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

ANANIAS HODI KGOPA

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.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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.

.....

Ananias Hodi Kgopa

Supervisor: **Dr. Brendan S. Wilhelmi**

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).

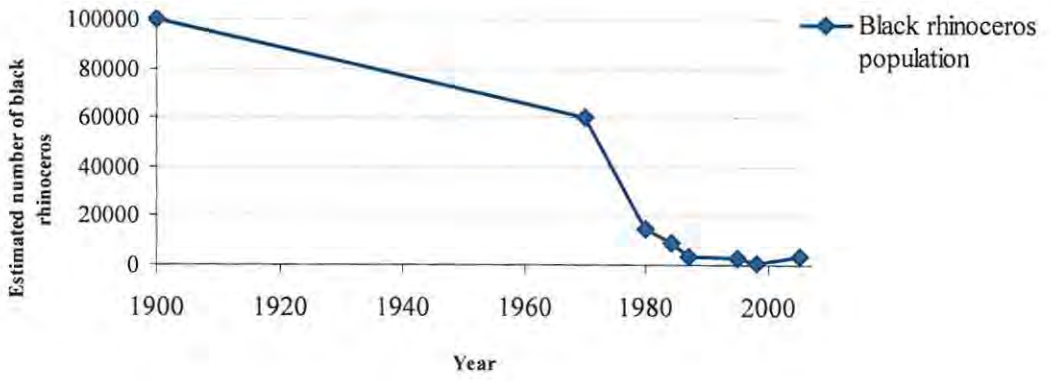


Figure 1.1: The decline of the black rhinoceros population in Africa (Emslie and Brooks, 1999).

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).

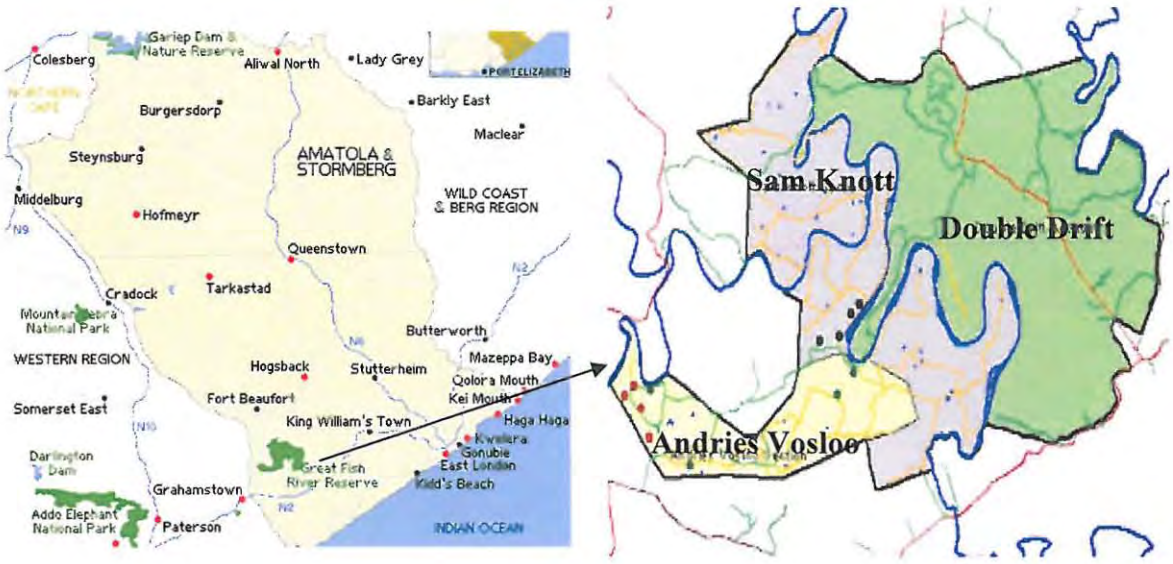


Figure 1.2: The three reserves that form the Great Fish River Reserve (Van Lieverloo and Schuiling, 2004).

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.

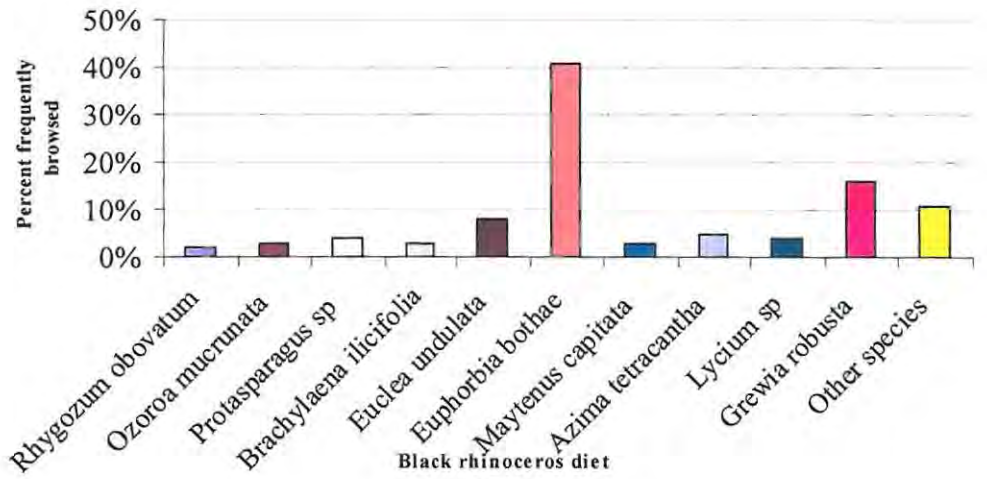


Figure 1.5: Tree and shrubs species most frequently browsed by black rhinoceros in the short *Euphorbia* thicket in the GFRR. The data is expressed as the percentages of bites recorded throughout the observation period (Brown *et al.*, 2003).

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).

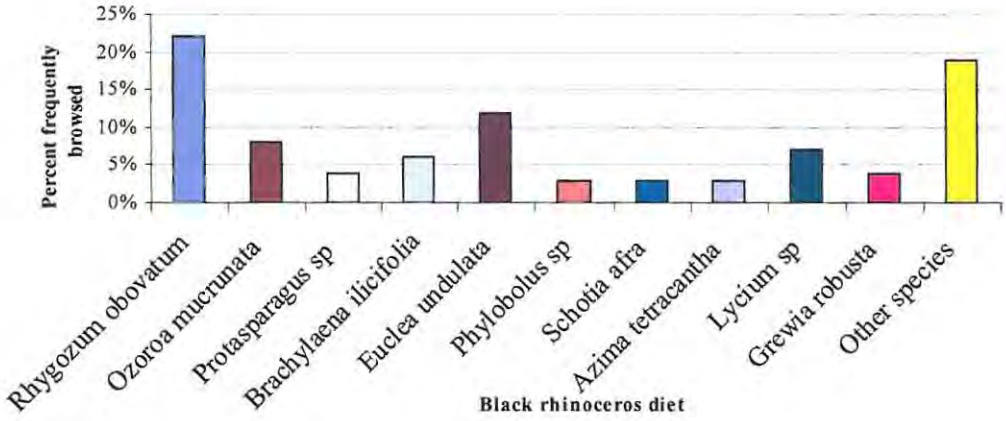


Figure 1.6: Tree and shrubs species most frequently browsed by the black rhinoceros in the medium *Portulacaria* thicket in the GFRR. The data is expressed as percentage of bites recorded throughout the observation period (Brown *et al.*, 2003).

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).

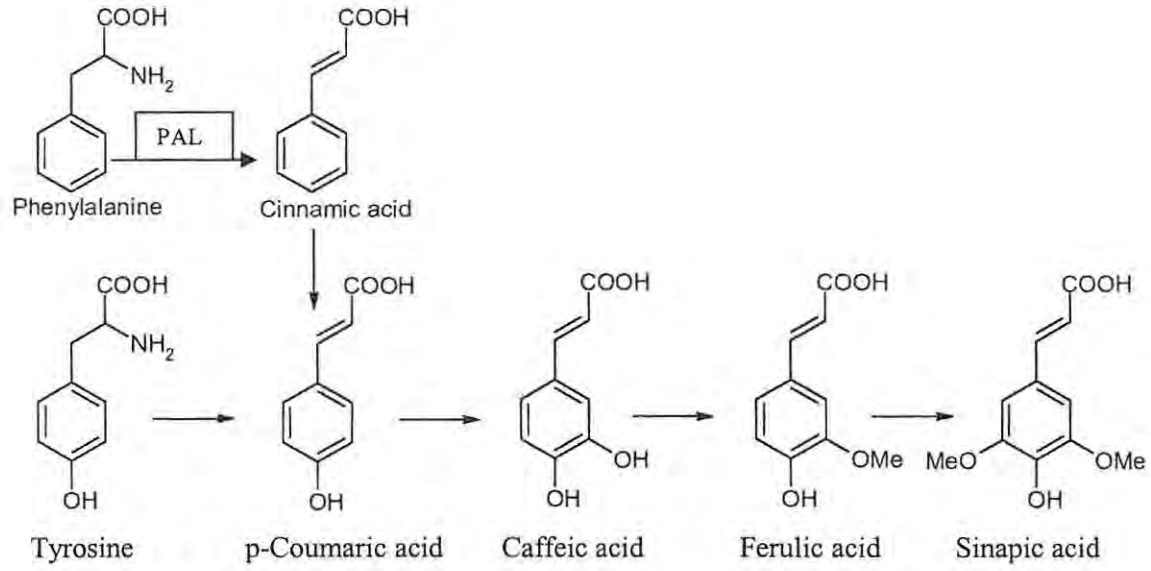


Figure 1.7: Biosynthesis of phenolic compounds via the shikimate pathway (Paixão *et al.*, 2007).

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.

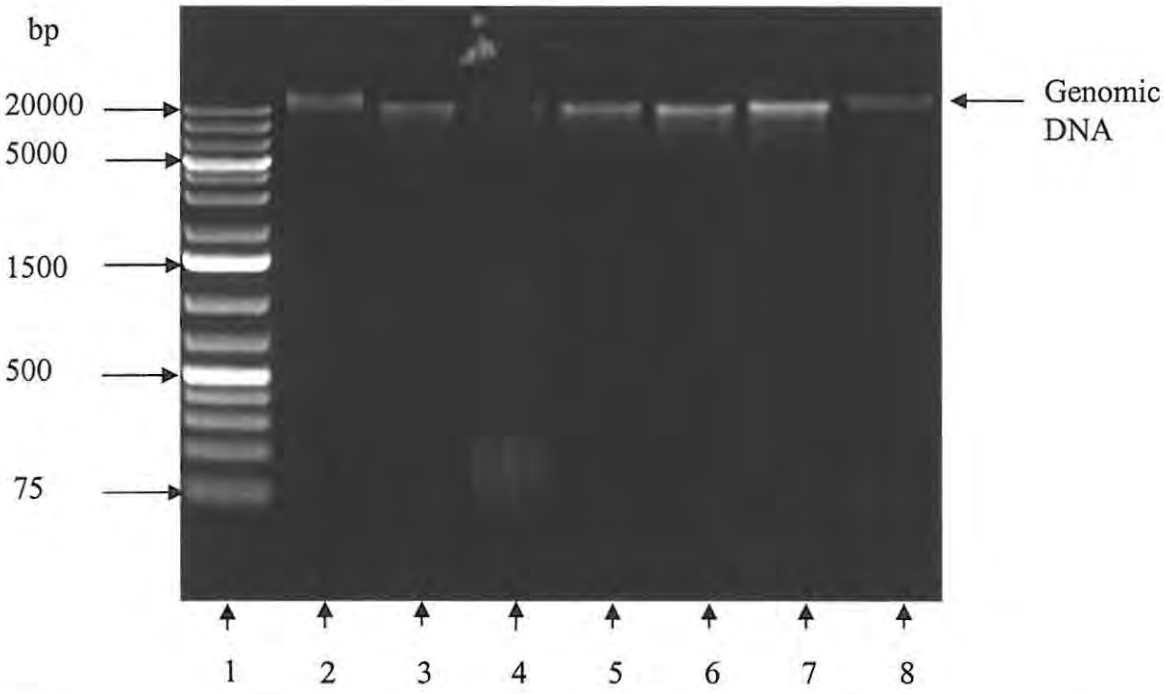


Figure 2.1: The genomic DNA extracts of selected plant species observed to be part of the diet of black rhinoceros of the GFRR. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: *Azima 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.

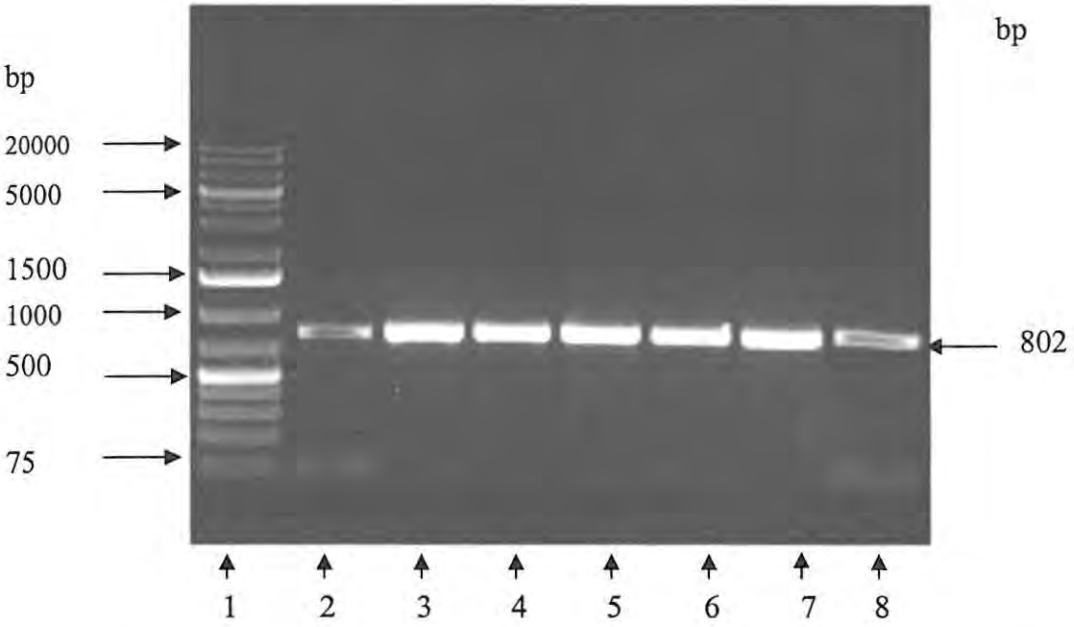


Figure 2.2: Amplification of the 802 bp *rbc*L gene from plants observed to be part of the diet of the black rhinoceros of the GFRR. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer. Lane1: 0.75 - 20 kb DNA ladder, Lane 2: *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.

