc*L gene sequences, using the MEGA program and is presented in Fig. 2.4.

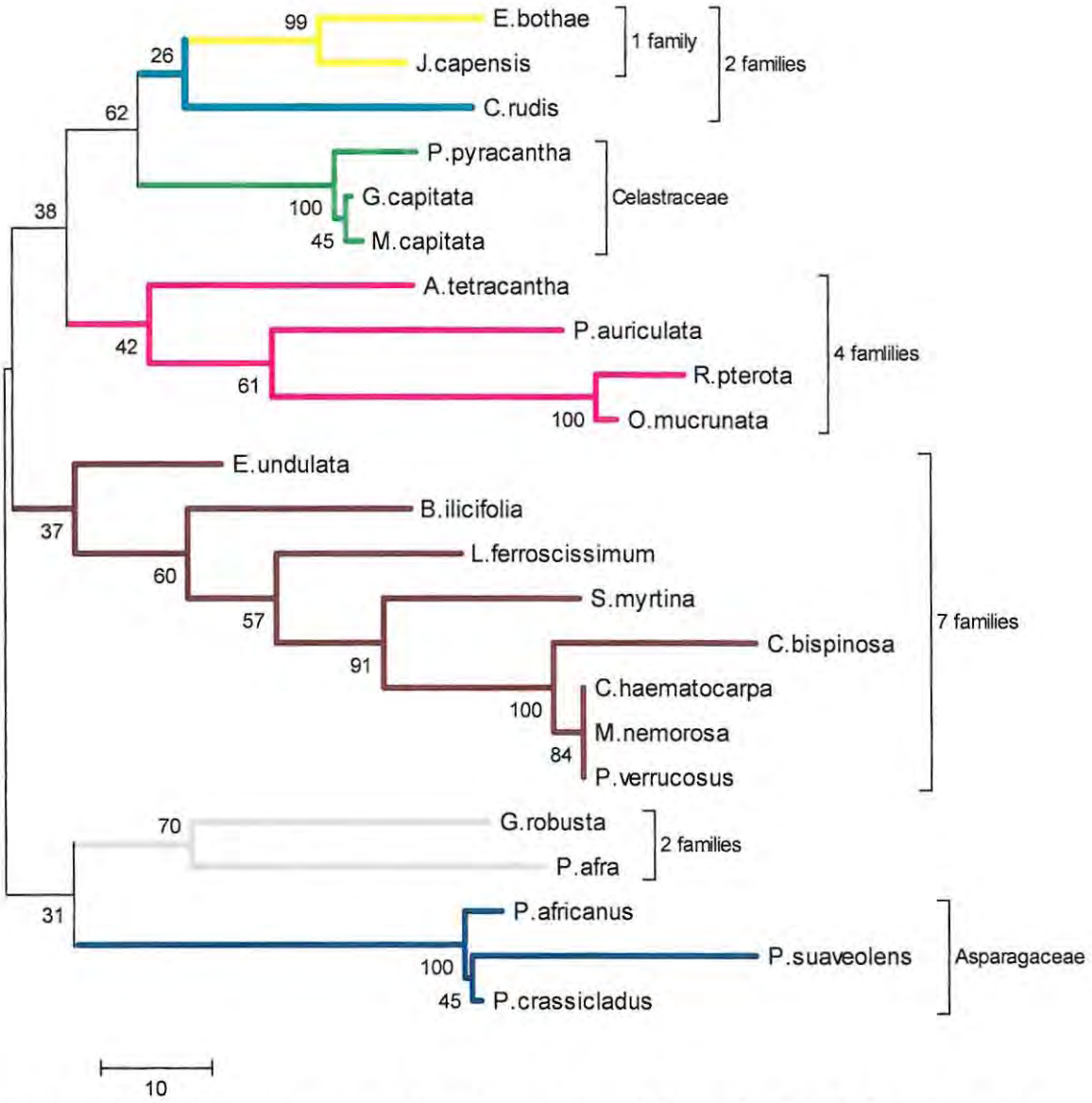

Figure 2.4: Phylogenetic tree of the plants collected from the GFRR based on the *rbc*L gene.

The *rbc*L gene of the majority of the plants investigated was highly conserved. From the phylogenetic tree, the molecular data generated did not always show the expected clustering of plants. For example, *P. verrucosus* from the Euphorbiaceae family diverged and was clustered in close proximity to plants from Apocynaceae and Celastraceae family.

2.3.6 Alignment of the amino acid sequences

The amino acid sequences derived from the translation of the nucleotide sequences of the *rbc*L gene of *M. capitata*, *G. capitata* and *P. pyracantha* from the family Celastraceae, were further assessed as depicted in fig. 2.5.

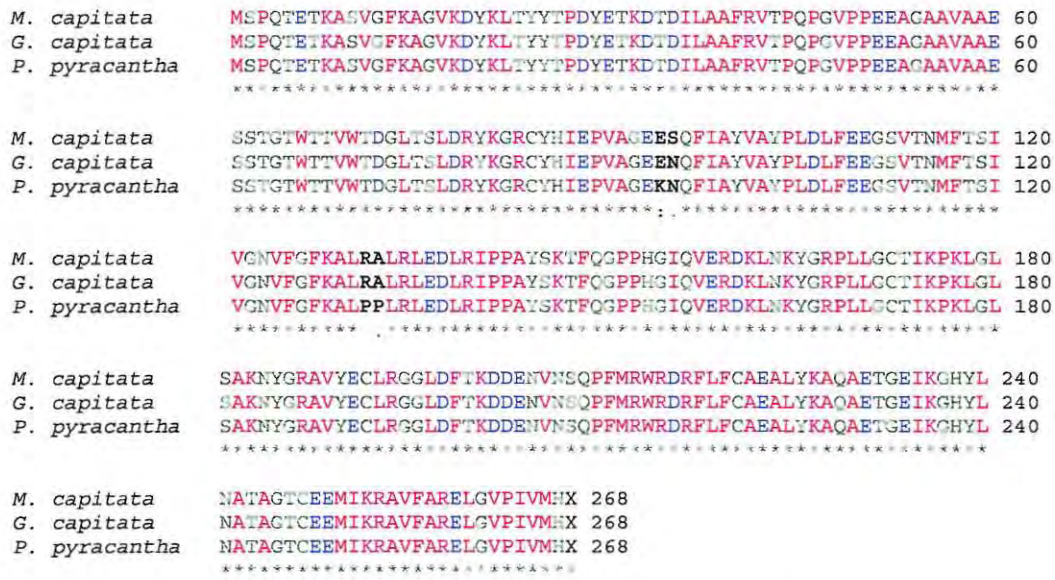

Figure 2.5: Alignment of the amino acid sequences derived from the translation of the *rbc*L gene sequences of *M. capitata*, *G. capitata* and *P. pyracantha* from the Celastraceae family.

Amino acid differences were noted at positions 94 and 95, and at positions 131 and 132.

2.4 Discussion

Partial *rbc*L gene sequences were generated for twenty-three plants collected from the GFRR. Sequences from these plants served as a reference collection from which unknown sequences could be identified.

DNA was extracted with a plant extraction kit, with the exception of *Phyllanthus verrucosus* from the Euphorbiaceae family. This plant produces a mucous material, which interfered with the extraction of DNA. The CTAB extraction procedure was used which enabled DNA extraction from this plant.

Partial *rbc*L gene (802 bp) amplification was performed using the DNA extracted from the GFRR plants. Amplification of this gene was a challenge for *P. verrucosus*, *A. tetracantha*, *E. undulata* and *M. nemorosa*. BSA was added at varying concentrations which allowed for amplification of the plant's DNA. BSA has been reported to prevent interferences during PCR by scavenging substances that inhibit *Taq* polymerase (Höss *et al.*, 1992; Iotti and Zambonelli, 2006; Bradley *et al.*, 2007).