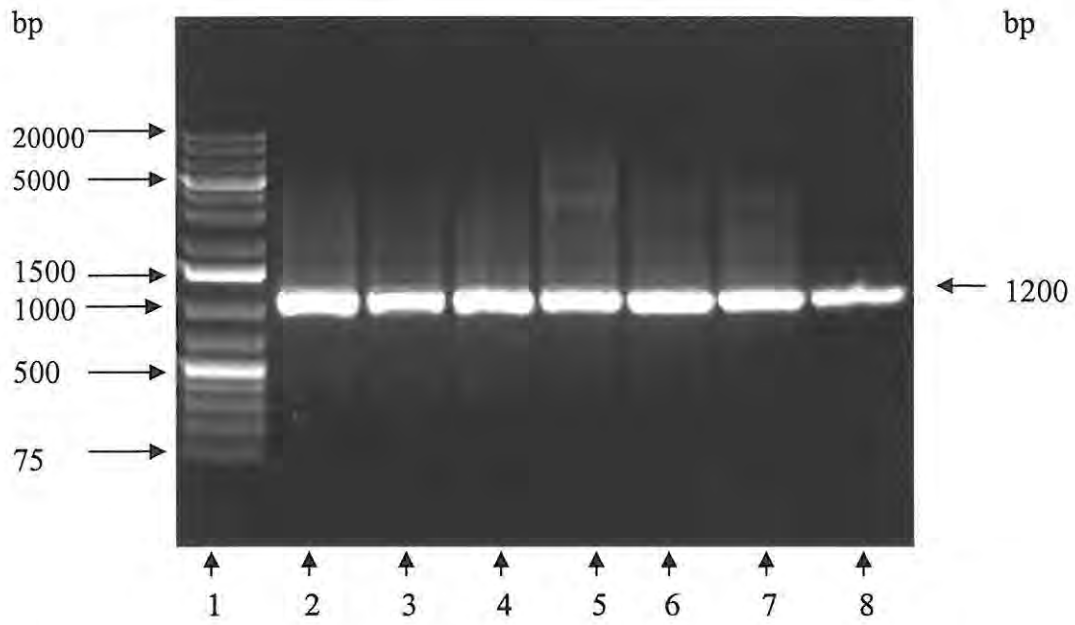


Figure 2.3: Amplification of the 802 bp *rbc*L gene insert cloned into the pGem-T Easy vector using M13 primers. The DNA was electrophoresed at 12V/cm for 25 min using 1% agarose gel stained with 0.4 µg/ml ethidium bromide in 1X TAE buffer. Lanel: 0.75 - 20 kb DNA ladder, Lane 2: *Azima tetracantha*, 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.

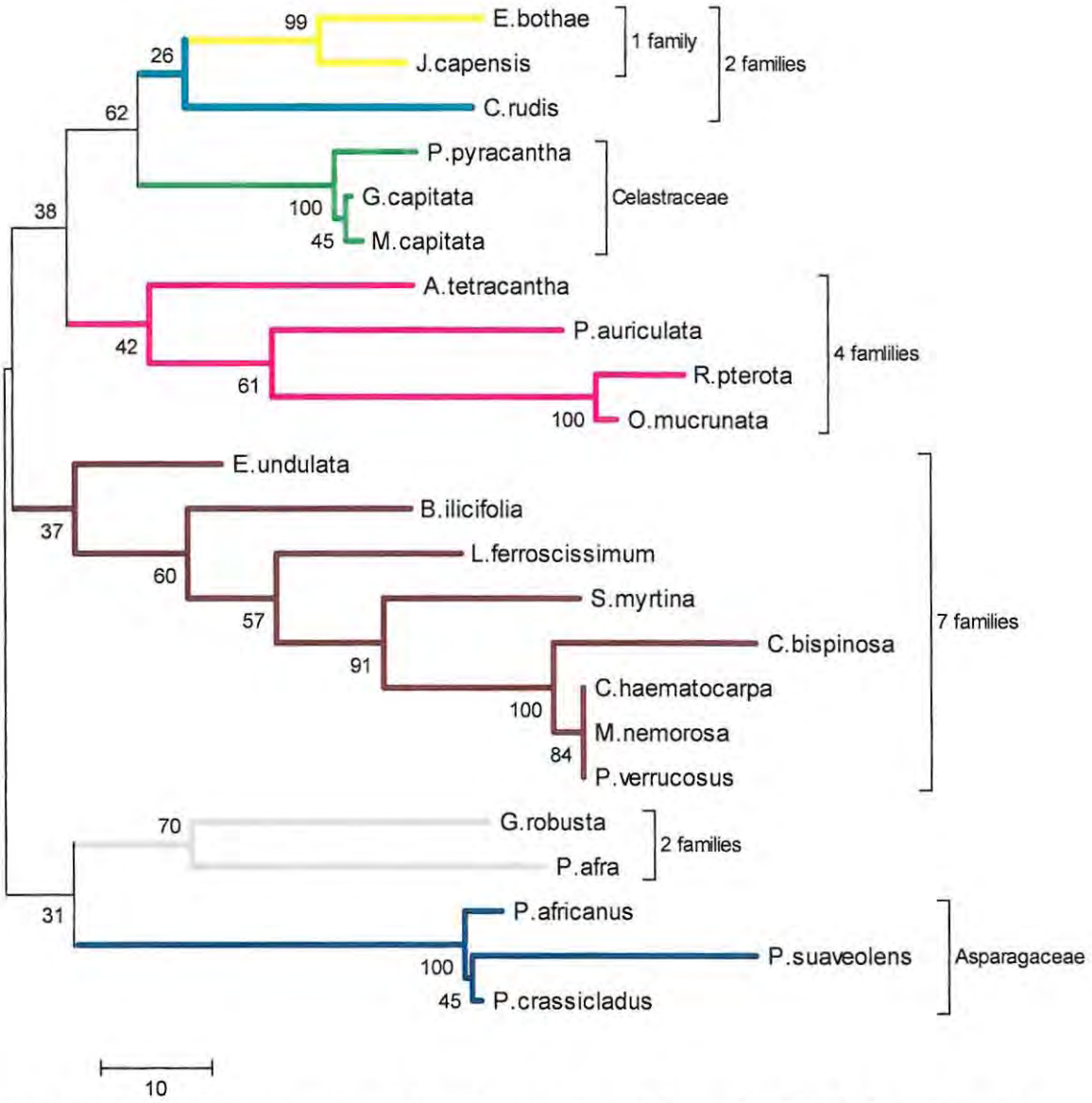


Figure 2.4: Phylogenetic tree of the plants collected from the GFRR based on the *rbc*L gene.

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.

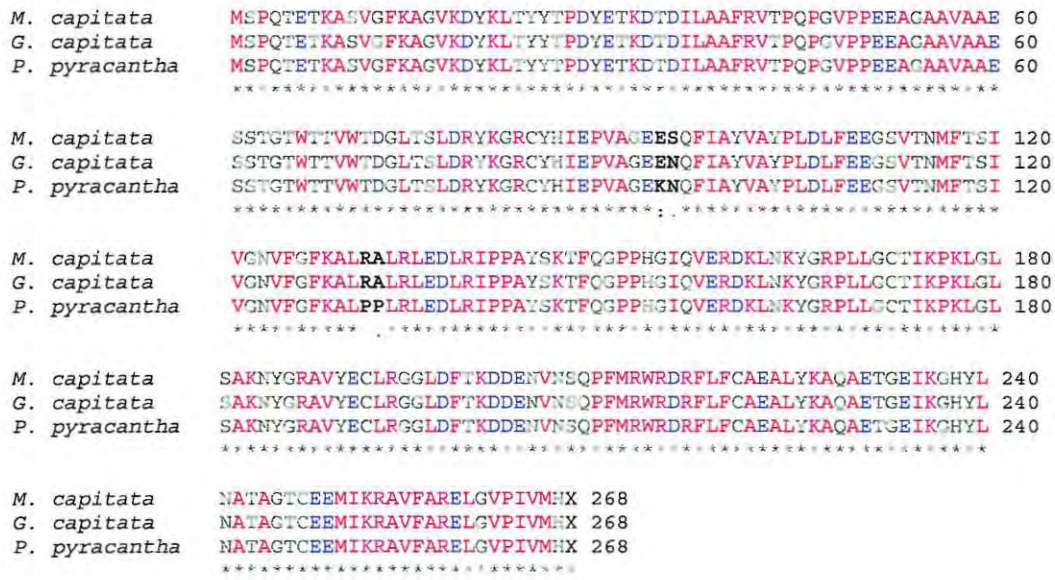


Figure 2.5: Alignment of the amino acid sequences derived from the translation of the *rbc*L gene sequences of *M. capitata*, *G. capitata* and *P. pyracantha* from the Celastraceae family.

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.

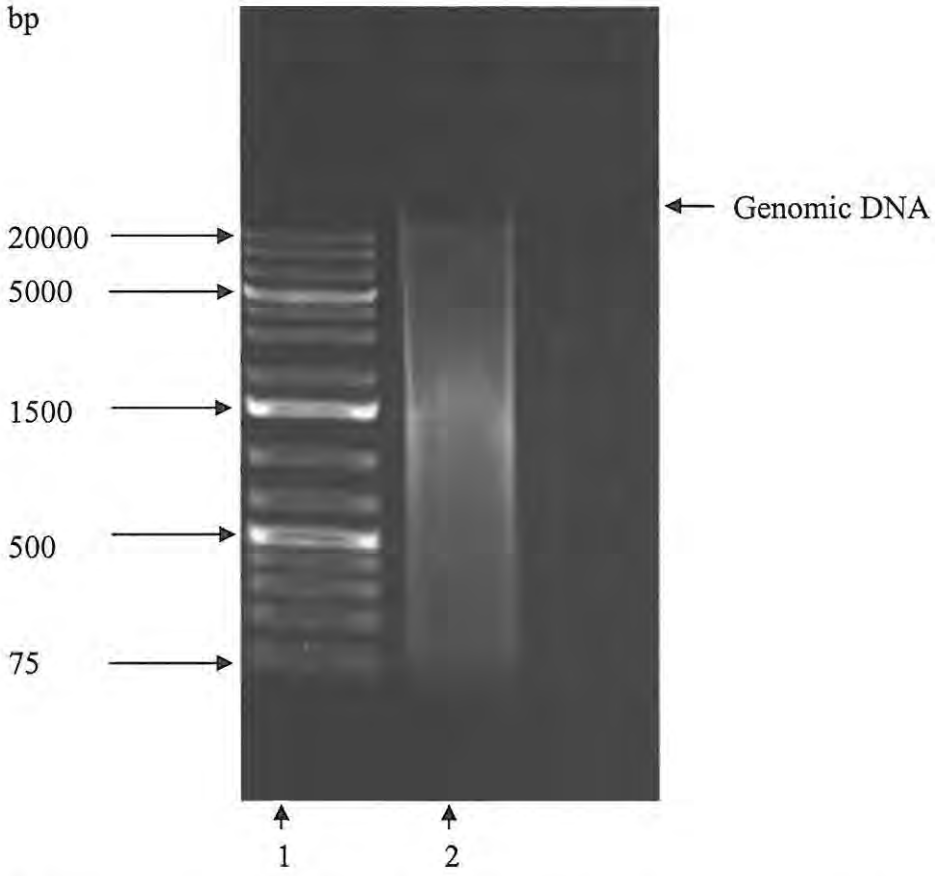


Figure 3.1: Genomic DNA extracted from the selected dung sample of the black rhinoceros of the GFRR using the CTAB extraction method. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in I X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder,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

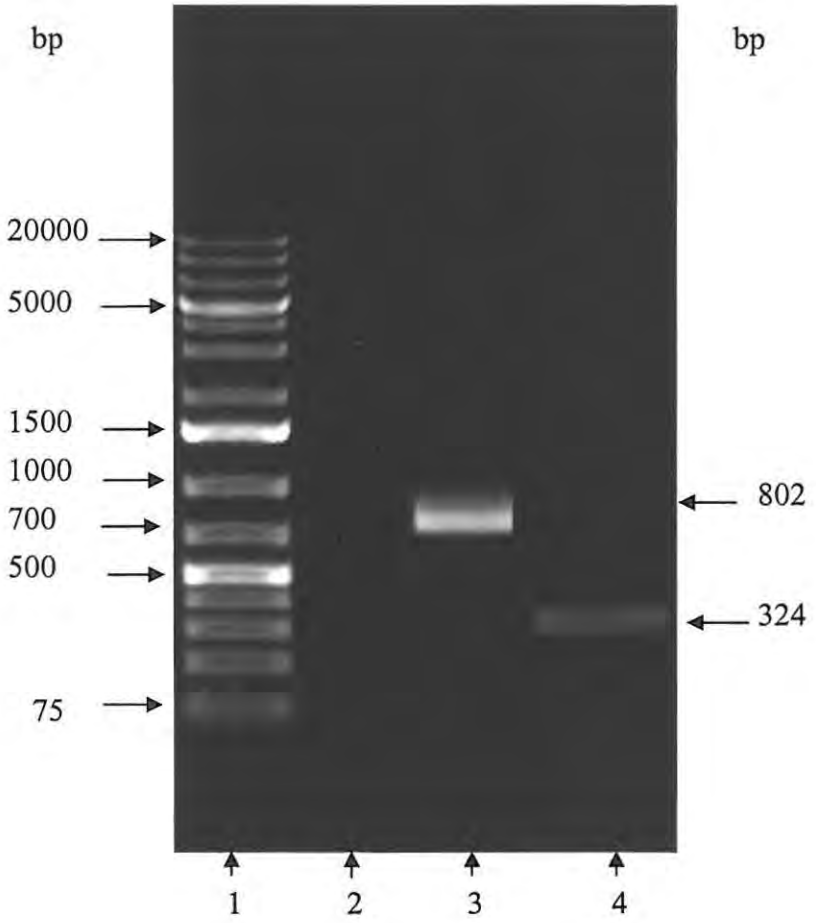


Figure 3.2: Dung amplification product of the *rbc*L gene (802 bp) from the black rhinoceros from the GFRR. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in I X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: negative control (no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

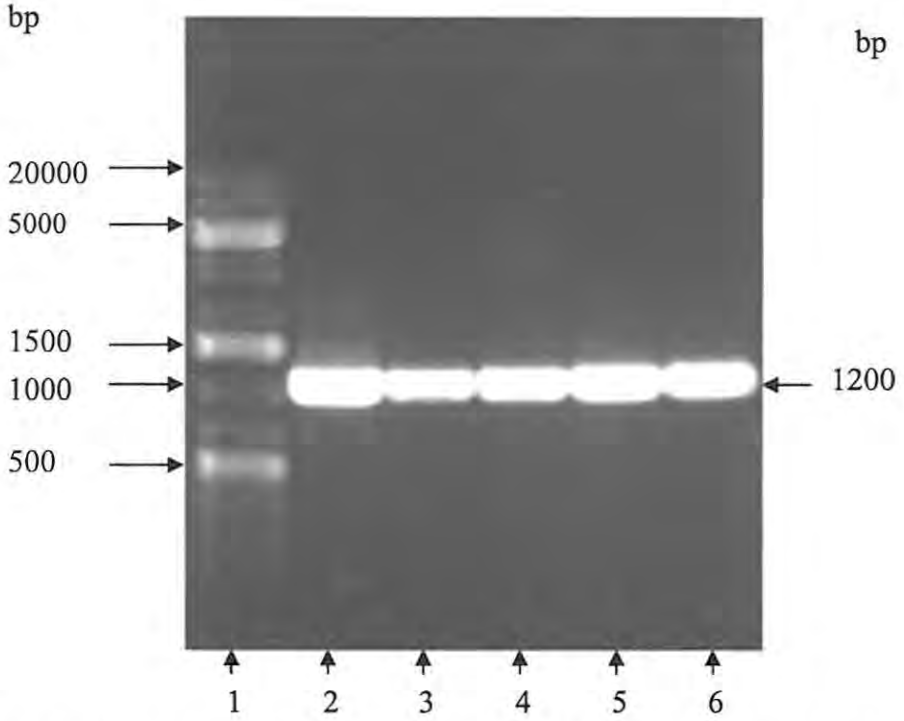


Figure 3.3: Amplification of the *rbc*L gene (802 bp) cloned into the pGem-T Easy vector using M13 primers. The DNA was electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromode in I X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2 to 6: Plasmid PCR products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

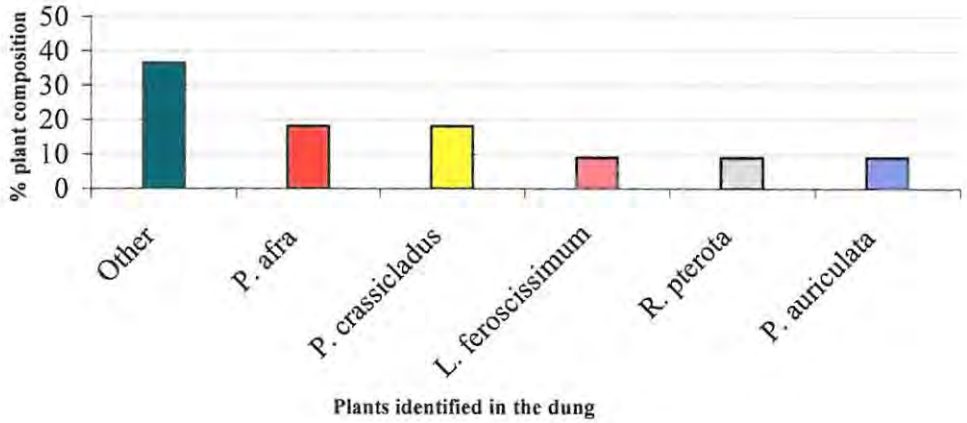


Figure 3.4: Plants species identified in the dung samples of the black rhinoceros using PCR, cloning and sequencing techniques.

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.

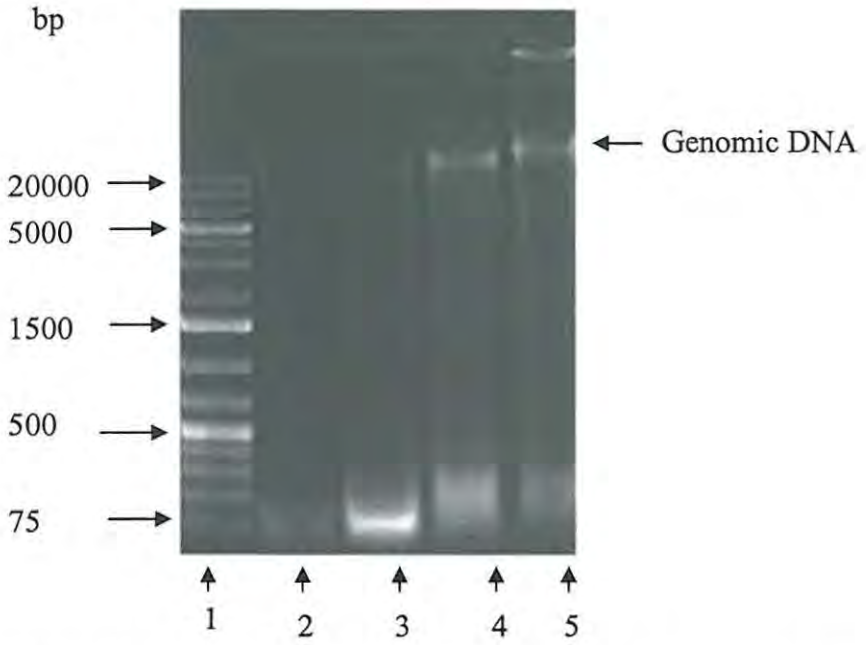


Figure 3.5: Resolution of the seasonal genomic DNA from four black rhinoceros dung samples. DNA was the electrophoresed at 12 V/cm for 25 min using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide in 1 X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: Summer dung sample, Lane 3: Autumn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

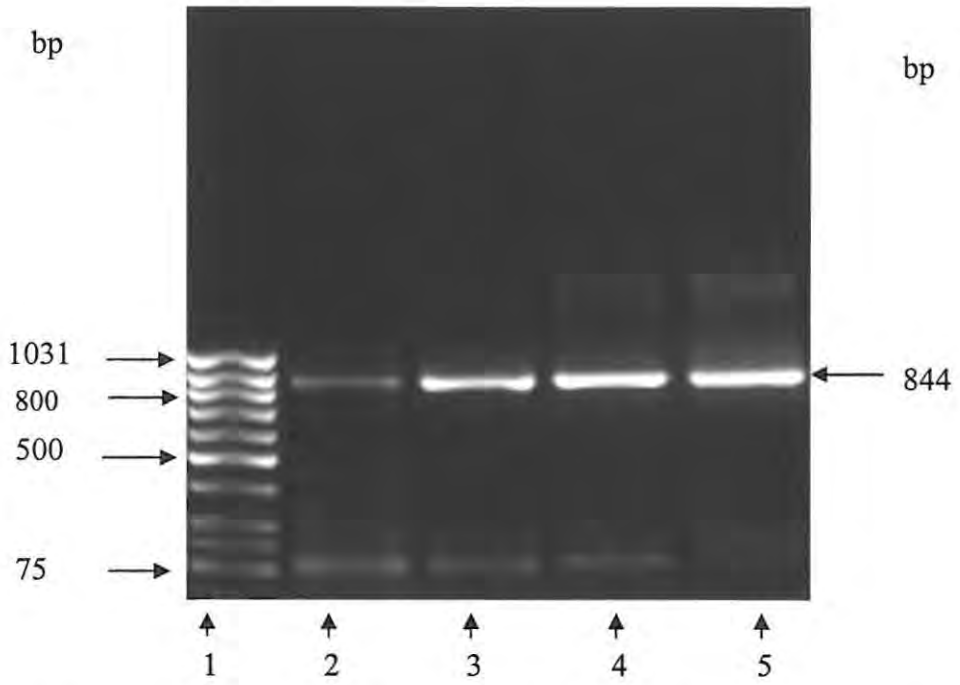


Figure 3.6: Amplification of a partial *rbc*L gene from the dung of the black rhinoceros using modified primers. DNA was resolved using 1% agarose gel and stained with 0.4 µg/ml ethidium bromide at 12 V/cm for 25 min in 1 X TAE buffer. Lane 1: 0.75 - 20 kb DNA ladder, Lane 2: Summer, Lane 3: Autumn,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

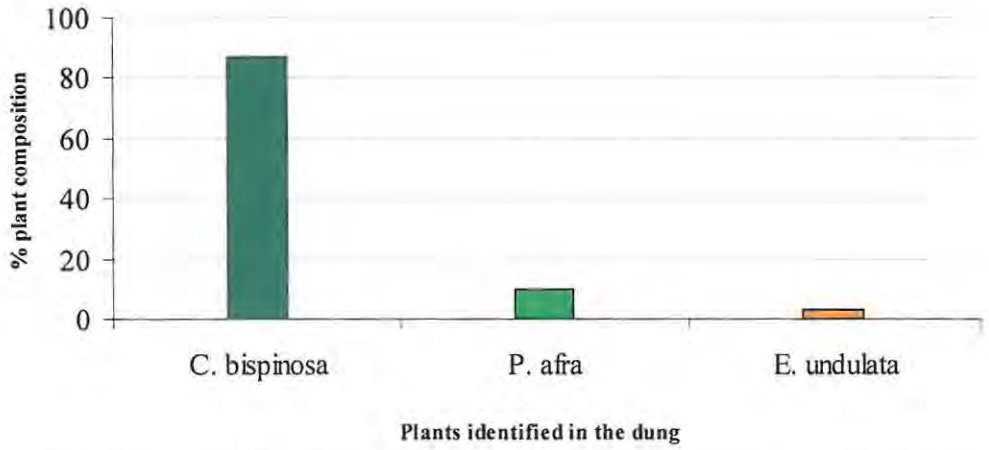


Figure 3.7: Identification of plants in a summer dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

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.

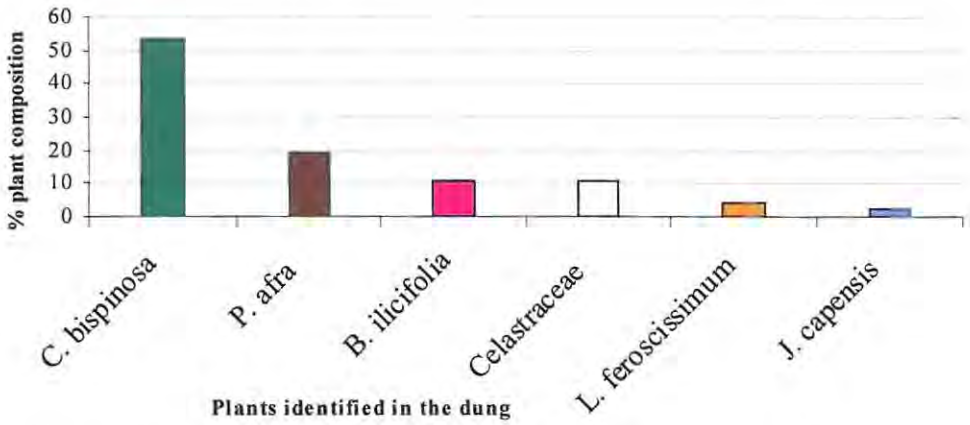


Figure 3.8: Identification of plants in an autumn dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

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.

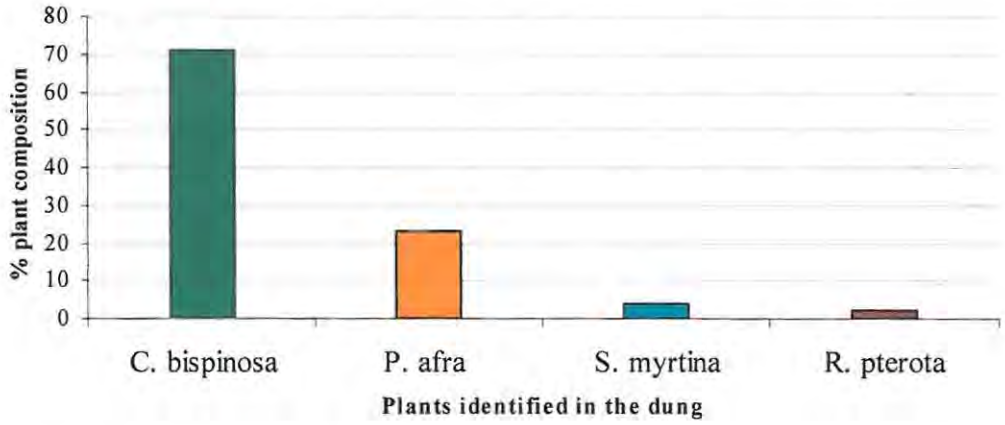


Figure 3.9: Identification of plants in a winter dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

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.

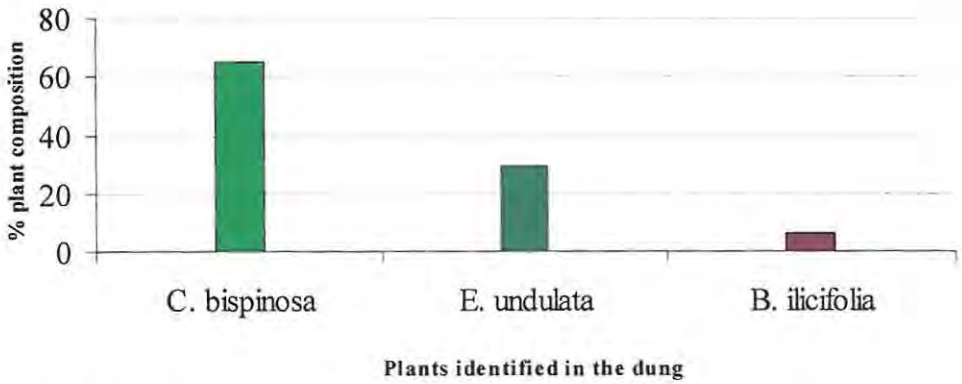


Figure 3.10: Identification of plants in a spring dung sample of the black rhinoceros from sequences generated by a Genome Sequencer FLX System (GS FLX).

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.

CHAPTER FOUR
ANTIOXIDANT CAPACITY OF DIETARY PLANTS OF THE BLACK RHINOCEROS

4.1 Introduction

The antioxidant activity of phenolic compounds is mainly due to the redox properties that allow them to act as reducing agents, oxidizing agents, hydrogen donators and metal chelators (Villaño *et al.*, 2005; Tawaha *et al.*, 2007). A compound can transfer a hydrogen atom or single electron to reduce an oxidant. The antioxidant activity of phenolic compounds depends mainly on the structural configuration of the molecule, such as the number and position of the available hydroxyl groups (Paixão *et al.*, 2007).

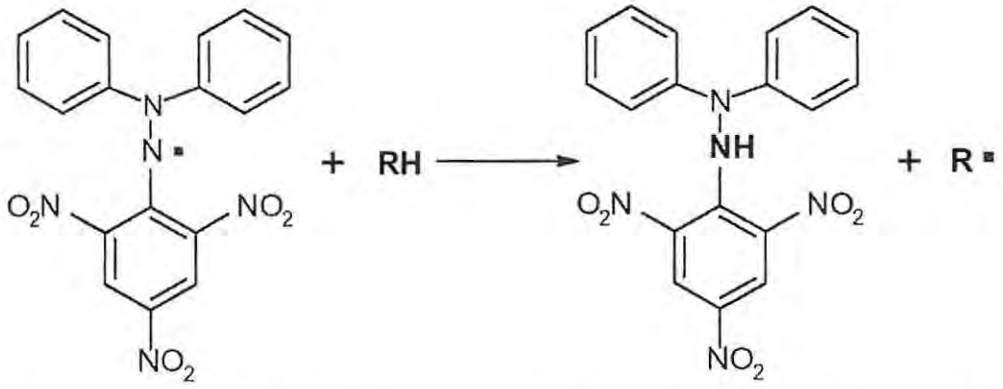
Many phenolic compounds have been assayed for their antioxidant capacity using different analytical methods such as 2,2-azinobis (3-ethyl benzothiazolium-6-sulfonic acid) ($ABTS^{\cdot+}$), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and the ferric reducing antioxidant potentials (FRAP) (Villaño *et al.*, 2005; Paixão *et al.*, 2007). These assays use different chemical reactions, principles and experimental conditions, which may lead to variable results when attempting to compare assays (Paixão *et al.*, 2007).