The *rbc*L gene sequences of the plants collected from the GFRR were compared to plant sequences deposited in the GenBank database using the BLASTn program. The majority of plant sequences showed relatively high number of mismatches, giving less than 100% taxonomic identification of plants to family, genus and species level. Of the twenty-three plants sequenced, *J. capensis*, *P. auriculata*, *P. afra* and *B. ilicifolia* sequences gave 100% similarity to plants in the Genbank database. *J. capensis*, *P. auriculata* and *P. afra* were identified correctly to family, genus and species level. The *B. ilicifolia* gave 100% identity to *T. camphoratus* from the same family. For this plant, a larger segment of the *rbc*L gene would need to be sequenced to allow differentiation.

The *rbc*L gene sequence from *P. auriculata* gave 100% identity to the three plant sequences namely, *P. auriculata*, *P. capensis* and *Rheum x cultorum*. The *P. auriculata* and *P. capensis* sequences are from the Plumbaginaceae family, and *R. x cultorum* was classified to the Polygonaceae family. As the *P. auriculata* plant sample was identified at the Selmer Shonland herbarium from its morphological

characteristics, the match to *P. capensis* and *Rheum x cultorum* were not correct. The *rbc*L gene sequence from *O. mucunata* gave a 99% match to two different plant sequences from the Anacardiaceae family. The *rbc*L gene sequences of *C. haematocarpa* (Apocynaceae family), *P. verrucosus* (Euphorbiaceae family) and *M. nemorosa* (Celastraceae family) were identified to *C. bispinosa* (Apocynaceae family). Each of these sequences had two mismatches and gave 99% identity to *C. bispinosa*. The information obtained from these sequences indicate that for correct taxonomic identification, a longer fragment, possibly greater than 1 kb, may be required for their discrimination (Kress *et al.*, 2005).

Of the twenty-three plants assessed, *C. bispinosa*, *P. pyracantha*, *P. auriculata*, *P. afra*, *L. feroscissimum*, *J. capensis* and *A. tetracantha* were identified to family, genus and species level. Sixteen of the *rbc*L gene sequences did not give full taxonomic identification and may not be represented in the GenBank database. Given these results, sequencing of the complete *rbc*L gene of a greater number of GFRR plants, and depositing all the sequences in the GenBank database would be most beneficial. The sequencing of an alternate plant specific portion of DNA may allow for more accurate genotypic characterization of the plants (Poinar *et al.*, 1998; Savolainen and Chase, 2003).

A phylogenetic tree of the plants collected from the GFRR was constructed. Plant sequences from the families Asparagaceae and Celastraceae respectively, were clustered in close proximity. However, of the plant sequences from the Euphorbiaceae family, *E. bothae* and *J. capensis* were assigned to the correct family. *P. verrucosus*, also from the Euphorbiaceae family matched 100% to the *rbc*L gene sequences from *C. haematocarpa* and *M. capitata*. These sequences were not discriminated by the 802 bp *rbc*L gene sequences. Although, the *rbc*L gene sequences were highly conserved between the *C. haematocarpa* and *C. bispinosa,* there were sufficient sequence variations to allow for their discrimination.

M. capitata, *G. capitata* and *P. pyracantha*, from the Celastraceae family, are morphologically similar and are difficult to distinguish in the field. The amino acid sequences of the three species were used to infer their phylogenetic relationship. When the amino acid sequences of the three plant species were assessed, differences were observed at the position 94 and 95. *M. capitata* and *G. capitata* had glutamic

acid at position 94. *P. pyracantha* had lysine at the same position. At position 95, *M. capitata* had asparagine, while both *P. pyracantha* and *G. capitata* had serine in this position. At positions 131 and 132, *M. capitata* and *G. capitata* had arginine and alanine, respectively. In the same positions, *P. pyracantha* had two proline amino acids. Taxonomists have recently reclassified (synonymized) certain *Maytenus* species as *Gymnosporia* species and our findings would seem to support this view.

As there were many discrepancies between the reference plants' *rbc*L gene sequences and the GenBank, the sequence data generated from the *rbc*L gene of GFRR plants was utilized as a reference collection for the identification of sequences generated from DNA extracted from black rhinoceros dung. This will be discussed in Chapter three.

CHAPTER THREE
MOLECULAR DETERMINATION OF PLANTS IN BLACK RHINOCEROS DUNG

3.1 Introduction

Establishing the diet of herbivores through observation is a relatively simple, but not always conclusive technique. Difficulties encountered when observing herbivores browsing or grazing are that the researcher may disturb the normal habitat, and that the wild animals being observed are dangerous. For these reasons, it is difficult to get sufficiently close to the animals for the collation of accurate data. The method also does not determine whether the feeding habits on a particular plant species are common or a rare event (Symondson, 2002). This is particularly relevant to observation of the black rhinoceros when studying their diet by counting twigs and leaves eaten from particular plants (Muya and Oguge, 2000).

Hansen *et al.* (1973) analyzed different herbivores' faeces to estimate their diet using microhistology. However, microhistological approaches have met with limited success due to the degree of digestion of plant material, which often prevents identification of plants consumed. Researchers have proposed the use of molecular techniques as an alternative for the assessment of herbivore diet (Höss *et al.*, 1992; Poinar *et al.*, 1998; Hofreiter *et al.*, 2003; Bradley *et al.*, 2007).

Amplification, cloning and sequencing have been used for DNA analysis from various sources such as faeces (Höss *et al.*, 1992; Poinar *et al.*, 1998; Hofreiter *et al.*, 2003; Bradley *et al.*, 2007). DNA-based faecal analysis has been used as a tool for the investigation of food habits and species identity in mammals (Jarman *et al.*, 2002; Kurose *et al.*, 2005). The DNA barcode approach has also been applied to the detection of the diet of predators such as endangered pigmy blue whale (*Balaenoptera musculus brevicauda*), Adelie penguin (*Pygoscelis adeliae*) and Steller sea lions (Jarman *et al.*, 2002; Deagle *et al.*, 2005). Currently, this approach has only received limited attention in studying the diet of herbivores. By targeting plant specific DNA,

which has sufficient variation, the identification of specific plants is possible (Bradley *et al.*, 2007). A number of researchers have attempted to determine the diet of herbivores using different DNA fragments (Poinar *et al.*, 1998; Bradley *et al.*, 2007; Matheson *et al.*, 2008).

The use of DNA based methods to determine the diet of herbivores was first illustrated by Höss *et al.* (1992) by using the *rbc*L gene as a DNA barcode to identify plant material found in the faeces of European brown bear. A 356 bp segment of the *rbc*L gene was amplified, followed by direct sequencing of the PCR product. The sequence was compared to 414 *rbc*L gene sequences and was identified as *Photinia villosa,* a plant of the Rosaceae family.

PCR based analysis of DNA sequences was also used to determine the origin of ancient dung which had been trapped in caves (Poinar *et al.*, 1998). Molecular analysis of the 12S rRNA gene showed that the dung originated from the extinct ground sloth (*Nothrotheriops shastensis*). Sequencing of a 183 bp segment of the *rbc*L gene from the dung revealed seven plant species when the sequences were compared to 2 300 *rbc*L gene sequences deposited in the GenBank database. Four plant species were identified to a family level, whereas the other three plants were identified to order level. However, it is worth noting that initial extractions from the dung did not yield DNA that could be amplified. Interference was possibly due to inhibition by reducing sugars that cross-linked with the DNA.

Hofreiter *et al.* (2003) revealed the diet of the extinct ground sloth (*Lagidium spp.*) using morphological characteristics and molecular techniques by analyzing their dung retrieved from caves. In analyzing the diet of this species, 110 bp *rbc*L gene sequences were amplified and sequenced. The dung revealed 13 plants sequences, ten of which were assigned to one order and seven different families. The study was limited due to the short length of the DNA sequenced, which limited plant identification. However, shorter DNA fragments are easier to amplify when analyzing DNA that is degraded through exposure to digestive systems and the environment.

Recently, Bradley *et al.* (2007) studied the diet of the wild western gorilla and colubus monkeys by molecular analysis of their faeces. A 157 bp fragment of the *rbc*L gene

was targeted for both the gorillas and the monkey studies, and a 350 bp fragment of the *ITS-2* region was sequenced for the monkey study. The *rbc*L analysis of the gorilla faeces identified 16 different plants to subclass, order and family level. Molecular analysis of monkey faeces identified four plant families. These plants were identified to species level by analysis of the *ITS-2* sequences.

Amplification, cloning and sequencing methods of DNA analysis from faeces has had some success. However, this technique is relatively expensive and yields limited data for the time and effort required. The demand for faster and more efficient sequencing techniques has led to the development of the next generation sequencer, namely the Roche (454) Genome Sequencer FLX System (GS FLX). The advantage of Genome Sequencing Technology is that larger amounts of DNA sequence data can be generated from a complex mixed sample. The technique supports the analysis of samples from a variety of starting materials, such as genomic DNA, PCR products and cDNA (Droege and Hill, 2008).

In this study, PCR products were generated from DNA extracted from dung using standard *rbc*L primers. A second round of PCR was then performed using modified *rbc*L primers. The forward primer was modified with a 19 bp oligonucleotide (adaptor A) complementary to the oligonucleotides attached to the beads used in GS FLX sequencing. The reverse primer was modified with a 19 bp oligonucleotide, which was used as the priming template for clonal amplification. Further, it was modified with four specific nucleotides, which allowed for sequencing of seasonal dung samples in a single reaction mix.

After PCR with the modified primers, single stranded DNA amplification products were mixed with excess Sepharose beads carrying oligonucleotides complementary to adaptor A. The beads were captured in individual microreactors for clonal amplification. Clonal amplification was achieved using a primer complementary to adaptor B. Sequencing was performed by the addition of DNA polymerase, ddNTPs, sulfurylase and luciferase, using pyrosequencing (Mardis, 2008). Correct nucleotide incorporation results in a light reaction generated by the sulfurylase and luciferase enzymes, which is recorded by the sequencing instrument. The signaling light

strength is proportional to the number of nucleotides incorporated in a single nucleotide flow.

In this study, a molecular technique was investigated for the analysis of the dung of black rhinoceros of the GFRR. The chloroplast specific *rbc*L gene was used as a DNA barcode to identify the plants from the dung. A brief study was done to check the feasibility of identifying plants in the dung, by DNA extraction, amplification, cloning and sequencing. Thereafter, a seasonal study was done on dung samples using GS FLX.

3.2 Materials and methods

3.2.1. The *rbc*L gene amplification, cloning and sequencing

3.2.1.1 Reagents

Tris base salt, ethylenediaminetetraacetic acid, sodium chloride, polyvinyl pyrrolidone, bovine serum albumin, sodium dodecyl sulfate, were purchased from Sigma Aldrich (Germany). Liquid nitrogen was obtained from Afrox (South Africa). Chloroform:isoamyl alcohol, propan-1-ol, ethanol, silica gel and acetic acid were purchased from Merck (South Africa).

3.2.1.2 Collection of black rhinoceros dung samples

Black rhinoceros dung samples were collected from the Great Fish River Reserve at different times throughout the year. Collected dung samples were placed in 10 x 25 cm zipper plastic bags containing silica gel and stored at –20°C prior to DNA extraction.

3.2.1.3 Genomic DNA extraction from dung sample

Dried black rhinoceros dung (0.5 g) was initially ground to a fine powder using a coffee grinder (Russel Hobbs 9714 satin), of which 0.1 g was further ground under liquid nitrogen using a mortar and pestle. Genomic DNA extraction was carried out following the CTAB method of Doyle and Doyle (1987) with modifications. The grounded powder was suspended in 610 µl CTAB extraction buffer (Appendix B4), 20 µl of 5 M NaCl and 70 µl of a 20% SDS solution. The reaction mixture was incubated at 65°C for 1 h in a water bath with occasional gentle mixing. The reaction mixture was cooled to room temperature before adding 10 µl of 10 mg/ml RNase A solution followed by incubating at 37°C for 10 min with mixing after 5 min. For DNA purification, 700 µl chloroform:isoamyl alcohol (24:1, v/v) was added to the mixture, followed by centrifugation (9 100 x g, 5 min at room temperature). The resulting aqueous phase collected was transferred into a new 1.5 ml microcentrifuge tube. DNA was precipitated by adding 0.7 volumes of cold propan-1-ol and incubated at –20°C overnight. The overnight sample was centrifuged (9 100 x g, 20 min at room temperature) and the resulting supernatant discarded. The pellet was centrifuged twice

(6 000 x g, 2 min at room temperature), with washes using 1 ml of 70% ethanol after each centrifugation step. The pellet was air dried for 15 min, resuspended in 50 µl ddH_2O and stored at –20°C. The genomic DNA extracted was electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer. The gel was visualized using translumination radiation using a gel documentation system (UviproChemi, United Kingdom).

3.2.1.4 Amplification of the rbcL gene (802 bp) from the dung samples

The same procedure used for amplification of the partial *rbc*L gene from plants was used for the amplification of partial *rbc*L gene (802 bp) from the dung sample as per Section 2.2.4.2. Primers used were as described in Section 2.2.4.1, Table 2.2.

3.2.1.5 Cloning

PCR products from a dung sample were cloned (Section 2.2.5.1), sequenced (Section 2.2.6) and analyzed following the procedure as described in section 2.2.7. Transformation of the plasmid construct to *E. coli* competent cells was performed as described in Appendix G. Plasmid preparation and purification were done as described in Section 2.2.5.3. PCR amplification for the plasmid constructs were performed as described in Section 2.2.5.4.

3.2.1.6. Sequencing of the rbcL gene from the dung

*rbc*L gene sequencing and analysis were performed as described in Sections 2.2.6 and 2.2.7.

3.2.2 Genome Sequencing Technology

3.2.2.1 Reagents

Chemicals used are listed in Section 3.2.1.1.

3.2.2.2 Genomic DNA Extraction from the black rhinoceros dung samples

Genomic DNA was extracted from four seasonal dung samples of the black rhinoceros (collected as described in Section 3.2.1.2), following the method described in Section 3.2.1.3.

3.2.2.3 Amplification of the partial rbcL gene from four seasonal dung samples

Amplification of the partial *rbc*L gene was performed as described in Section 2.2.4.2, using the PCR the primers described in Section 2.2.4.1 (Table 2.2). The amplification products were diluted 100-fold with $dddH_2O$. These diluted PCR products were reamplified with modified primers as shown in Table 3.1.

Table 3.1: Modified primers used for the amplification of the *rbc*L gene from four seasonal dung samples of the black rhinoceros of the GFRR.

	5' → 3'	
1For-A	GCCTCCCTCGCGCCATCAG	39
Rev 802-B-TGAC	GCCTTGCCAGCCCGCTCAGTGAC	44
Rev 802-B-ACTG	GCCTTGCCAGCCCGCTCAGACTG	44
Rev-802-B-CATG	GCCTTGCCAGCCCGCTCAGCATG	44
Rev-802-B-CATC	GCCTTGCCAGCCCGCTCAGCATC	44

Both the forward and the reverse primers were used for the second round of amplification of the *rbc*L gene. Adaptor A on the forward primer is shown in violet. Adaptor B on the reverse primers is shown in blue. The four-nucleotide key sequences are shown in pink.

3.2.2.4 DNA sequencing and analysis

The DNA of the four seasonal dung samples of the black rhinoceros was analyzed by pyrosequencing using a Roche (454) GS FLX sequencer at Inqaba Biotechnologies (Pretoria, South Africa).

3.3 Results

3.3.1 Dung DNA analysis

3.3.1.1 Extraction of DNA from dung

Genomic DNA was extracted from the dung sample using the procedure of Doyle and Doyle (1987), followed by modifications as in Fig. 3.1.