Certain limitations such as slow reaction rates (Brand-Williams *et al.*, 1995), solubility problems of certain antioxidants and possible interference from organic acids may be encountered when these assays are used (Fukumoto and Mazza, 2000). Furthermore, absolute values of antioxidant activities may vary from one study to another, causing difficulty when comparing single compounds even when the same method is used (Re *et al.*, 1999; Paixão *et al.*, 2007). Another difficulty is that the antioxidant activities of pure phenolic compounds are expressed in different terms and are therefore difficult to compare (Villaño *et al.*, 2005; Paixão *et al.*, 2007). The main limitation in using antioxidant activity assays is that they may not be representative of antioxidant activities *in vivo* (Villaño *et al.*, 2007; Choi *et al.*, 2007). However, antioxidant activity assays are useful as indicators of the antioxidant capacity of dietary components prior to consumption (Villaño *et al.*, 2007).

Certain assays utilize free radical chromogens, which are quenched in the presence of antioxidants and may lead to a decrease in absorbance. Widely used chromogens are $ABTS^{\cdot+}$ and $DPPH^{\cdot}$, both of which show excellent stability under certain assay conditions and also show important differences in response to different antioxidants (Arnao, 2000; Samarth *et al.*, 2008). Alternatively, the Folin-Ciocalteu assay has been used to measure the total phenolics in various natural products and beverages (Davalos *et al.*, 2003; Katalinic *et al.*, 2006; Tawaha *et al.*, 2007; Paixão *et al.*, 2007; Villaño *et al.*, 2007). The FRAP assay directly measures the redox potential of an antioxidant (Halvorsen *et al.*, 2002).

4.2 The $DPPH^{\cdot}$ method

DPPH is a violet-coloured stable free radical that was discovered by Goldschmidt and Renn (1922) (cited by Ionita, 2005) and is used as a colorimetric reagent for redox reactions. This reagent does not react with oxygen and can be kept indefinitely with little decomposition. It has been used in a variety of investigations such as the determination of antioxidant properties of amines, phenols and natural compounds such as vitamins, plant extracts and medicinal drugs. When the DPPH radical is reduced, its decolourisation can be measured at room temperature and at a wavelength of 515 nm (Brand-Williams *et al.*, 1995; Arnao, 2000; Argolo *et al.*, 2004; Chen *et al.*, 2006; Paixão *et al.*, 2007). The reduction of the $DPPH^{\cdot}$ depends on the ability of a substance or a complex mixture of substances to donate either hydrogen atoms or electrons to the radical. Reduction of $DPPH^{\cdot}$ may lead to the formation of a free radical ($R^{\cdot}$) and a reduced form of $DPPH^{\cdot}$ as shown in Fig. 4.1. The free radical produced can undergo further reactions if it is not completely eliminated. The decolourization of the $DPPH^{\cdot}$ is an indication of the number of DPPH molecules reduced by the antioxidant molecule (Arnao, 2000; Paixão *et al.*, 2007).



diphenylpycrylhydrazyl (radical) diphenylpycrylhydrazine (nonradical)

Figure 4.1: Structure of a DPPH radical undergoing reduction by an antioxidant molecule (adapted from Paixão *et al.*, 2007).

The DPPH radical method is rapid and simple, when the DPPH$^{\cdot}$ is dissolved in an organic solvent, which does not interfere with the DPPH assay. Interference could lead to underestimation of the effectiveness of certain antioxidant compounds in the reaction mixture (Brand-Williams *et al.*, 1995; Arnao, 2000; Paixão *et al.*, 2007).

The mechanism by which antioxidants react with the DPPH$^{\cdot}$ may differ and the scavenging activity of different compounds depends on the structure of the antioxidant (Brand-Williams *et al.*, 1995). Furthermore, certain antioxidants react quickly while others react more slowly with DPPH$^{\cdot}$ due to its stability. As a result, kinetic assays may be useful for slow reacting compounds although the reaction rate may not be linear (Brand-Williams *et al.*, 1995; Bondet *et al.*, 1997; Paixão *et al.*, 2007).

4.3 The $ABTS^{\cdot+}$ method

The ABTS assay is rapid and requires limited technical experience to enable the processing of a large number of samples (Paixão *et al.*, 2007). $ABTS^{\cdot+}$ is a stable cation chromogen free radical, which can be generated using either manganese dioxide or potassium persulfate (Miller and Rice-Evans, 1997a; Denis *et al.*, 2004). This radical is soluble in both aqueous and organic solutions, thus, both hydrophobic and hydrophilic compounds can be measured readily (Arnao, 2000; Re *et al.*, 1999; Nenaids *et al.*, 2004). This radical can be employed against a wide range of

compounds such as plant extracts, beverages and biological fluids (Re *et al.*, 1999; Nenaids *et al.*, 2004; Villaño *et al.*, 2005; Katalinic *et al.*, 2006).

The ABTS radical assay is based on the ability of an antioxidant to donate a hydrogen atom to the radical cation. The reduction of blue-green $ABTS^{\cdot+}$ cation is measured by a decrease in absorption at a wavelength of 734 nm (Miller and Rice-Evans, 1997a & b; Re *et al.*, 1999; Nenaids *et al.*, 2004). Different researchers have modified the assay by dissolving the $ABTS^{\cdot+}$ in inorganic solvents or phosphate buffered saline (PBS). This may be the reason for the lack of consistency of the results between various laboratories (Nenaids *et al.*, 2004; Villaño *et al.*, 2005). Furthermore, an antioxidant reduces $ABTS^{\cdot+}$ in a manner dependent on the time of the reaction, on the antioxidant activity of the sample and concentration of antioxidant (Miller and Rice-Evans, 1997a & b; Re *et al.*, 1999).

4.4 The FRAP assay

This assay directly measures reductants in a sample (Halvorsen *et al.*, 2002). The method was initially used to measure plasma antioxidants (Benzie and Strain, 1996) and was further used to assay pure compounds (Pulido *et al.*, 2000). Many phenolic compounds, including plant extracts can be assayed for their ability to reduce ferric ion-2,4,6-tri-2-pyridyl-s-triazine (Fe^{3+}-TPTZ) complex to ferrous ion-2,4,6-tri-2-pyridyl-s-triazine (Fe^{2+}-TPTZ) complex. This method takes advantage of the oxidation-reduction abilities of reductants and is applicable to both aqueous and alcohol extracts of different plants (Pulido *et al.*, 2000; Wong *et al.*, 2006). However, a possible limitation of this method is that it does not react with thiols found in plants, which emphasizes the lack of accuracy for certain crude samples (Halvorsen *et al.*, 2002).

4.5 The Folin-Ciocalteu assay

This assay is rapid, reproducible and can be used to assess the phenolic content of a wide range of samples at the same time (Paixão *et al.*, 2007; Tawaha *et al*, 2007). In principle, the assay measures the ability of various phenolic compounds to reduce a

phosphotungstate-phosphomolybdate complex, which results in the formation of blue coloured reaction products that can be measured at 765 nm (Paixão *et al.*, 2007). The reduction of the phosphotungstate-phosphomolybdate complex depends on the number of phenolic hydroxyl groups available (Singleton and Rossie, 1965; Katalinic *et al.*, 2006; Tawaha *et al.*, 2007).

Not all the phenolic compounds in an extract may contribute equally to the overall reduction of the Folin reagent as certain extracts with a high number of phenolic compounds may give either low or high values (Tawaha *et al.*, 2007). A limitation of this assay is that it is not specific when estimating the total phenolic content in a mixed sample (Paixão *et al.*, 2007). Furthermore, interference from sugars, lipids and chlorophyll may overexpress the total phenolic compounds obtained in a given sample under certain circumstances (Luximon-Ramma *et al.*, 2002; Dávalos *et al.*, 2003).

In the present investigation, plants found to be part of the diet of the black rhinoceros by molecular analysis of dung and by observation studies, were assessed for their antioxidant capacity using DPPH, ABTS and FRAP assays. The Folin-Ciocalteu method was used to measure the total phenolic content of these plants. This study attempted to ascertain a possible relationship between black rhinoceros browse and the antioxidant capacity of this browse.

4.6 Materials and methods

4.6.1 Reagents

Methanol, hydrochloric acid, acetic acid, sodium chloride, potassium chloride, sodium phosphate, potassium dihydrogen phosphate and sodium carbonate (anhydrous) were purchased from Merck (South Africa). 2,2-diphenyl-1-picryl-hydrazyl, gallic acid, Folin–Ciocalteu's phenol reagent, 2,2-azinobis (3-ethyl benzothiazolium-6-sulfonic acid), potassium persulfate, ferric chloride, 2,4,6-trypyridil-*s*-triazine and ferrous sulfate heptahydrate were purchased from Sigma Aldrich (Germany).

4.6.2 Preparation of plant extracts for antioxidant activity assays

Leaves of twenty-five plant species reported to form part of the diet of the black rhinoceros were collected from the GFRR. These plants were assessed for their free radical scavenging activities, ferric reducing abilities and for total phenolic content. The plants were identified and classified at the Selmer Schonland Herbarium in Grahamstown.

The leaves of the plants collected were placed in separate Ziploc plastic bags (10 x 25 cm) with silica gel distributed between the layers of the leaves and each sample was stored at –20°C. Extraction of the fresh plant material was carried out as soon as possible after collection. Plants leaves (1 g wet weight per sample) were placed in 3 ml of 80% methanol and ground using a mortar and a pestle. The homogenates were transferred to 25 ml tubes and shaken in the dark at 20°C for 48 h. The homogenates were stored in the dark at -20°C for 48 h to ensure maximum extraction of phenolics (Awika *et al.*, 2003). Samples were equilibrated to room temperature and centrifuged (15 500 x g, 15 min at room temperature) and the supernatant decanted. Each residue was extracted twice (3 ml 80% methanol) and centrifuged (15 500 x g, 5 min at room temperature) until the extracts were clear. The concentrations of the extracts were measured as the actual dry weight of plant material (1 g wet weight dried at 60°C for 12 h) per volume, as described by Halvorsen *et al.* (2002).

4.6.3 Preparation of working reagents for the assay of antioxidant capacity and phenolic content

4.6.3.1 The DPPH˙ reagent

The study investigated the antioxidant activities of different plant extracts using DPPH˙ (25 mg/l), prepared fresh in 80% methanol using 45 ml eppendorf tubes and protected from light. The assay for DPPH˙ scavenging activity was initially developed using an extract from *Carissa haematocarpa*. The stability of the DPPH˙ radical solution was monitored throughout the experiments and the initial absorbance of DPPH˙ was ± 0.600.

4.6.3.2 The $ABTS^{\cdot+}$ reagent

$ABTS^{\cdot+}$ was generated by oxidation of ABTS salts with potassium persulfate (1:0.5, volume/volume (v/v); both were prepared in ddH_2O. A 7 mM ABTS solution was added to 2.45 mM of potassium persulfate. The reaction mixture was left to stand in the dark at room temperature for 12 h before performing the antioxidant assays. Prior to analysis of these extracts, the $ABTS^{\cdot+}$ stock solution was diluted with PBS, pH 7.4, containing 150 mM NaCl and dissolved to an absorbance of 0.700 ± 0.012 at 734 nm. The $ABTS^{\cdot+}$ cation solution was equilibrated at 30°C for 30 min and kept at 4°C until required. The ABTS absorbance was monitored for stability throughout the analysis.

4.6.3.3 The FRAP reagent

The FRAP reagent was prepared daily (for compatibility with the previous analysis) by mixing 300 mM acetate buffer (pH 3.6), 10 mM of 2,4,6-TPTZ solution in 40 mM hydrochloric acid and 20 mM of ferric chloride (Fe_3Cl) solution in proportions of 10:1:1 (v/v/v), respectively. The FRAP reagent was used at 37°C.

4.6.3.4 Folin-Ciocalteu reagent

For the preparation of the standard stock solution, 0.05 g of gallic acid was dissolved in 80% methanol to a final volume of 10 ml and was stored at 4°C. For the preparation of working solutions of gallic acid, the following volumes: 0, 10, 20, 30, 50 and 100 µL were pipetted separately and diluted to a total volume of 1 ml using 80% methanol. A standard calibration curve was constructed using six concentrations: 0, 50, 100, 150, 250, 500 mg/l gallic acid. A 0.2 N Folin-Ciocalteu reagent was prepared

in ddH_2O. Anhydrous sodium carbonate (Na_2CO_3) was prepared as a 20% solution by dissolving 20 g of salt in 80 ml of ddH_2O. The solution was heated for 30 min at 60°C, cooled to room temperature and the volume made up to 100 ml.

4.6.4 Antioxidants and total phenolic assays

4.6.4.1 The DPPH radical scavenging activity assay

The effect of plant extracts on the $DPPH^{\cdot}$ absorbance was estimated following the procedure described by Brand-Williams *et al.* (1995) and Dávalos *et al.* (2003), with modification. Stock solutions from the crude extracts were prepared as 1 mg/ml working solutions in 80% methanol. Separate concentrations in a range between 0 and 1000 µg/ml of an ascorbic acid standard and plant extracts were prepared. Assays were performed in a microtitre plate reader and absorbance read on a Powerwave spectrophotometer (Bio-Tek Instruments, Inc). Briefly, to a 96-well microtitre plate, 5.2 µl of plant extract was added to 194.8 µl of the 25 mg/l of DPPH radical. The decrease in absorbance was measured at 515 nm for 30 min at 1 min intervals at room temperature. The procedure was followed for all plant extracts, a methanol blank and ascorbic acid controls. For each concentration (0, 250, 500, 750 and 1 000 µg/ml) of the samples under the study, the reaction kinetics were plotted and the percentage of the $DPPH^{\cdot+}$ remaining was calculated as follows:

$$\% \ DPPH^{\cdot}_{rem} = [(A_{515\ nm})_{Sample}/(A_{515\ nm})_{Blank}] \times 100$$

where, $(A_{515\ nm})_{Sample}$ is the absorbance of the test sample and $(A_{515\ nm})_{Blank}$ is the absorbance of the methanol blank sample.

4.6.4.2 The ABTS radical cation antioxidant activity assay

The ABTS radical cation decolourization assay was performed, with minor modifications, according to Re *et al.* (1999). The ABTS radical was warmed to 30°C before use. After incubation of 200 µl $ABTS^{\cdot+}$ cation with 2 µl plant extract at varying concentrations between 0 and 1 000 µg/ml, the absorbance reading was taken immediately for 6 min at 30°C using the Powerwave spectrophotometer (Bio-Tek Instruments, Inc.). The ascorbic acid standard was assessed as described above, and ddH_2O was used as a blank. The percent antioxidant activity was calculated using the following equation:

% decolourization = $[(A_0-A_x)/(A_0) \times 100]$,

where, A_0 is the absorbance of the blank solution, and A_x is the absorbance of the test sample (Lima *et al.*, 2005).