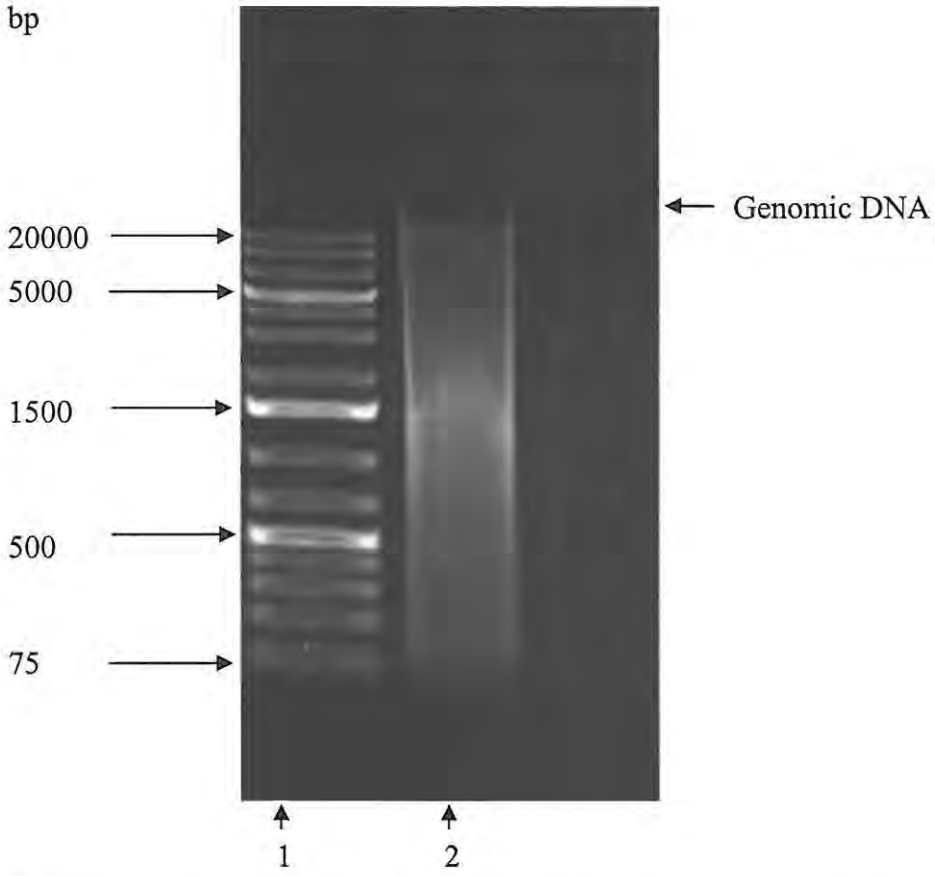

Figure 3.1: Genomic DNA extracted from the selected dung sample of the black rhinoceros of the GFRR using the CTAB extraction method. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in I X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: Genomic DNA from the dung sample.

The gel indicates a highly degraded DNA.

3.3.1.2 Amplification of the partial rbcL gene from the dung sample

Selected dung sample DNA was amplified for the partial *rbc*L gene. A positive and a negative control were used to monitor the PCR reaction conditions as presented in Fig. 3.2.

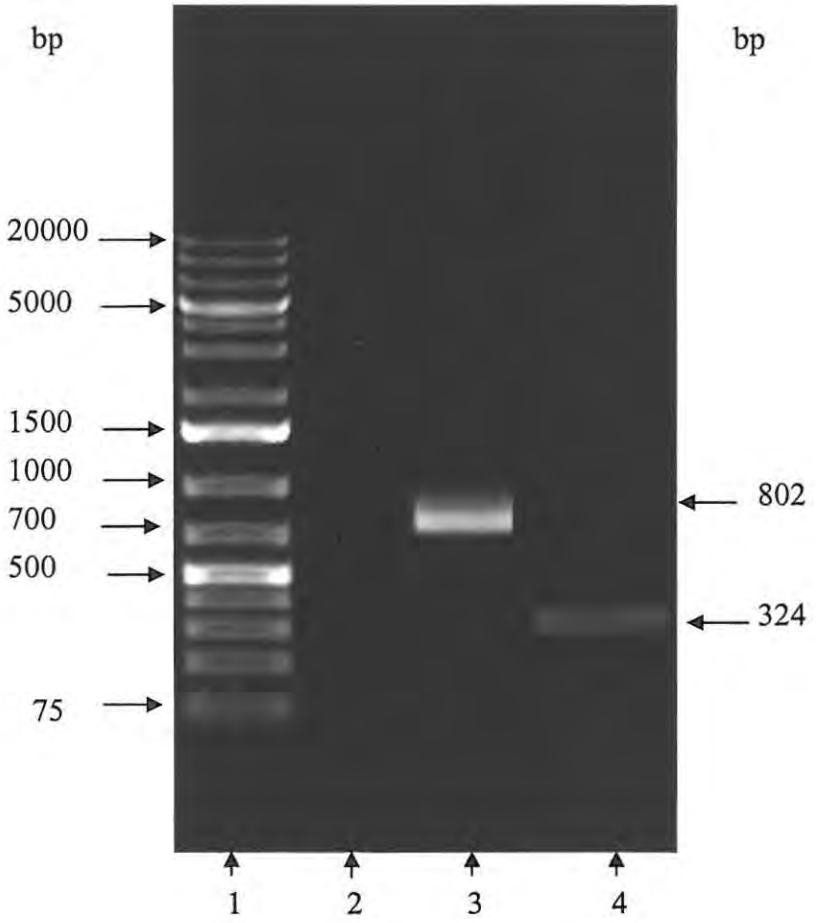

Figure 3.2: Dung amplification product of the *rbc*L gene (802 bp) from the black rhinoceros from the GFRR. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in I X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: negative control (no DNA template), Lane 3: 802 bp PCR product and Lane 4: positive control (plasmid PCR product, 324 bp).

The partial *rbc*L gene amplified from the dung sample was ca. 802 bp, as expected for plant DNA.

3.3.1.3 Plasmid PCR for the rbcL gene (802 bp)

Randomly selected clones from the dung sample were amplified from plasmid containing the partial *rbc*L gene. Five plasmid PCR products from selected clones are shown in Fig. 3.3.

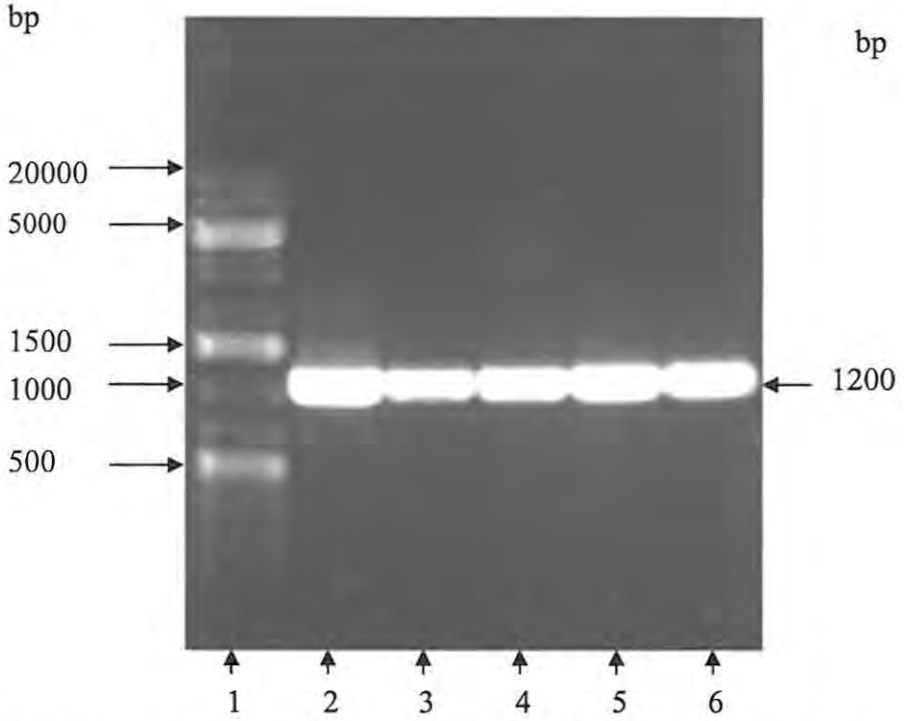

Figure 3.3: Amplification of the *rbc*L gene (802 bp) cloned into the pGem-T Easy vector using M13 primers. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromode in I X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2 to 6: Plasmid PCR products from the randomly selected clones.

High intensity bands of the *rbc*L gene from the dung clones were noted. The size of the band is 1.2 kb due to the overlapping nucleotides from the vector that flank the region of insert.

3.3.1.4 rbcL gene sequencing from dung

Following genomic DNA extraction and cloning, eleven clones were selected randomly and the 802 bp *rbc*L gene sequenced bidirectionally. Both the forward and the reverse clone sequences were aligned and edited using Bioedit software program. *rbc*L clone sequences were compared to the plant sequences deposited in the

GenBank database using the BLASTn program (Poinar *et al.*, 1998; Hofreiter *et al.*, 2003; Bradley *et al.*, 2007) as shown in Table 3.2.

Table 3.2: Comparison of clone sequences from the selected dung sample against sequences in the GenBank database.

Sequence number	Plant family	Accession number	Genus	Species	% Match
One	Aizoaceae	M97889	*Lithops spp.*		99
		AM234787	*Carpobrotus*	*edulis*	
		AM234792	*Lampranthus*	*filicaulis*	
Two	Apocynaceae	AJ419738	*Carissa*	*bispinosa*	99
Three	Asparagaceae	AM234843	*Asparagus*	*capensis*	99
Four	Anacardiaceae	AY510148	*Cotinus*	*coggygria*	99
Five	Acanthaceae	AM234781	*Monechma*	*spartioides*	99
Six	Apocynaceae	AJ419738	*Carissa*	*bispinosa*	99
Seven	Asparagaceae	AY149374	*Asparagus*	*officinales*	98
Eight	Apocynaceae	AJ419738	*Carissa*	*bispinosa*	99
Nine	Aizoaceae	M97889	*Lithops sp.*		99
		AM234787	*Carpobrotus*	*edulis*	
Ten	Apocynaceae	AJ419738	*Carissa*	*bispinosa*	98
Eleven	Plumbaginaceae	EU002283	*Plumbago*	*auriculata*	100
	Polygonaceae	M77702	*Rheum x*	*cultorum*	
	Plumbaginaceae	Y16906	*Plumbago*	*capensis*	

Clone eleven showed 100% identities to two sequences from the Plumbaginaceae and one sequence to Polygonaceae family. The sequences showed no mismatches and gaps. Sequence number three showed one mismatch to the Asparagaceae family and could be assigned as correct. Nine of these sequences had more than one mismatch when compared to sequences in the GenBank database, and their identifications were inconclusive. The clone sequences were compared to the *rbc*L gene reference sequences of the GFRR plants. The results of these comparisons are presented in Appendix M, and a summary is presented in Fig. 3.4.

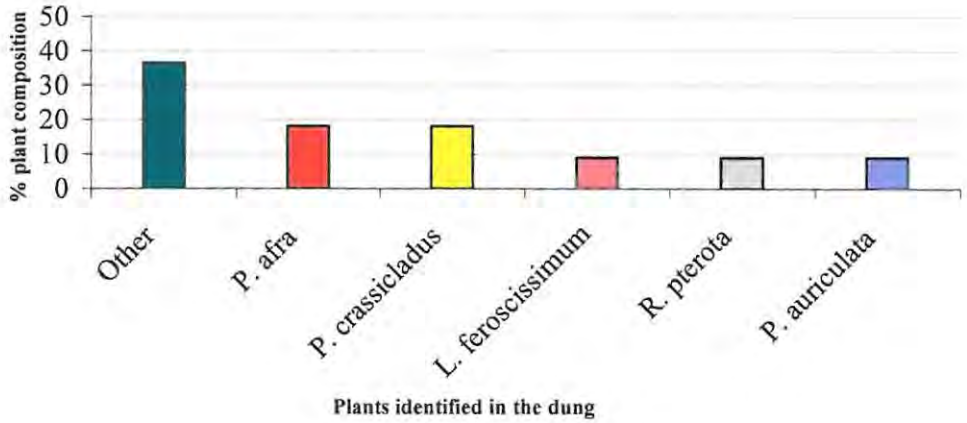

Figure 3.4: Plants species identified in the dung samples of the black rhinoceros using PCR, cloning and sequencing techniques.

Clone one and nine were identified as *P. afra*. Clone two, six, eight and ten were matched to *P. verrucosus*, *C. haematocarpa* and *M. nemorosa*, and could not be identified conclusively as they matched more than one plant species. Clone three and seven were matched to *P. crassicladus*. Clone four, five and eleven were identified as *R. pterota*, *L. feroscissimum*, *P. auriculata*, respectively.

3.3.2 The Genome Sequencing Technology

3.3.2.1 Genomic DNA from four seasonal dung samples

Genomic DNA from four seasonal dung samples was extracted. Genomic DNA from these dung samples was highly degraded, and is presented in Fig. 3.5.

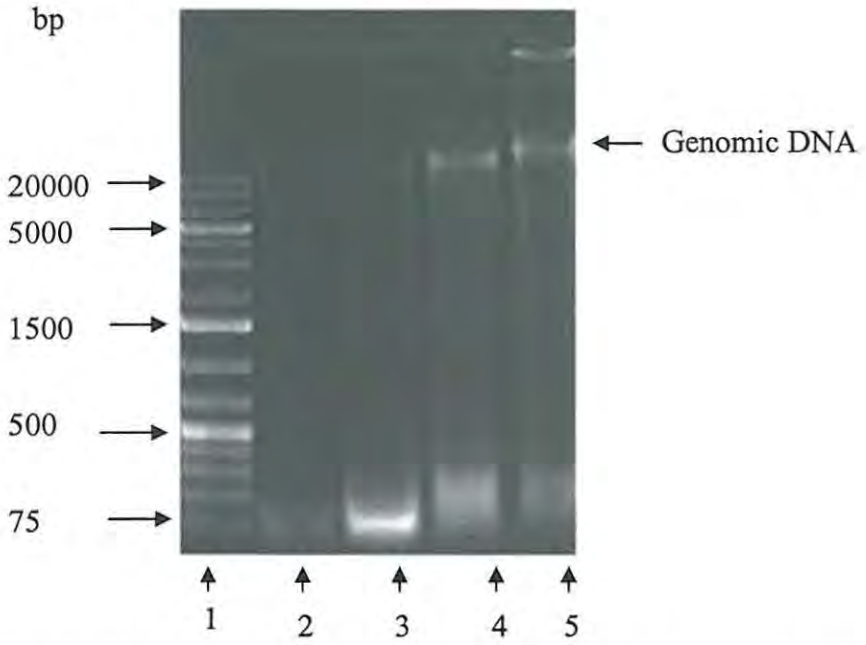

Figure 3.5: Resolution of the seasonal genomic DNA from four black rhinoceros dung samples. DNA was the electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: Summer dung sample, Lane 3: Autumn dung sample, Lane 4: Winter dung sample and Lane 5: Spring dung sample.

3.3.2.2 PCR amplification of partial rbcL gene from four seasonal dung samples

The diluted PCR products of the partial *rbc*L gene were re-amplified with modified primers as described in Table 3.1. PCR products after amplification with the modified primers are presented in Fig. 3.6.