4.6.4.3 Ferric reducing antioxidant power (FRAP) assay

The total antioxidant capacity of each plant extract was determined according the original procedure of Benzie and Strain (1996) using the FRAP assay as modified by Wong *et al.* (2006). In brief, 200 µl of FRAP reagent was heated to 37°C, followed by the addition and mixing of 20 µl of ddH_2O and 6.67 µl of plant extract. Absorbance readings were taken immediately at 593 nm. The temperature was maintained at 37°C and the reaction monitored for 30 min at 1 min intervals. A methanolic solution of ferrous sulfate (0.028 - 0.28 mg/l) was used to generate a linear calibration curve. The results were expressed as mg Fe (II)/g of dry weight of plant material. Ascorbic acid was used as a reference standard in the assay.

4.6.4.4 Assays of the total phenolic content of the plant extracts

Total phenolic content was estimated by the Folin-Ciocalteu colourimetric method, based on the procedure of Waterhouse (no date), using gallic acid as the standard phenolic compound. The method in brief was as follows: for each calibration, 20 µl of the standard phenolic compound, plant extracts and the blank reagent were pipetted into separate tubes, and to each tube, 1.58 ml of ddH_2O was added, followed by the addition of 100 µl of Folin-Ciocalteu reagent. The reaction mixtures were gently mixed and incubated at room temperature for 8 min, after which 250 µl of Na_2CO_3 anhydrous (20% solution) was added and mixed gently by pipetting. The reaction solutions were incubated at 40°C for 30 min with continuous shaking at 100 rpm. The absorbance readings of the resulting blue coloured solutions were measured at 765 nm using the Powerwave spectrophotometer. The results were expressed as gallic acid equivalents (GAE)/g dry weight.

4.6.5 Statistical analysis

All experiments were performed in triplicate for separately prepared sample concentrations. The data are expressed as mean ± standard deviations (SD).

4.7 Results

Due to the relatively high sample number, selected results of plants showing high, medium and low antioxidant activities are represented graphically. A complete data set of the different antioxidant assays is tabulated for all plants at a concentration of 1 mg/ml. Three antioxidant assays are reported.

4.7.1 DPPH radical scavenging activity assay

Each plant extract was tested for the free radical scavenging activities against the DPPH radical using 0, 250, 500, 750 and 1 000 µg/ml as working concentrations. A dose-response characteristic profile of three selected methanolic plant extracts of the twenty-five plants investigated on the scavenging of the DPPH$^{\cdot}$ is presented in Fig. 4.2.

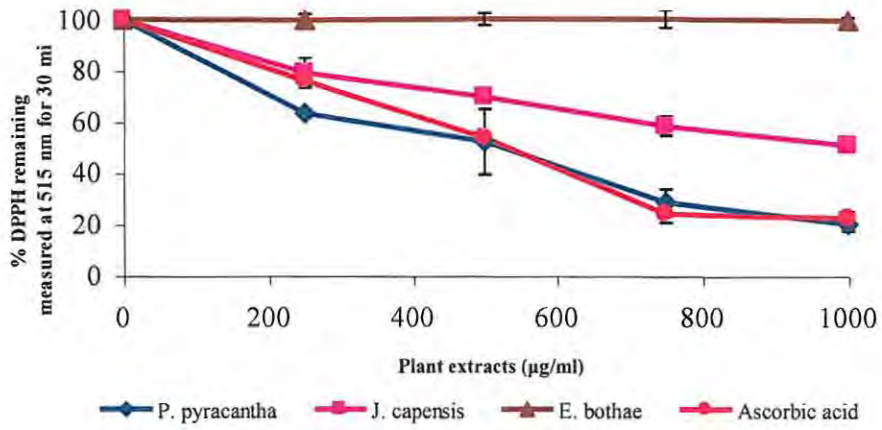


Figure 4.2: Dose-response profiles of three selected plant extracts on the scavenging of DPPH radicals. Ascorbic acid was used as a reference standard. The results are the mean of triplicate determinations ± SD.

The ascorbic acid and *P. pyracantha* had similar scavenging abilities of the DPPH$^{\cdot}$ at 1 000 µg/ml. *P. pyracantha* extracts had higher antioxidant activity relative to other plants investigated, and showed a higher activity at 250 µg/ml compared to ascorbic acid at the same concentration. Similar trends were observed for the other plant species investigated, however, these activities were lower than *P. pyracantha*.

As an example, *O. mucrunata*, is presented in Fig. 4.3 showing the kinetics of DPPH˙ scavenging at the concentrations investigated over 30 min.

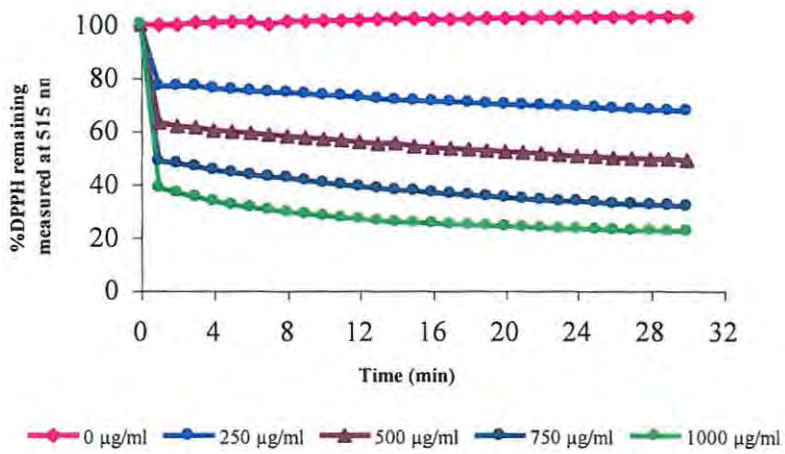


Figure 4.3: Kinetic reaction showing the percent DPPH˙ remaining when monitored for 30 min at different concentrations of *O. mucrunata* extract. The results are the mean of triplicate determinations.

The scavenging of DPPH˙ was most noticeable in the first minute of the assay. The plant extract continued to scavenge the DPPH˙ at a much slower rate, and the reactions were relatively stable after 30 min. A 30 min incubation time was chosen for the DPPH assay for the remaining plants. The scavenging ability was highest at the higher extract concentrations.

The plant antioxidant activities, as determined by the DPPH assay at 1 000 µg/ml, are presented in Table 4.1.

Table 4.1: Percentage DPPH radical remaining after incubation with extracts from various plants reported as part of the diet of black rhinoceroses of the GFRR evaluated at 1 000 µg/ml for 30 min.

Plant family	Plant species	% DPPH˙ remaining
Celastraceae	***Putterlickia pyracantha***	**20.15 ± 2.65**
Anacardiaceae	***Ozoroa mucrunata***	**21.51 ± 4.18**
Euphorbiaceae	***Phyllanthus verrucosus***	**23.43 ± 2.79**
Celastraceae	***Maytenus capitata***	**26.77 ± 1.36**
Ebenaceae	***Euclea undulata***	**33.83 ± 1.64**
Euphorbiaceae	***Jatropha capensis***	**50.83 ± 0.96**
Fabaceae	*Schotia afra*	62.45 ± 1.92
Apocynaceae	*Carissa bispinosa*	63.62 ± 2.37
Celastraceae	*Maytenus heterophylla*	77.50 ± 1.50
Tiliaceae	*Grewia robusta*	77.65 ± 3.76
Anacardiaceae	*Rhus pterota*	82.82 ± 2.29
Apocynaceae	*Carissa haematocarpa*	87.09 ± 4.21
Portulacaceae	*Portulacaria afra*	87.34 ± 2.12
Asteraceae	*Tarchonanthus camphoratus*	89.73 ± 5.10
Salvadoraceae	*Azima tetracantha*	90.38 ± 1.28
Solanaceae	*Lycium ferocissimum*	91.19 ± 2.62
Plumbaginaceae	*Plumbago auriculata*	91.43 ± 0.35
Asteraceae	*Brachylaena ilicifolia*	91.76 ± 4.11
Capparaceae	*Capparis sepiaria*	91.97 ± 1.76
Asparagaceae	*Protasparagus crassicladus*	92.06 ± 1.32
Bignoniaceae	*Rhygozum obovatum*	92.21 ± 3.25
Asparagaceae	*Protasparagus africanus*	93.84 ± 0.50
Boraginaceae	*Ehretia rigida*	94.92 ± 3.02
Asparagaceae	*Protasparagus suaveolens*	95.97 ± 0.63
Euphorbiaceae	*Euphorbia bothae*	99.71 ± 1.30
Positive control	**Ascorbic acid**	**22.34 ± 3.13**

The mean values of triplicate assays ± SD. Plant are listed in the decreasing order of the antioxidant activity. The plants, which scavenged DPPH˙ at ≥ 50%, are shown in bold.

The percentage DPPH$^{\cdot}$ remaining in the reaction mixture ranged from 99.71% to 20.05%. The plants with the highest antioxidant activities were *P. pyracantha* (Celastraceae) > *O. mucrunata* (Anacardiaceae) > *P. verrucosus* (Euphorbiaceae) > *M. capitata* (Celastraceae) > *E. undulata* (Ebenaceae) and *J. capensis* (Euphorbiaceae). The ascorbic acid had a value of 22.34% ± 3.13.

4.7.2 ABTS radical cation decolourization assay

The ability of plant extracts and the ascorbic acid to scavenge the ABTS$^{\cdot+}$ was monitored for 6 min using the concentrations of 0, 250, 500, 750 and 1 000 µg/ml. Fig 4.4 shows the dose-response characteristics of three selected plant extracts.

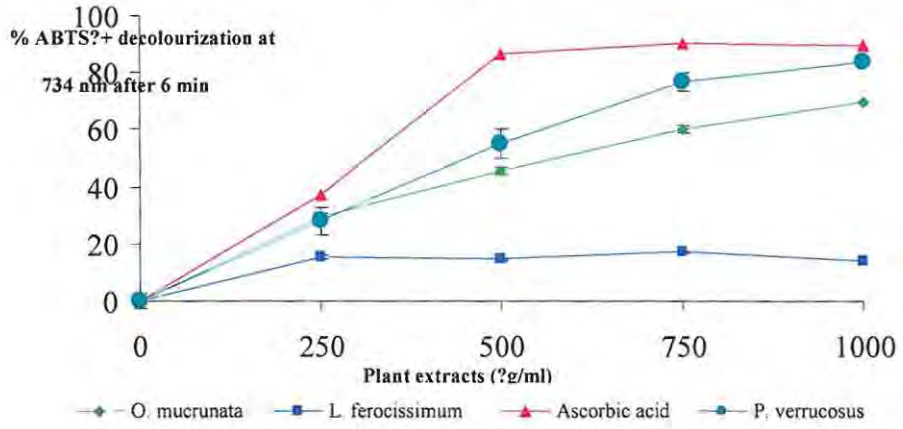


Figure 4.4: Effect of concentration on the decolourization of ABTS$^{\cdot+}$ absorbance by the plant extracts. The reaction was monitored for 6 min and the results are the mean of triplicate determinations.

Plant extracts showed an increasing antioxidant activity with an increase in the concentration, with the ascorbic acid completely scavenging the ABTS$^{\cdot+}$ at 500 µg/ml. The majority of the plant extracts had a limited effect on the scavenging of the ABTS$^{\cdot+}$ (Table 4.2). *O. mucrunata* was used as an example to represent kinetic profiles of antioxidant activities against the ABTS$^{\cdot+}$ when evaluating incubation time at different concentrations as shown in Fig. 4.5.

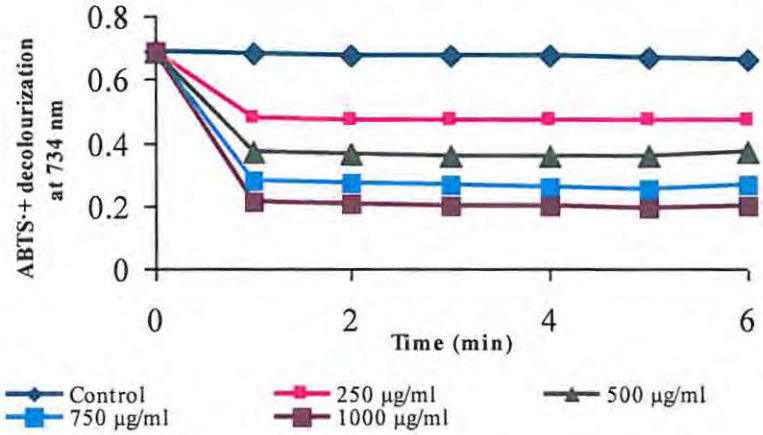


Figure 4.5: Decolourization of $ABTS^{\cdot+}$ by methanolic extracts of *O. mucrunata* when monitored for 6 min with 1 min intervals. The results are the mean of triplicate assays.

Each concentration of the extract reacted similarly with the $ABTS^{\cdot+}$ and completed the reaction in 1 min. The degree of scavenging of the $ABTS^{\cdot+}$ increased with increasing concentration of the extracts.

Three different plant extracts, each showing high, medium and low $ABTS^{\cdot+}$ scavenging activity are presented in Fig. 4.6. The antioxidant activities were evaluated at 1000 µg/ml for 6 min with 1 min intervals.

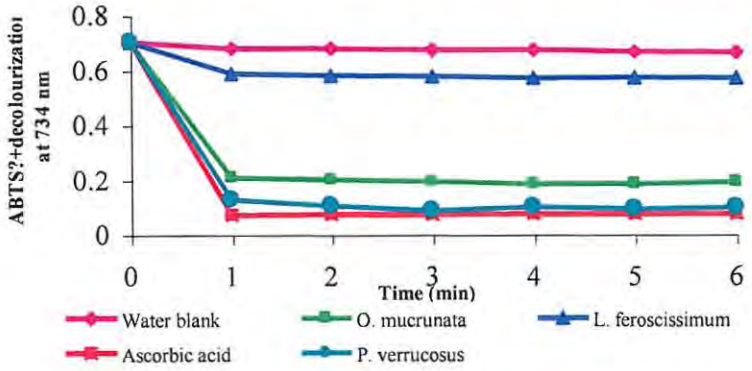


Figure 4.6: Decolourization of $ABTS^{\cdot+}$ absorbance by methanolic extracts of *O. mucrunata, P. verrucosus* and *L. ferocissimum*. Ascorbic acid was used as a positive control and water blank was as a negative control. The results are the mean of triplicate assays.

The percentage decolourization of the $ABTS^{\cdot+}$ absorbance of the twenty-five plants investigated is presented in Table 4.2. Each plant extract (1 000 µg/ml) was evaluated for scavenging activities.

Table 4.2: Percentage of ABTS radical scavenged by various plants reported to be part of the diet of black rhinoceroses of the GFRR evaluated at 1 000 µg/ml for 6 min.