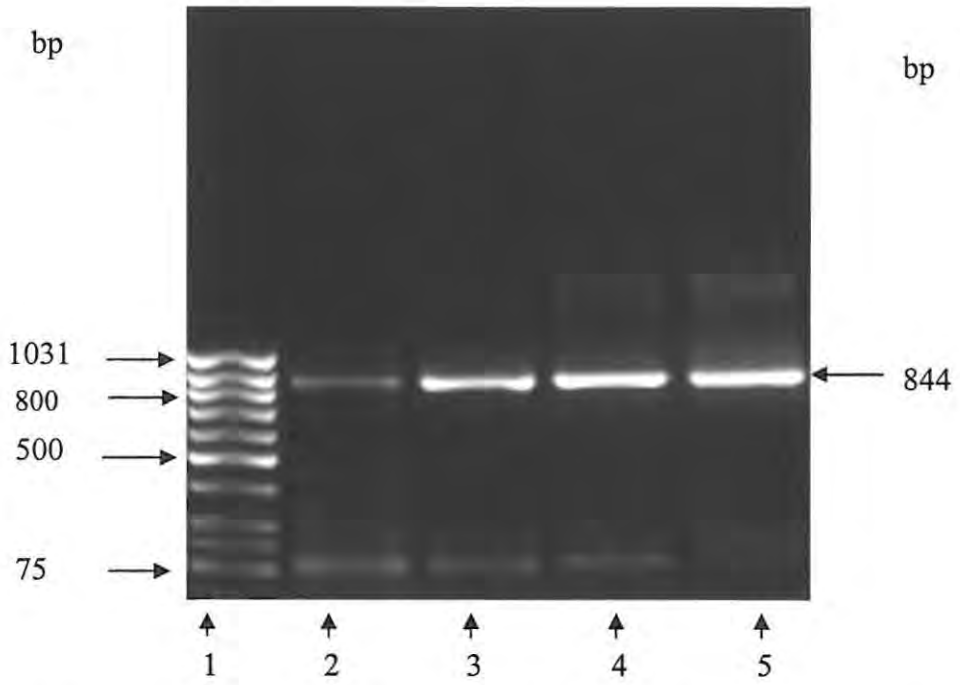

Figure 3.6: Amplification of a partial *rbc*L gene from the dung of the black rhinoceros using modified primers. DNA was resolved using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide at 12 V/cm for 25 min in 1 X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: Summer, Lane 3: Autumn, Lane 4: Winter and Lane 5: Spring PCR products.

The expected size of the PCR products was 844 bp, due to the addition of the adaptor nucleotides.

3.3.2.3 Sequencing of seasonal dung samples of the black rhinoceros

The seasonal dung sequences were identified by comparison to the GenBank and GFRR sequence databases.

3.3.3 Analysis of the *rbc*L gene sequences from a summer dung sample

3.3.3.1 Comparison of a summer dung rbcL gene sequences to plant sequences of the GenBank database

The *rbc*L gene sequences from this study were generated using genome sequencing technology as described in section 3.1. Thirty-one *rbc*L gene sequences were recovered from the summer dung sample, and the results of a GenBank BLASTn are presented in Table 3.3.

Table 3.3: Plant species with the closest match to the *rbc*L gene sequences of the summer dung sample as determined by a BLASTn comparison to the GenBank database.

No. of Sequences	Plant family	Accession no.	Genus	Species	% Match
Three	**Aizoaceae**		Five different plants		97
Twenty-five	**Apocynaceae**	AJ419738	*Carissa*	*bispinosa*	92 to 99
One	**Apocynaceae**	X91758	*Acokanthera*	*oblongifolia*	95
One	**Apocynaceae**		Two different plants		97
One	**Vitaceae**	AJ419723	*Rhoicissus*	*rhomboides*	98

Sequences showed variable lengths between 240 and 280 bp. Sequences were identified to family, genus to species level. Of the thirty-one sequences recovered, 27 were identified as Apocynaceae family, of which 25 were matched to *C. bispinosa,* one to *O. oblongifolia* and one was inconclusive. One sequence was identified as Vitaceae family, and the closest match was to *R. rhomboides*. One sequence identified as Apocynaceae family could not be identified conclusively as it matched equally to two plant species. Three sequences were identified as Aizoaceae, but were inconclusive at genus and species level.

3.3.3.2 rbcL gene sequences obtained from a summer dung sample compared to plant sequences in the GFRR database

When compared to plant sequences in the GFRR database, dung sequences were identified to plant family, genus and species level. These results are presented in Appendix N1 and are summarized in Fig. 3.7.

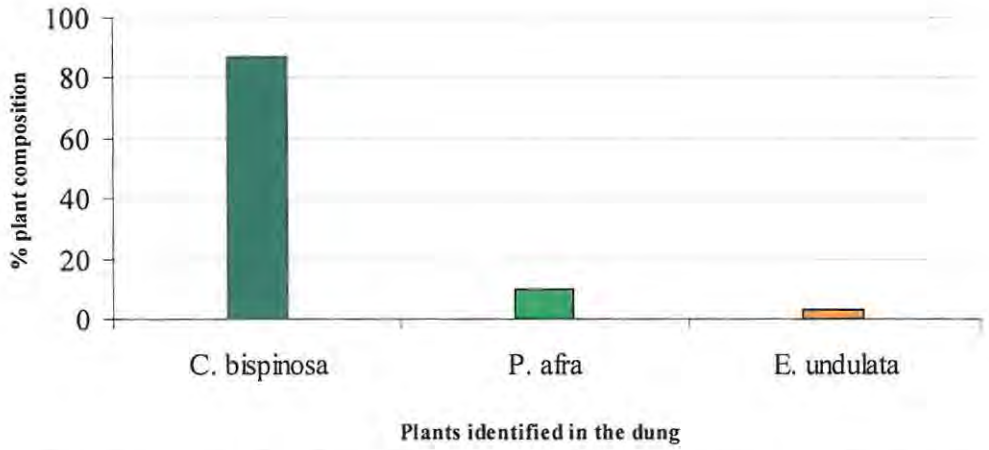

Figure 3.7: Identification of plants in a summer dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

The Apocynaceae family was represented by *C. bispinosa* (27), the Ebenaceae family was represented by *E. undulata* (1) and the Portulacaceae family was represented by *P. afra* (3).

3.3.4. Analysis of the *rbc*L gene sequences from an autumn dung sample

3.3.4.1 Comparison of an autumn dung sample rbcL gene sequences to plant sequences in the GenBank database

A total of forty-seven *rbc*L gene sequences were obtained from an autumn dung sample. The results of a GenBank BLASTn are presented in Table 3.4.

Table 3.4: Plant species with the closest match to the *rbc*L gene sequences of the autumn dung sample as determined by a BLASTn comparison to the GenBank database.

No. of sequences	Plant family	Accession no	Genus	Species	% Match
One	**Acanthaceae**	L01886	*Barleria*	*prionitis*	98
Five	**Aizoaceae**	AF132100	*Galenia*	*pubescens*	96 to 99
Two	**Aizoaceae**	AM234789	*Disphyma*	*crassifolium*	94
One	**Aizoaceae**	AM234792.1	*Lampranthus*	*filicaulis*	97
One	**Aizoaceae**	AF132094	*Tetragonia*	*tetragonioides*	95
Four	**Aizoaceae**	-	Sixteen different plants		98 to 99
Seven	**Amaranthaceae**	AY270061	*Atriplex spp.*		95 to 100
One	**Amaranthaceae**	AY270122	*Pupalia*	*lappacea*	98
One	**Asteraceae**	EU385023	*Tarchonanthus*	*camphoratus*	98
One	**Amaranthaceae**	AY270113	*Oreobliton*	*thesioides*	94
	Stegnospermataceae	M62571	*Stegnosperma*	*halimifolium*	
	Aizoaceae	AM234797	*Tetragonia spp.*		
One	**Amaranthaceae**	AY270062	*Atriplex spp..*		95
	Orobanchaceae	AY563940	*Aureolaria*	*pedicularia*	95
	Aizoaceae	AM234789	*Disphyma*	*crassifolium*	95
Sixteen	**Apocynaceae**	AJ419738	*Carissa*	*bispinosa*	96 to 98
Two	**Celastraceae**	AM234959	*Putterlickia*	*pyracantha*	96 to 98
One	**Euphorbiaceae**	AY794824	*Euphorbia*	*abyssinica*	98
One	**Humiriaceae**	AB233889	*Humiria*	*balsamifera var. balsamifera*	94
One	**Rubiaceae**	-	Six different plants		93
One	**Schlegeliaceae**	AY919278	*Synapsis*	*ilicifolia*	93

Sequences are represented by twelve families in a ratio of Acanthaceae (1): Aizoaceae (9): Amaranthaceae (8): Asteraceae (1): Apocynaceae (16): Stegnospermataceae (1): Orobanchaceae (1): Rubiaceae (1): Humiriaceae (1): Celastraceae (2): Schlegeliaceae (1): Euphorbiaceae (1). Two sequences could not be identified conclusively as they matched more than one family and plant at genus and species level. The *rbc*L gene sequences from Rubiaceae (1) and Aizoaceae (4) could not be identified conclusively as they matched more than one plant genus and species.

3.3.4.2 *rbcL gene sequences obtained from an autumn dung sample compared to plant sequences in the GFRR database*

When compared to plant sequences in the GFRR database, dung sequences were identified to plant family, genus and species level. These results are presented in Appendix N2, and are summarized in Fig. 3.8.

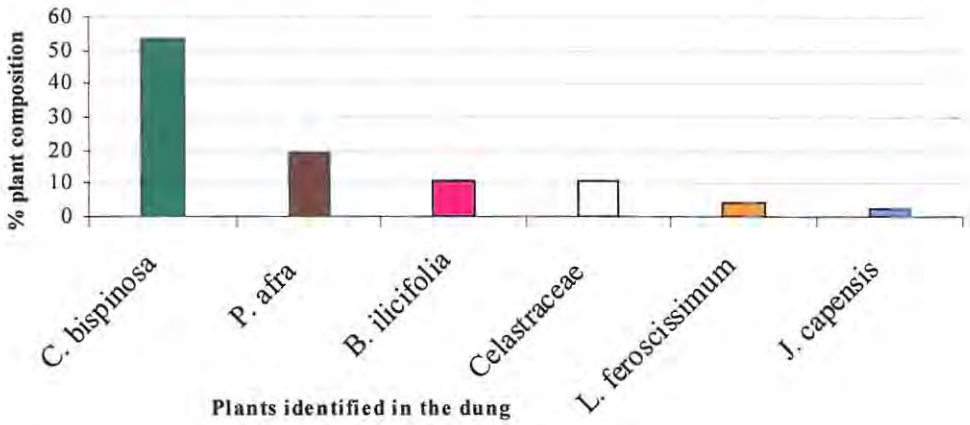

Figure 3.8: Identification of plants in an autumn dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

Five plants were identified when analysing an autumn dung sample. The Asteraceae was represented by *B. ilicifolia* (5), the Apocynaceae was represented by *C. bispinosa* (25), Euphorbiaceae was represented by *J. capensis* (1), Solanaceae was represented by *L. feroscissimum* (2) and the Portulacaceae was represented by *P. afra* (9). *M. capitata*, *P. pyracantha* and *G. capitata* were identified to the Celastraceae family, however could not be differentiated and therefore the genera and species of these sequences was inconclusive.

3.3.5. Analysis of the *rbc*L gene sequences from a winter dung sample

3.3.5.1 Comparison of a winter dung sample rbcL gene sequences to plant sequences in the GenBank database

Forty-eight *rbc*L gene sequences were recovered from a winter dung sample, and the results of a GenBank BLASTn are presented in Table 3.5.

Table 3.5: Plant species with the closest match to the *rbc*L gene sequences of the winter dung sample as determined by a BLASTn comparison to the GenBank database.

No. of sequences	Plant family	Accession no.	Genus	Species	% Match
One	**Aizoaceae**	AM234789	*Disphyma*	*crassifolium*	96.0
One	**Aizoaceae**	AF132099	*Galenia*	*pubescens*	97.0
Eight	**Aizoaceae**	-	Twelve different plants		98 to100
One	**Anacardiaceae**	-	Three different plants		98
Twenty-six	**Apocynaceae**	AJ419738	*Carissa*	*bispinosa*	94 to 98
One	**Apocynaceae**	X91766	*Picralima*	*nitida*	98
Two	**Apocynaceae**	X91758	*Acocanthera*	*oblongifolia*	95 to 96
Three	**Apocynaceae**	-	Twelve different plants		97 to 98
One	**Bignoniaceae**	AM234922	*Rhygozum*	*obovatum*	95
One	**Portulacaceae**	AM235080.1	*Portulacaria*	*afra*	97
One	**Rubiaceae**	AJ286695	*Codia*	*rudis*	96
One	**Rubiaceae**	-	Six different plants		94
One	**Hectorellaceae**	EF551347	*Hectorella*	*caespitosa*	95
	Portulacaceae	AM235080.1	*Portulacaria*	*afra*	94

The Aizoaceae family was represented by 10 sequences, of which 8 could not be identified conclusively as they matched with more than one plant at genus and species level. The Apocynaceae family was represented by 32 sequences, of which 26 sequences were matched to *C. bispinosa*, one matched to *P. nitida*, two matched to *A. oblongifolia* and three could not be identified conclusively as they matched with more than one plant at genus and species level. The Portulacaceae and Rubiaceae families were each represented by two sequences. Bignoniaceae and Anacardiaceae families

were each represented by one sequence. One sequence was identified to Hectorellaceae and Portulacaceae, and could not be identified conclusively as it matched more than one plant at genus and species level. The abundant plant was *C. bispinosa*.

3.3.5.2 rbcL gene sequences obtained from a winter dung sample compared to plant sequences in the GFRR database

The *rbc*L gene sequences amplified from a winter dung sample were compared to plant sequences in the GFFR database. The best percent match data is presented in Appendix N3, and is summarized in Fig. 3.9.

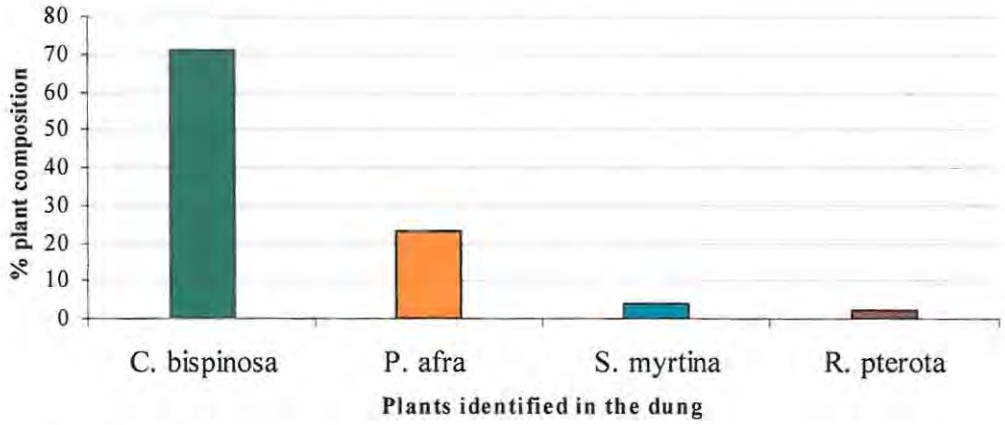

Figure 3.9: Identification of plants in a winter dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

The Apocynaceae family was represented by *C. bispinosa* (34), the Anacardiaceae family was represented by *R. pterota* (1), the Portulacaceae was represented by *P. afra* (11) and the Rhamnaceae family was represented by *S. myrtina* (2). Of the plant families identified, Apocynaceae was the most abundant.

3.3.6 Analysis of the *rbc*L gene sequences from a spring dung sample

3.3.6.1 Comparison of a spring dung sample rbcL gene sequences to plant sequences in the GenBank database

Seventeen *rbc*L gene sequences were obtained from a spring dung sample of the black rhinoceros. Sequences were compared to sequences in the GenBank database for identity and the results are shown in Table 3.6.

Table 3.6: Plant species with the closest match to the *rbc*L gene sequences of the spring dung sample as determined by a BLASTn comparison to the GenBank database.

No. of sequences	Plant family	Accession no.	Genus	Species	% Match
Five	**Aizoaceae**	-	Five different plants		99
Eleven	**Apocynaceae**	AJ419738	*Carissa*	*bispinosa*	96 to 98
One	**Asteraceae**	EU385023	*Tarchonanthus*	*camphoratus*	98

The Aizoaceae family was represented by 5 sequences and could not be identified conclusively to genus and species. Eleven sequences were identified to the Apocynaceae family, represented by *C. bispinosa*. One sequence representing Asteraceae was identified to *T. camphoratus*.

3.3.6.2 rbcL gene sequences obtained from a spring dung sample compared to plant sequences in the GFRR database

The *rbc*L gene sequences amplified from a spring dung sample were compared to plant sequences in the GFFR database. The best percentage match data is presented in Appendix N4, and is summarized in Fig. 3.10.