Plant family	Plant species	% ABTS decolourization
Euphorbiaceae	***Phyllanthus verrucosus***	**84.05 ± 3.27**
Celastraceae	***Putterlickia pyracantha***	**80.46 ± 2.15**
Anacardiaceae	***Ozoroa mucrunata***	**70.19 ± 3.62**
Euphorbiaceae	***Jatropha capensis***	**47.26 ± 11.55**
Ebenaceae	***Euclea undulata***	**44.02 ± 6.96**
Celastraceae	***Maytenus capitata***	**41.14 ± 2.85**
Anacardiaceae	*Rhus pterota*	34.26 ± 2.26
Fabaceae	*Schotia afra*	20.17 ± 3.90
Portulacaceae	*Portulacaria afra*	20.03 ± 1.40
Celastraceae	*Maytenus heterophylla*	18.17 ± 3.24
Apocynaceae	*Carissa bispinosa*	17.92 ± 1.99
Asparagaceae	*Protasparagus crassicladus*	14.72 ± 1.92
Tiliaceae	*Grewia robusta*	14.64 ± 1.61
Solanaceae	*Lycium ferocissimum*	14.04 ± 5.26
Asteraceae	*Brachylaena ilicifolia*	10.56 ± 1.70
Plumbaginaceae	*Plumbago auriculata*	10.45 ± 1.98
Cappararaceae	*Capparis sepiaria*	8.53 ± 0.32
Bignoniaceae	*Rhygozum obovatum*	6.38 ± 1.64
Boraginaceae	*Ehretia rigida*	5.75 ± 3.39
Asteraceae	*Tarchonanthus camphoratus*	5.32 ± 3.04
Apocynaceae	*Carissa haematocarpa*	4.68 ± 1.65
Asparagaceae	*Protasparagus africanus*	4.58 ± 0.25
Salvadoraceae	*Azima tetracantha*	4.13 ± 1.65
Asparagaceae	*Protasparagus suaveolens*	4.11 ± 4.17
Euphorbiaceae	*Euphorbia bothae*	2.24 ± 2.01
Positive control	Ascorbic acid	89.3 ± 2.25

The results are mean values of triplicate assays ± SD. Plants are listed in decreasing order of the antioxidant activities. The plants, which scavenged $ABTS^{\cdot+}$ at ≥ 40%, are shown in bold.

The percentage of the $ABTS^{\cdot+}$ scavenged due to antioxidant activities of the plant extracts ranged from 84.05% to 2.24%. Plant extracts showing the strongest antioxidant activities were *P. verrucosus* (84.05 ± 3.27), *P. pyracantha* (80.46 ± 2.15) and *O. mucrunata* (70.19 ± 3.62).

4.7.3 Ferric reducing antioxidant power assay

The plant extracts were also investigated for their ability to reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. A linear calibration curve of ferrous sulfate in the range of 0.028-0.28 mg/l was constructed (Appendix P) to determine the reduction of Fe^{3+}-TPTZ. The ferric reducing potentials were initially measured using different extract concentrations over 30 min, and a representative sample of the plant extracts is shown in Fig. 4.7.

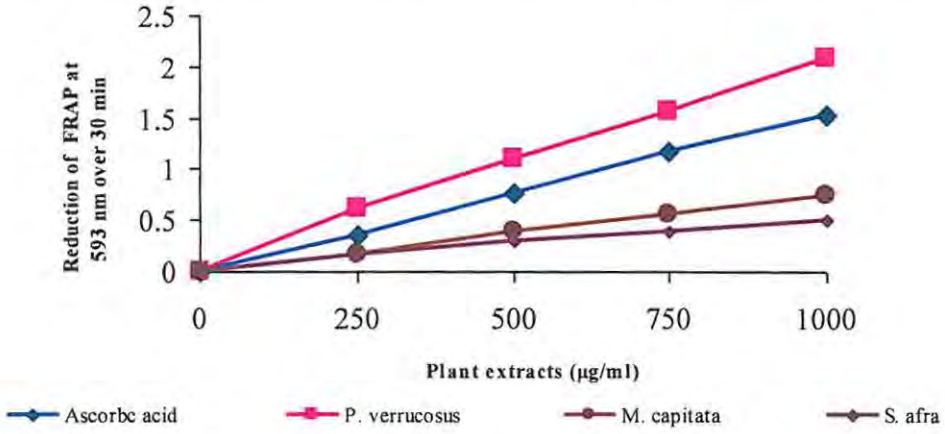


Figure 4.7: Concentration-dependent activities of the three-selected plant extracts on the reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. Ascorbic acid was used as the reference standard. The results are the mean of triplicate assays.

The profile shows that an increasing concentration of plant extract leads to greater reduction of the Fe^{3+}-TPTZ. The greatest activity was shown by *P. verrucosus*.

The kinetic reactions of each 1 000 µg/ml plant extract, when monitored at 593 nm for 30 min, are presented in Fig. 4.8.

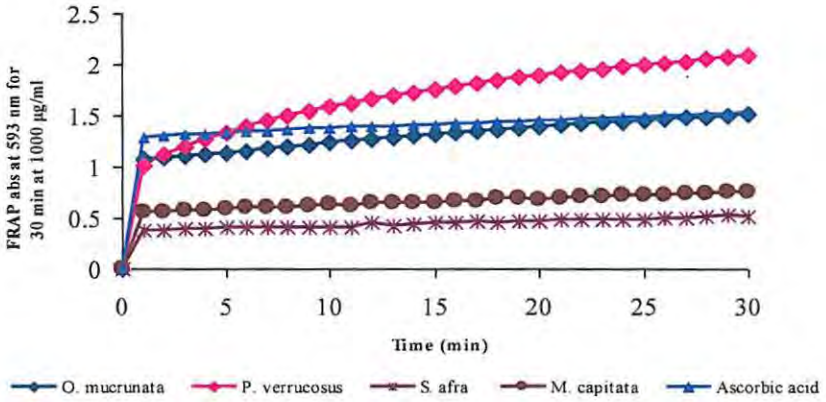


Figure 4.8: Kinetic reactions of each 1 000 µg/ml plant extract on the reduction of the Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex when monitored for 30 min. Ascorbic acid was used as a reference standard. The results are the mean of triplicate assays.

High reduction potential was observed for *P. verrucosus* and the *O. mucrunata*. Both the reactions were time dependent. Ascorbic acid standard completed the reduction reaction rapidly (within 1 min).

All plant extracts reacted rapidly with the FRAP reagent in the first minute, but varied in ferric reducing capacities. Plant extracts of 1 000 µg/ml were used to assess the ferric reducing capacities for 30 min and the results are presented in Table 4.3.

Table 4.3: Ferric reducing antioxidant power (FRAP) assay of GFRR plant extracts evaluated 1 000 µg/ml for 30 min.

Plant family	Plant species	mg Fe (II)/g dry weight
Euphorbiaceae	***Phyllanthus verrucosus***	**62.92 ± 2.50**
Anacardiaceae	***Ozoroa mucrunata***	**45.55 ± 5.97**
Celastraceae	***Putterlickia pyracantha***	**45.13 ± 3.20**
Ebenaceae	***Euclea undulata***	**24.84 ± 1.35**
Celastraceae	***Maytenus capitata***	**23.00 ± 2.01**
Euphorbiaceae	***Jatropha capensis***	**18.89 ± 0.66**
Fabaceae	*Schotia afra*	15.65 ± 0.69
Apocynaceae	*Carissa bispinosa*	14.36 ± 0.13
Celastraceae	*Maytenus heterophylla*	11.12 ± 0.30
Tiliaceae	*Grewia robusta*	8.80 ± 0.85
Anacardiaceae	*Rhus pterota*	7.36 ± 0.36
Asparagaceae	*Protosparagus crassicladus*	5.87 ± 0.84
Bignoniaceae	*Rhygozum obovatum*	5.32 ± 0.88
Asteraceae	*Tarchonanthus camphorates*	4.89 ± 0.59
Asparagaceae	*Protosparagus suaveolens*	4.68 ± 0.54
Capparaceae	*Capparis sepiaria*	4.50 ± 0.62
Apocynaceae	*Carissa haematocarpa*	3.99 ± 0.35
Portulacaceae	*Portulacaria afra*	3.95 ± 0.48
Boraginaceae	*Ehretia rigida*	3.82 ± 0.26
Plumbaginaceae	*Plumbago auriculata*	3.57 ± 0.08
Salvadoraceae	*Azima tetracantha*	3.16 ± 0.82
Asparagaceae	*Protosparagus africanus*	2.97 ± 0.65
Asteraceae	*Brachylaena ilicifolia*	2.27 ± 0.59
Euphorbiaceae	*Euphorbia bothae*	2.05 ± 1.54
Solanaceae	*Lycium ferocissimum*	0.20 ± 0.02
Positive control	Ascorbic acid	46.10± 3.36

The results are represented by the mean triplicate assays ± SD. Plants are listed in decreasing order of Fe^{3+}-TPTZ reducing capacity. Plants with the highest reducing capacity are shown in bold.

The strongest antioxidant capacities were exhibited by *P. verrucosus* > *O. mucrunata* > *P. pyracantha* > *E. undulata* and *M. capitata*.

4.7.4 Total phenolic content of plant extracts

The total phenolic content of the twenty-five plant extracts was studied using the Folin-Ciocalteu assay, and the results are presented in Table 4.4.

A linear calibration curve of gallic acid, in the range between 0 and 500 mg/l was constructed as shown in Appendix Q. There was a wide variation in the total phenolic content of the plant samples investigated, which is shown in Table 4.4. The phenolics ranged from 5.66 to 33.87 GAE/g dry weight. *P. verrucosus* > *J. capensis* > *M. capitata* > *E. undulata* > *C. bispinosa* > *O. mucrunata and P. crassicladus* showed the highest total phenolic content (> 20 GAE/g dry weight).

Table 4.4: Total phenolic content found in various plants reported as part of the diet of the black rhinoceros of the GFRR as determined by the Folin-Ciocalteu assay.

Plant family	Genus and species	GAE/g dry weight
Euphorbiaceae	***Phyllanthus verrucosus***	**33.87 ± 1.73**
Euphorbiaceae	***Jatropha capensis***	**30.97 ± 0.12**
Celastraceae	***Maytenus capitata***	**24.81 ± 1.21**
Ebenaceae	***Euclea undulata***	**24.05 ± 0.18**
Apocynaceae	***Carissa bispinosa***	**23.90 ± 0.77**
Anacardiaceae	***Ozoroa mucrunata***	**22.89 ± 2.29**
Asparagaceae	***Protasparagus crassicladus***	**22.01 ± 0.86**
Anacardiaceae	*Rhus pterota*	19.57 ± 0.84
Fabaceae	*Schortia afra*	17.89 ± 0.46
Apocynaceae	*Carissa haematocarpa*	17.42 ± 0.55
Celastraceae	*Maytenus heterophylla*	17.29 ± 1.21
Plumbaginaceae	*Plumbago auriculata*	16.42 ± 3.39
Asparagaceae	*Protasparagus suaveolens*	15.79 ± 0.75
Bignoniaceae	*Rhygozum obovatum*	15.04 ± 1.46
Asteraceae	*Tarchonanthus camphoratus*	14.33 ± 1.74
Tiliaceae	*Grewia robusta*	14.11 ± 0.70
Euphorbiaceae	*Euphorbia bothae*	13.45 ± 1.06
Celastraceae	*Putterlickia pyracantha*	13.40 ± 1.53
Capparaceae	*Capparis sepiaria*	12.29 ± 1.74
Asteraceae	*Brachylaena ilicifolia*	12.10 ± 0.99
Portulacaceae	*Portulacaria afra*	10.61 ± 1.01
Boraginaceae	*Ehretia rigida*	10.40 ± 1.10
Asparagaceae	*Protasparagus africanus*	10.03 ± 1.41
Salvadoraceae	*Azima tetracantha*	7.65 ± 0.87
Solanaceae	*Lycium ferocissimum*	5.66 ± 0.40

The results are represented as the mean of triplicate assays ± SD. Plants are listed in the decreasing order of the phenolic contents. GAE = gallic acid equivalents (mg/l).

4.7.5 Correlation of antioxidant activities of the twenty-five plant extracts with total phenolic content

The total phenolic content of each plant extract was plotted against their antioxidant activity when investigated using the DPPH, ABTS and FRAP assayed as shown in Fig. 4.10 A, B and C.

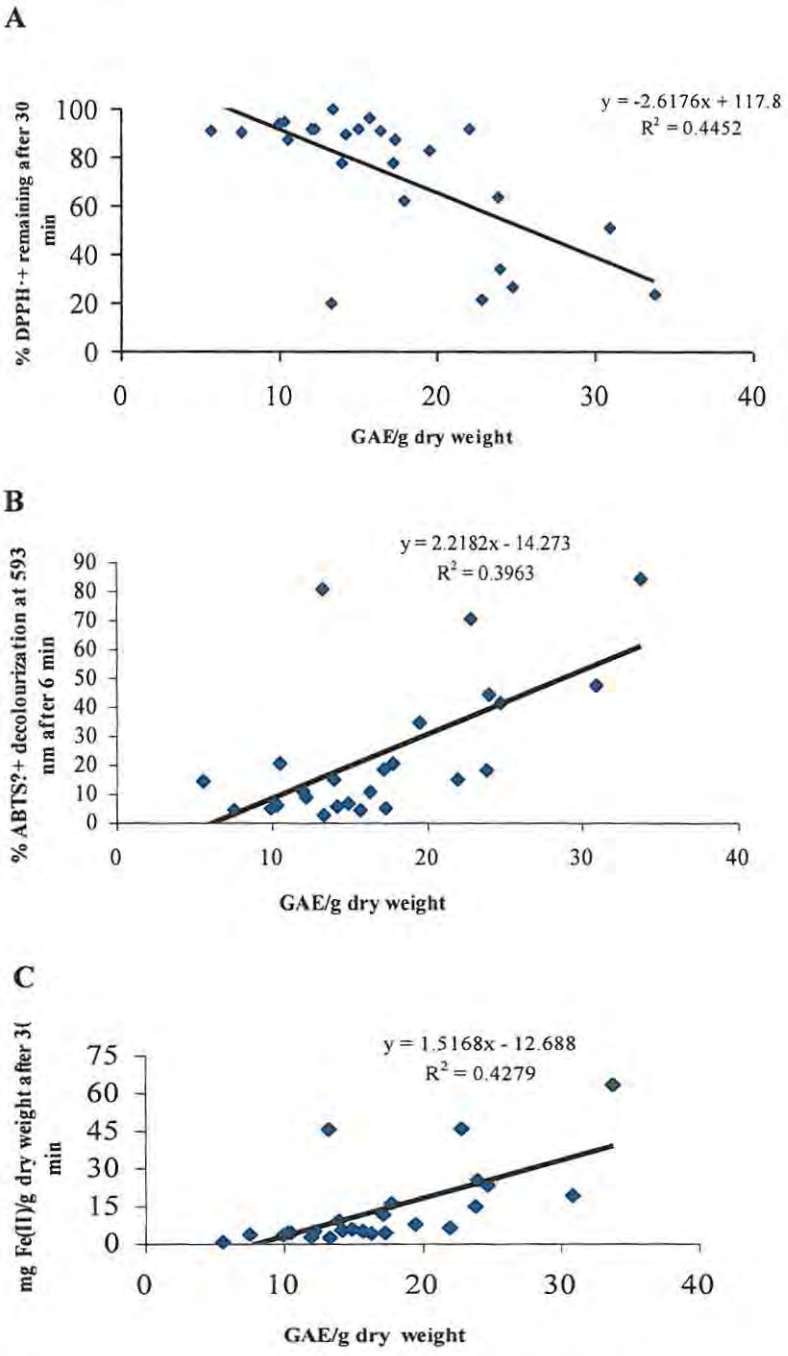


Figure 4.9: Correlation between the total phenolic content and the antioxidant activity of the twenty-five plants evaluated using the A) DPPH, B) ABTS and C) FRAP assays.

The correlation coefficient of the graphs depicted in Fig. 4.9 suggests a low level of correlation between antioxidant activity and phenolic content. However, the graphical representations of the antioxidant assays against total phenolic content indicate that the majority of plants with a low antioxidant activity also have a low phenolic content. Although less pronounced, plants with high antioxidant activities also have relatively high total phenolic content. The exception is *P. pyracantha* which in all three assays had a high antioxidant capacity, but a relatively low total phenolic content of 13.4 GAE/g dry weight.

4.8 Discussion

4.8.1 Preparation of plant extracts

The present study has investigated the antioxidant capacity and the total phenolic content of twenty-five plants of the GFRR, many of which form part of the diet of the black rhinoceros of the GFRR. In order to prepare plant extracts for antioxidant assays, researchers have used different procedures such as freeze-drying or using a rotary evaporator to concentrate plant extracts (Karawita *et al.*, 2005; Chen *et al.*, 2006; Suddhuraju and Becker, 2007). These procedures could result in a loss of active compounds. Furthermore, since antioxidant compounds are water or lipid soluble, or bound to plant cell walls, their recovery depends on the solvent used for extraction (Karawita *et al.*, 2005; Chen *et al.*, 2006; Suddhuraju and Becker, 2007). It is also important to consider how the extraction procedure could influence the qualitative estimation of antioxidant capacities from plant materials (Prakash, 2001; Choi *et al.*, 2007). This is particularly important when different assays are used to measure the antioxidant capacities of plant extracts, as it could result in incorrect interpretation of the antioxidant activity found in the extracts (Prakash, 2001; Karawita *et al.*, 2005; Chen *et al.*, 2006; Suddhuraju and Becker, 2007; Choi *et al.*, 2007).

This study has used a simple methanol extraction as used by Karawita *et al.* (2005) and Wong *et al.* (2006). Extraction from plants such as *E. bothae*, was problematic, and may have led to an underestimation of the antioxidant capacity of these plants.

4.8.2 The DPPH$^{\cdot}$ assay

Free radicals can be produced through normal metabolic processes or by phagocytes, and can be deleterious when produced in large quantities in biological systems. For this reason, antioxidant molecules may be useful in inhibiting the reactivity of these radicals (Cheeseman and Slater, 1993; Martinez-Cayuela, 1995), but the activity of antioxidants against free radicals in biological systems is not entirely clear. As a result, exogenous free radicals have become useful in assessing the antioxidant activities of samples *in vitro* as representative of a biological system (Brand-Williams, 1995; Chen *et al.*, 2006). This has led to a variety of assays to test for the antioxidant

activities of pure compounds and plant extracts using radicals such as DPPH (Chen *et al.*, 2006, Paixão *et al.*, 2007).

Little information is available on the antioxidant capacities of plants forming part of the diet of the black rhinoceros of the GFRR. This study has investigated the antioxidant activities of these plant extracts against the DPPH$^{\cdot}$. The degree of the DPPH$^{\cdot}$ decolourization depends on the nature of antioxidants in the samples (Brand-Williams, 1995) and the stability of DPPH$^{\cdot}$ in reacting with antioxidants in plant extracts was similar to the studies of Brand-Williams *et al.* (1995) and Bondet *et al.* (1997). The present study resulted in using a reaction time of 30 min to allow for any slow reacting antioxidants in the plant extracts to react with the DPPH$^{\cdot}$.

By using a 30 min reaction time, differences in the scavenging capacities of plant extracts against the DPPH$^{\cdot}$ were noticed when assessed in four concentration ranges as shown in Fig. 4.2. Most plants reached the steady state when assayed for 30 min at 1 000 µg/ml shown in Fig. 4.3. Therefore, this concentration was used as the standard concentration for further analysis of radical scavenging activities of plant extracts. The convenience of using one concentration for assessing antioxidant activities of samples is in accordance with the study of Pulido *et al.* (2000) and Mosquera *et al.* (2007).

The percentage of the remaining DPPH$^{\cdot}$, after scavenging by the plant extracts, ranged between 99.71% and 20.05% and demonstrated that if extracts react for 30 min, most reached steady state. Plants having the best antioxidant activities are shown in a decreasing order in Table 4.1. The best six plants were *P. pyracantha* > *O. mucrunata* > *P. verrucosus* > *M. capitata* > *E. undulata* > *J. capensis*. *P. pyracantha* and *O. mucrunata* exhibited higher antioxidant activity than the ascorbic acid at the concentration tested. Among all plant families investigated, two species from the Celastraceae (*P. pyracantha* and *M. capitata*) and two species from Euphorbiaceae (*P. verrucosus* and *J. capensis*) exhibited strong radical scavenging activities. The results suggest that different plant extracts have a variety of antioxidant compounds with different scavenging activities and reaction rates against DPPH$^{\cdot}$. These results are in agreement with the study of Mosquera *et al.* (2007), where plants showing high

scavenging activities against the DPPH radical often were from the family Euphorbiaceae.

The higher antioxidant activity of some plants, when compared to the ascorbic acid at the concentration investigated, would best be explained if compounds in the extracts were isolated and characterized individually. The scavenging activity of the plant extracts is most likely due to the presence of phenolics and flavonoids, which are able to donate hydrogen or electrons. However, detailed mechanisms of the scavenging abilities of plants extracts are not clear and remain for further investigations (Kefalas *et al.*, 2003; Rai *et al.*, 2006). The results obtained in this study suggest that the $DPPH^{\cdot}$ assay is useful in assessing the antioxidant activities of plants browsed by the black rhinoceros.

4.8.3 The $ABTS^{\cdot+}$ assay

The $ABTS^{\cdot+}$ method has been used widely to evaluate the radical scavenging activity of antioxidants of plant extracts as well as pure of compounds. The method is based on the ability of antioxidant molecules to donate hydrogen to the ABTS radical (Miller and Rice-Evans, 1997a and b; Lima *et al.*, 2005; Choi *et al.*, 2007).

The present study investigated the ability of select plants, forming part of the diet of the GFRR black rhinoceros, to scavenge $ABTS^{\cdot+}$. The scavenged $ABTS^{\cdot+}$ is presented as the percentage decrease of absorbance at 734 nm in Fig. 4.4. An example of selected plant extracts showing an increased response to different concentrations over time is shown in Fig. 4.5.

Some studies have experienced a biphasic reaction with the initial reaction being due to the most active compounds reacting rapidly with the radical. The remaining compounds which are less reactive in the sample, give a second slower reaction (Re *et al.*, 1999; Villaño *et al.*, 2004). Due to the plant extracts being relatively crude, and potentially containing a range of unknown antioxidants, this phenomenon may be occurring in these investigations.

The antioxidant activities of the plant extracts against the $ABTS^{\cdot+}$ ranged from 84.05% to 2.24% as shown in Table 4.2. The strongest antioxidant activity was

obtained in the plant extracts from three different families. These were *P. verrucosus* (84.05% ± 3.27), *P. pyracantha* (80.46% ± 2.15) and *O. mucrunata* (70.19% ± 3.62). Most plant extracts contain flavonoids, which may contribute to a wide range of antioxidant activities (Garcia-Alonso *et al.*, 2004; Samarth *et al.*, 2008). According to Shimoi *et al.* (1996), plant flavonoids, which show antioxidant activity *in vitro*, may contribute as antioxidants *in vivo*.

This investigation prepared the $ABTS^{\cdot+}$ in an aqueous solution. Therefore, the hydrophobic compounds in the plant extracts may be underestimated for their antioxidant activities. For this reason, it would be informative to know which compounds contribute to the antioxidant activities. However, the $ABTS^{\cdot+}$ assay gave comparable results to the other antioxidant assays used to assess the antioxidant activities of the selected GFRR plants.

4.8.4 The FRAP assay

The FRAP assay was initially used to test for plasma antioxidant capacity (Benzie and Strain, 1996). This assay was modified to measure the reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex by plant extracts and pure compounds (Pulido *et al.*, 2000; Wong *et al.*, 2006). The ability of antioxidants to increase the absorbance of the FRAP reagent depends on an oxidation-reduction reaction changing the colourless Fe^{3+}-TPTZ to a blue coloured Fe^{2+}-TPTZ complex (Katalinic *et al.*, 2006; Suddhuraju and Becker, 2007). The antioxidant activity is related to the reducing potentials of the test compounds (Firuzi *et al.*, 2005; Paixão *et al.*, 2007).

The present study investigated the ferric reducing antioxidant capacities of GFRR plants. This assay is known to produce conflicting results depending on the solvent used (Pulido *et al.*, 2000). This study standardized the experimental procedure by dissolving the test samples in 80% methanol as described by Firuzi *et al.* (2005).

The plants that showed the strongest ferric reducing capacities were *P. verrucosus* > *O. mucrunata* > *P. pyracantha* > *E. undulata* and *M. capitata*. The ascorbic acid standard showed high ferric reducing capacities when compared to the majority of the plant extracts, but showed less reducing capacity when compared to *P. verrucosus*.

4.8.5 Total phenolic content

This study estimated the total phenolic content in each of the twenty-five plant extracts using the Folin-Ciocalteu assay because phenolic compounds are reported to be good sources of antioxidant activities (Duan *et al.*, 2006; Katalinic *et al.*, 2006). Methanol was used for the phenolic extractions, and for this reason, most compounds extracted in this study were hydrophilic.

Bandoniene and Murkovic (2002) showed that plants contain a diverse group of phenolic compounds such as phenolic acids, hydroxycinnamic acid derivatives and flavonoids. According to Singleton and Rossie (1965), phenolic compounds respond differently in the Folin-Ciocalteu assay due to the number of available phenolic hydroxyl groups that could be oxidized. The total phenolic content obtained from the twenty-five plants investigated ranged from 5.66 to 33.87 GAE/g dry weight. The results are presented in Table 4.4, which indicates seven plants having a phenolic content >20 GAE/g dry weight.

E. undulata, *M. capitata*, *O. mucrunata* and *P. crassicladus* are preferred browse of the black rhinoceros (Brown *et al.* 2003), which had a high phenolic content as shown in the present study. Plants such as *P. verrucosus*, *J. capensis* and *P. pyracantha*, which also had a high phenolic content, have not been reported as preferred browse of the black rhinoceros. Muya and Oguge (2000) reported that the black rhinoceros prefers plants with a low quantity of secondary chemical substances.

It is very difficult to conclude that the obtained values of phenolic compounds in this study are due entirely to the oxidizable phenolic hydroxyl groups present in the plant extracts as certain substances such as chlorophyll, lipids, aromatic amines and sugar derivatives might influence the assays (Singleton and Rossie, 1965; Luximon-Ramma *et al.*, 2002; Dávalos *et al.*, 2003). Furthermore, the influence of the phenolic content found in the preferred diet of the black rhinoceros needs further investigations to determine its effect.

4.8.6 Correlation between antioxidant activities, phenolic content and black rhinoceros browse

Antioxidant studies typically use different analytical methods to determine antioxidant activity due to the differences in the chemical reactions involved in the assays (Paixão *et al.*, 2007). This study has established the *P. verrucosus, O. mucrunata, E. undulata, M. capitata* and *J. capensis* as the best five plants exhibiting high antioxidant activities and phenolic content between the four assays. Extracts exhibiting high antioxidant activities by one method showed good antioxidant activity by the other methods. This is also applicable to plants with low antioxidant activities and phenolic contents when evaluated alone (Fukumoto and Mazza, 2000). An exception is the plant extract *P. pyracantha* which showed high antioxidant activity for all three antioxidant assays, but had a relatively low total phenolic content. *P. crassicladus* and *C. bispinosa* had total phenolic contents of 22.01 and 23.90 GAE/g dry weight, respectively. However, both these plant extracts had low antioxidant activities when assayed by the three antioxidant assays.

Ausland *et al.* (2002) and Brown *et al.* (2003) reported *E. bothae, G. robusta, J. capensis, P. auriculata, A. tetracantha, E. undulata, O. mucrunata* and *R. obovatum* as the preferred plants in the diet of the black rhinoceros. In our study using the *rbc*L gene as a molecular marker, *C. bispinosa* was the most frequently identified plant in the dung. Of the preferred browse, three plant species, namely *J. capensis, E. undulata* and *O. mucrunata* were identified as having high antioxidant activity. The *C. bispinosa* showed a relatively a high total phenolic content when compared to the other plants investigated. However, most of the plants identified as the black rhinoceros browse in the GFRR had low antioxidant and total phenolic content. From the results of this study, and the diet preferences observed by Ausland *et al.* (2002) and Brown *et al.* (2003), it might be considered that the black rhinoceros prefers plants with low antioxidant activities. However, this cannot be stated with any certainty. The black rhinoceros appears to select plants that give it a balanced diet, which may be the reason for the successful propagation of this species on the GFRR.

CHAPTER FIVE
CONCLUSION

A large increase in the number of black rhinoceroses, from only four introduced to the GFRR in 1986, motivated the present study to investigate molecular methods of diet determination, and the evaluation of the nutritional value of their browse. As an attempt to contribute to the conservation and management of the black rhinoceros in the GFRR, this study adopted molecular and biochemical approaches to characterize the diet of the black rhinoceros.

Initially plant DNA was amplified and the *rbc*L gene partially sequenced from a variety of plants collected in the GFRR. These plants were chosen based partly on the results of observational studies of browsing black rhinoceros. The *rbc*L sequences of these plants served as a reference collection from which unknown sequences obtained later in the study could be compared and identified. Difficulties in extracting DNA from certain plants were overcome by using a modified CTAB extraction protocol. The addition of BSA to PCR reaction mixtures allowed for the PCR amplification of extracted DNA samples containing *Taq* polymerase inhibitors.

Of the twenty-three plants sequenced, sixteen were not identified when compared to plant sequences in the GenBank database. The sequences generated in this study will be deposited in the GenBank database once the plant specimens have been lodged with the Selmer Schonland Herbarium in Grahamstown, which will be done in the near future. The 802 bp *rbc*L gene sequence was unable to discriminate between *C. haematocarpa*, *M. nemorosa* and *P. verrucosus*, as these sequences proved to be identical. For the discrimination of these plants, a sequence greater than 1 kb may be required to yield sufficient variation, as suggested by Kress *et al.* (2005).

A proof of concept study of a single dung sample identified *C. bispinosa*, *P. afra, P. crassicladus, P. auriculata*, *R. pterota* and *L. ferocissimum*. Extraction of DNA from dung is challenging as the DNA is often degraded. Extracting a representative sample is difficult to ascertain, especially from a heterogeneous dung sample. However, in this study, the *rbc*L gene from DNA extracted from dung was amplified,

cloned and sequenced. Ideally, when using this approach, a larger number of clones should be sequenced to allow for a more conclusive result. However, this is labour intensive and time consuming, therefore in this study, it was decided to explore GS FLX sequencing.