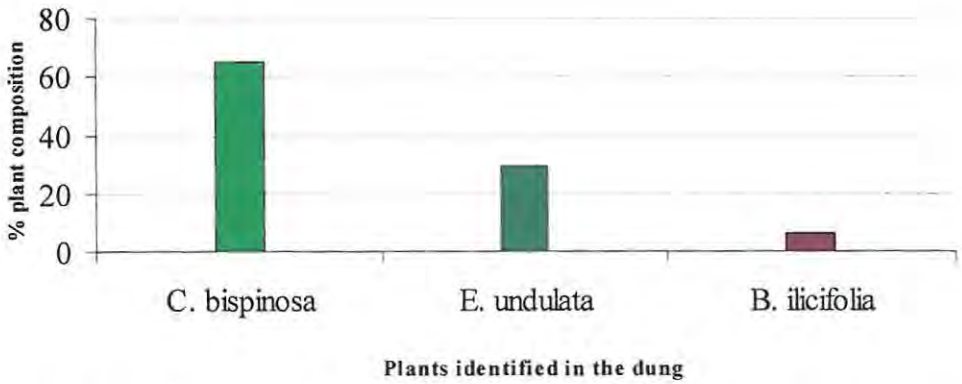

Figure 3.10: Identification of plants in a spring dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

The Apocynaceae family was represented by four sequences and was identified to *C. bispinosa*. Five sequences were identified to the Ebenaceae family and were represented by *E. undulata*. One sequence was identified to Asteraceae family and was represented by *B. ilicifolia*.

3.3.7 A summary of plants identified in the black rhinoceros dung samples by GS FLX analysis.

Table 3.7: Percentage plant composition in black rhinoceros dung over four seasons.

Plants in a year	Summer	Autumn	Winter	Spring
B. ilicifolia	-	10.6	-	5.9
J. capensis	-	4.3	-	-
L. feroscissimum	-	2.1		-
R. pterota	-	-	2.1	-
S. myrtina	-	-	4.2	-
Celastraceae	-	10.6	-	-
C. bispinosa	87.1	53.2	70.8	64.7
P. afra	9.67	19.1	22.9	-
E. undulata	3.22	-	-	29.4

Eight different plants, from different families, were identified from black rhinoceros dung using GS FLX sequencing. In all seasons, *C. bispinosa* was the most abundant plant identified in the dung.

3.4 Discussion

Seasonal dung samples of the black rhinoceros were collected and the plant composition was determined using DNA-based techniques. The potential of DNA-based techniques to study animal diet using faeces was highlighted by Höss *et al.* (1992) who studied the diet of European brown bears. Poinar *et al.* (1998) analyzed the faeces of the extinct ground sloth to determine its diet. Bradley *et al.* (2007) studied the diet of primates by DNA analysis of their faeces. Recently, Matheson *et al.* (2008) detected plant materials in the diet of insects by DNA analysis of their gut contents. These studies motivated our investigations in the development of DNA-based techniques for the determination of the diet of the endangered black rhinoceros of the GFRR. Molecular analysis of faeces for diet determination has the potential to provide a noninvasive and scientifically more accurate alternative method to microhistology and observation.

3.4.1 Extraction, amplification, cloning and sequencing from dung samples

Extraction of DNA from dung samples of the black rhinoceros was a problem throughout the experiments and DNA was extracted several times before recovery. This has been reported elsewhere in studies by Huber *et al.* (2002 and 2003). The CTAB extraction procedure was used for DNA isolation from the dung samples of the black rhinoceros. This method is recommended when extracting DNA from faeces as it allows flexibility for the removal of contaminating compounds (Huber *et al.*, 2002 and 2003; Remya *et al.*, 2004). Sufficient grinding was found to be an important factor for successful isolation of DNA from the dung.

The *rbc*L gene was amplified from the extracted DNA using PCR. The targeted DNA was plant specific, as the dung contained DNA from microorganisms, the host animal as well as plants. It had to be sufficiently conserved to give accurate phylogenetic information, but had to have sufficient variation to provide differentiation, particularly to a species level. The target DNA template is usually relatively short, due to degradation of DNA extracted from feaces. There should also be a sufficiently large sequence database available for meaningful comparison (Bradley *et al.*, 2007).

This study amplified 802 bp of the *rbc*L chloroplast gene from dung samples. Due to DNA degradation in faeces, other researchers have amplified shorter DNA fragments (Höss *et al.*, 1992). Plant material is generally poorly digested in the black rhinoceros, and although the DNA extracted from black rhinoceros dung was degraded, it was possible to amplify a relatively large portion of the *rbc*L gene. However, amplification was initially problematic, which may have been due to co-extracted phenolic compounds that can limit the activity of the *Taq* polymerase (Iotti and Zambonelli, 2006). Addition of BSA to the PCR mix led to successful amplification. BSA is reported to scavenge *Taq* polymerase inhibitors (Höss *et al.*, 1992; Iotti and Zambonelli, 2006; Bradley *et al.*, 2007). The amount of DNA template was also an important factor and was increased as required for successful amplification of the *rbc*L gene.

Only eleven clones, containing the *rbc*L gene, were sequenced to show proof of concept. Each sequence was taken to represent a sequence of a particular plant present in the dung. These sequences were taxonomically identified by comparison to sequences in the GenBank database and our own reference sequences of GFRR plant DNA. Clone sequences were initially compared against sequences in the GenBank database for identification as presented in Table 3.1. Six plant families identified from the GenBank sequence database were Aizoaceae (2), Apocynaceae (4), Asparagaceae (2), Acanthaceae (1) and Anacardiaceae (1). Due to the relatively high number of nucleotide mismatches, possibly due to plant sequences not being available in the GenBank database, the sequences were compared to an internally generated GFRR database (Chapter two). The sequences were analyzed by pairwise alignment algorithm (http://www.ebi.ac.uk/Tools/emboss/align/index). Although much of the data was inconclusive, *P. afra*, *P. crassicladus*, *P. auriculata*, *R. pterota* and *L. feroscissimum* were identified in the black rhinoceros dung. This study was followed up with a GS FLX sequencing technology investigation, which had the potential to generate substantially more data.

3.4.2 FLX genome sequencing technology

FLX genome sequencing technology has the advantage of sequencing individual DNA fragments from a complex mixture of samples without bacterial cloning

(Droege and Hill, 2008). The *rbc*L sequences generated from the seasonal dung samples ranged from 240 and 280 bp.

The majority of plant sequences were not identified using the GenBank database, and the *rbc*L gene sequences were compared by alignment to the sequences generated from GFRR plants. Seasonal differences of plant species browsed by the black rhinoceros were noted. Three plant species were identified in the summer, six in the autumn, four in the winter and three in the spring dung samples. Contrary to previous studies, *C. bispinosa* was identified as the most abundant plant in all seasons. This plant has not been reported in the diet of GFRR black rhinoceros. *P. afra* was identified in all the dung samples, except the spring dung sample. *P. afra* was reported as a minor plant browsed by the black rhinoceros (Brown *et al.*, 2003; IJdema and de Boer, 2008). *E. undulata* was found in both the summer and spring dung samples. Brown *et al.* (2003) reported *E. undulata* as preferred browse in the medium *Portulacaria* thicket. Other plants observed by Brown *et al.* (2003) and identified in the dung include *B. ilicifolia*, *L. feroscissimum*, and plants from Celastraceae family.

Ausland and Sveipe (2000) and Brown *et al.* (2003) reported *Euphobia bothae* as the preferred plant in the diet of black rhinoceros of the GFRR. This plant was not identified in any of the dung samples analyzed in this study. It is most likely that more robust plants survive the digestion process less degraded, and therefore may be over represented in the dung. Although *E. bothae* is a favourite of the black rhinoceros, it occurs infrequently in the study area.

Plants identified in this study from dung, but not reported by Brown *et al.* (2003) as preferred browse include *S. myrtina*, *C. bispinosa* and *R. pterota*. It is not clear which method is the most effective. However, our investigation indicates that the molecular approach to determine diet may provide a complementary to micro-analysis, observations, etc. We recommend that the molecular approach is used in conjunction with observation and microhistological techniques to determine the diet of herbivores.