GS FLX sequencing has the potential to generate a large number of sequences from a single sample. Sequence data was obtained on dung samples collected t four different seasons and *C. bispinosa* was identified as the most prevalent plant in these dung samples. Although GS FLX generated a larger data set, a higher number of sequences were expected. The reason offered for these disappointing results is that the clonal DNA amplification product was too long. In future, primers should be designed to amplify a suitably variable segment of the *rbc*L gene, approximately 450 bp in length.

The main reason for using the *rbc*L gene in this study was that it is plant specific and being a chloroplast gene it is highly conserved, yet has sufficient variation to allow for differentiation between plants. Although the DNA from dung was often degraded, the template was of sufficient quality for PCR amplification of the 802 bp of the *rbc*L gene. Future studies should utilize primers that amplify a shorter segment of DNA, and which covers a more variable region of the *rbc*L gene. It is most likely that this variable region would be on the 3′ end of the gene. It may be necessary also to design primers for specific plants, or families of plants, due to sequence variation between the plants. This would probably lead to investigations using multiplex PCR.

The PCR may have shown bias when amplifying plants from dung samples. For example, *E. bothae* was identified as the preferred browse of the black rhinoceros by Ausland and Sviepe, (2000) and by Brown *et al.* (2003) yet this plant was not identified in the dung samples analyzed by molecular techniques. In contrast, the *C. bispinosa* was identified in all four seasonal dung samples, but was not identified as a significant component of the diet from observation studies. The *C. bispinosa* is tough and woody while *E. bothae* is a succulent plant with soft tissues, making it possible that its DNA did not survive digestion. The identification of succulent soft tissue plants such as *E. bothae* in dung may be a limitation of the molecular approach.

Although certain plants were identified using both observational and molecular techniques, other plants such as *C. bispinosa* were only identified in this molecular study. As these methods identified different plants browsed by the black rhinoceros, it may be valuable to use both of these methods together for the identification of plants in the diet. It may also be of value to identify other plant specific genes or regions of DNA, to allow for an increased ability to differentiate between plants present in the black rhinoceros dung.

Plants identified in the dung or by observation studies of the black rhinoceros were assessed for antioxidant capacities and their total phenolic content. An important finding of this study is illustrated clearly from an investigation of the relative position of certain plant species in Tables 4.1 – 4.3 which present the results of three different antioxidant assays. It is particularly evident that it is the same six plants that have the highest antioxidant activity in each assay and, furthermore, five of these plants also show the highest phenolic content (Table 4.4). This is in spite of each assay being chemically different. Dávalos *et al.* (2003) reported on the antioxidant capacity of seven different commercial dietary antioxidant supplements evaluated by three different methodologies and showed different antioxidant activity patterns, depending on the method, yet a range of samples showed the same overall pattern. Other studies report that antioxidant activities of particular compounds may vary from one study to another, even when the same assay is used making comparative data difficult (Re *et al.*, 1999; Paixao *et al.*, 2007). No one method is entirely suitable for predicting antioxidant capacity of an extract and the use of more than one method is recommended, suggesting the use of caution in the interpretation of results (Luximon-Ramma *et al.*, 2002).

A comparison of the major plant species indicates that only a few plants with high antioxidant capacity and phenolic content are favoured by the black rhinoceros. The black rhinoceros appear to prefer plants of relatively low antioxidant and total phenolic content. However, based upon these studies alone, it would be premature to suggest that the black rhinoceros selects plants for its diet based mainly on these criteria.

REFERENCES

Amin, R., Bramer, M., & Emslie, R. (2003). Intelligent data analysis for conservation: experiments with rhino horn fingerprint identification. *Knowledge-Based Systems, **16**,* 329-336.

Anderson, I., & Buckland, A. (2008). Structure and function of Rubisco. *Plant Physiology and Biochemistry, **46**,* 275-291.

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods in testing antioxidant activity. *Analyst, **127**,* 183-198.

Argolo, A. C. C., Sant, A. E. G., Pletsch, M., & Coelho, L. C. B. B. (2004). Antioxidant activity of leaf extracts from *Bauhinia monandra. Bioresource Technology, **95**,* 229-233.

Arnao, M. B. (2000). Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. *Trends in Food Science and Technology, **11**,* 419-421.

Ashley, M. V., Melnick, D. J., & Western, D. (1990). Conservation genetics of the black rhinoceros (*Diceros bicornis*): Evidence from the mitochondrial DNA of the three populations. *Conservation Biology, **4**,* 71-77.

Ausland, C., & Sviepe, A. M. (2000). Foraging behavior of black rhinoceros (*Diceros bicornis minor*) in the Great Fish River Reserve, South Africa. *Master of Science dissertation*, Department of Animal Science, Agricultural University of Norway. Oslo.

References

Ausland, C., Sviepe, A. M., Ganqa, N., Raats, J., & Palmer, R. A. (2002). Feeding behaviour of the black rhinoceros (*Diceros bicornis*) in the Great Fish River Reserve. *Proceedings of the Fifth International Wildlife Ranching Conference*, University of Pretoria, Pretoria. 63-67; cited from Brown, D. H., Lent, P. C., Trollope, W. S. W., & Palmer A. R. (2003). Browse selection of black rhinoceros (*Diceros bicornis*) in two vegetation types of the Eastern Cape Province, South Africa, with particular reference to Euphorbiaceae. *Proceedings of the VIIth International Rangelands Congress*, 509-512.

Bandoniene, D., & Murkovic, M. (2002). Online-HPLC-DPPH screening for evaluation of radical phenol extracted from apples (*Malus domestica* L). *Journal of Agriculture and Food Chemistry*, ***50***, 2482-2487.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of 'Antioxidant Power": The FRAP assay. *Analytical Biochemistry*, ***239***, 70-76.

Berger, J. (1994). Science, conservation and black rhinoceros. *Journal of Mammalogy*, ***75***, 298-308.

Bergman, B., & Auer, B (1993), "Easy preps": A fast and easy minipreparation for analysis of recombinant clones in *E. coli*. *Biotechnology*, ***14***, 527-528

Bondet, V., Brand-Williams, W., & Berset, C. (1997). Kinetic and mechanisms of antioxidant activity using the DPPH$^{\cdot}$ free radical method. *Libensm.–Wiss.u-Technology*, ***30***, 609-615.

Boudet, A-M. (2007). Evolution and current status of research in phenolic compounds. *Phytochemistry*, ***68***, 2722-2735.

Bradley, B. J., Stiller, M., Doran-Sheehy, D. M., Harris, T., Chapman, C. A., Vigilant, L., & Poinar, H. (2007). Plant DNA sequences from feces: Potential means for assessing diets of wild primates. *American Journal of Primatology*, ***69***, 1-7.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Libensm.–Wiss.u-Technology*, ***28***, 25-30.

References

Brown, D. H., Lent, P. C., Trollope, W. S. W., & Palmer A. R. (2003). Browse selection of black rhinoceros (*Diceros bicornis*) in two vegetation types of the Eastern Cape Province, South Africa, with particular reference to Euphorbiaceae. *Proceedings of the VIIth International Rangelands Congress*, 509-512.

Bulte, E. H., & Horan, R. D. (2003). Habitat conservation, wildlife extraction and agricultural expansion. *Journal of Environmental Economics and Management*, ***45***, 109-12.

Cheeseman, K. H., & Slater, T. F. (1993). An introduction to free radical biochemistry. *British Medical Bulletin*, ***49***, 481-493.

Chen, F-A., Wu, A-B., Shieh, P., Kuo, D-H., & Hsieh, C-Y. (2006). Evaluation of the antioxidant activity of *Ruellia tuberose*. *Food Chemistry*, ***94***, 14-18.

Choi, Y., Jeong, H-S., & Lee, J. (2007). Antioxidant activity of methanolic extracts from grains consumed in Korea. *Food Chemistry*, ***103***, 130-138.

Cohn, J. P. (1988). Captive breeding for conservation. *Bioscience*, ***38***, 312-316.

Curtis, S. E., & Clegg, M. T. (1984). Molecular evolution of chloroplast DNA sequences. *Molecular Biology Evolution*, ***1***, 291-301.

Dávalos, A., Gómez-Cordovés, C., & Bartolome, B. (2003). Commercial dietary supplements assayed for their antioxidant activity by different methodologies. *Journal of Agriculture and Food Chemistry*, ***51***, 2512-2519.

De Garine-Witchatitsky, M., Fritz, H., Gordon, I. J., & Illius, A. W. (2004). Bush selection along foraging pathways by sympatric impala and greater kudu. *Ecologia*, ***141***, 66-75.

Deagle, B. E., Tollit, D. J., Jarman, S. N., Hindell, M. A., Trites, A. W., & Gales, N. J. (2005). Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Molecular Ecology*, ***14***, 1831-1842.

References

Denis, A., Boubekeur, K., Molinié, P., Léone, P., & Palvadeau, P. (2004). Synthesis, crystal structure determination and physical characterization of two new hybrids inorganic-organic salts associating the organic anion $ABTS^{2-}$ and its radical anion $ABTS^{\cdot-}$ to the transition metal cations Fe^{3+} or Cu^{2+}:$Fe_2O(ABTS)_2(H_2O)_{16}$ and $Cu(ABTS)_2.(H_2O)_8$. *Journal of Molecular Structure,* ***689***, 25-32.

Dickman, C. R., & Huang, C. (1988). The reliability of fecal analysis as a method for determining the diet of insectivorous mammals. *Journal of Mammalogy*, ***69***, 108-113.

Dierenfeld, E. S. (1994). Vitamin in exotics: Effects, evaluation and ecology. Nutrition through the life cycle. *Journal of Nutrition*, ***124***, 2579S-2518S.

Dierenfeld, E. S. (1997). Symposium on 'nutrition of wild and captive wild animals'. *Proceedings of the Nutrition Society*, ***56***, 989-999.

Dierenfeld, E. S., du Toit, R., & Miller, R. E. (1988). Vitamin E in captive and wild black rhinoceros (*Diceros bicornis*). Short communications. *Journal of Wildlife Diseases*, ***24***, 547-550.

Doyle, J. J., & Doyle, J. J. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, ***19***, 11-15; cited from Bulani, S. (2007). Determination of the botanical composition of black rhinoceros (*Diceros bicornis*) dung using the *rbc*L gene as a molecular marker, and analysis of antioxidant and phenolic content of its browse. *Master of Science thesis*. Rhodes University, South Africa.

Droege, M., & Hill, B. (2008). The Genome Sequencer FLX^{TM} System-Longer reads more applications, straightforward bioinformatics and more complete data sets. *Journal of Biotechnology*, ***136***, 3-10.

Duan, X-J., Zhang, W-W., Li, X-M., & Wang, B-G. (2006). Evaluation of antioxidant property of extracts and fractions obtained from a red alga, *Polysiphonia urceolata*. *Food Chemistry*, ***95***, 37-43.

References

Duncan, A. J., Ginane, C., Elston, D. A., Kunaver, A., & Gordon, I. J. (2006). How do herbivores trade-off the positive and negative consequences of diet selection decision? *Animal Behavior*, ***71***, 93-99.

Dunn, J. M., Hearne, J. W., & McArthur, L. (no date). A simple individual based model of black rhinoceros in Africa. Department of Mathematical and Geospatial Sciences. Royal Melbourne Institute of Technology University. 2196-22012.

Emslie, R., & Brooks, M. (1999). African Rhino: Status Survey and conservation action plan. IUCN/SSC African Rhino Specialist Group. Switzerland and Cambridge, UK, (pp ix-92).

Fike, B. (2007). Eastern Cape Wildlife Management. *Colloquium. Personal Communication*, Rhodes University, South Africa. Date: 25.06.2007.

Firuzi, O., Lacanna, A., Petrucci, R., Marrosu, G., & Saso, L. (2005). Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. *Biochimica et Biophysica Acta*, ***1721***, 174-184.

Fitzgerald, A. E., & Waddington, D. C. (1979). Comparison of two methods of fecal analysis of herbivore diet. *Journal of Wildlife Management*, ***43***, 368-473.

Flynn, R. W., & Abdullah, M. T. (1984). Distribution and status of the Sumatran rhinoceros in Peninsular Malaysia. *Biological Conservation*, ***28***, 253-273.

Fromme, A. (2005). Current research: DNA Barcode synopsis. *Journal of Plos Biology*, ***1***, 1-3.

Fukumoto, L. R., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agriculture and Food Chemistry*, ***48***, 3597-3604.

Ganqa, N. M., & Scogings, P. F. (2007). Forage quality, twig diameter, and growth habit of woody plants browsed by black rhinoceros in semi-arid subtropical thicket, South Africa. *Journal of Arid Environments*, ***70***, 183-188.

References

Garcia-Alonso, M., Pascual-Teresa, S., Santos-Buelga, C., & Rivas-Gonzalo, J. C. (2004). Evaluation of the antioxidant properties of fruits. *Food Chemistry*, ***84***, 13-18.

Gavin, M. C. (2007). Foraging in the fallows: Hunting patterns across a successional continuum in the Peruvian Amazon. *Biological Conservation, **134***, 64-72.

Gielly, L., & Taberlet, P. (1994). The use of chloroplast DNA to resolve plant phylogenies: Noncoding versus *rbc*L sequences. *Molecular Biology Evolution*, ***11***, 769-777.

Goldschmidt, S., & Renn, K. (1922). Zweiwertiger Stickstoff: Über das α, α-Diphenyl-β-trinitrophenyl-hydrazyl. (IV). Mitteilung über Amin-Oxydation. *Berichte der Deutschen Chemischen Gesellschaft (A and B Series),* ***55,*** 628 – 643; cited from Ionita, P. (2005). Is DPPH stable free radical a good scavenger for oxygen active species? *Chemistry Paper*, ***59***, 11-16.

Graffam, W., Dierenfeld, E. S., Pattillo, G., & Bass, L. (1997). Evaluation of eight of native Texas browses as suitable forage substitutes for black rhinoceros (*Diceros bicornis*). *Proceedings of the Second Conference of the Nutrition Advisory Group American and Aquarium Association on Zoo and Wildlife Nutrition*, Fort Worth Zoo. Texas.

Gutteridge, S., & Gatenby, A. A. (1995). Rubisco synthesis, assembly, mechanism, and regulation. *The Plant Cell*, *7*, 809-819.

Halliwell, B. (2005). Free radicals and other reactive species in disease. *Encyclopedia of Life Sciences*, National University of Singapore. Singapore. John Willy & Sons, Ltd.

Hall-Martin, A. J., Erasmus, T., & Botha, B. P. (1982). Seasonal variation of diet and faeces composition of black rhinoceros *Diceros bicornis* in The Addo Elephant National Park. *Koedoe*, ***25,*** 63-82.

References

Halvorsen, B. L., Holte, K., Myhrstad, M. C. W., Barikmo, I., Hvattum, E., Remberg, S. F., Wold, A-B., Haffner, K., Baugerød, H., Anderson, L. F., Moskaug, J. Ø., Jacobs Jr, D. R., & Blomhoff, R. (2002). A systematic screening of total antioxidant in dietary plants. *Journal of Nutrition*, ***132***, 461-471.

Hansen, R. M., Peden, D. G., & Rice, R. W. (1973). Discerned fragments in feces indicate diet overlap. *Journal of Range Management*, ***26***, 102-105.

Harley, E. H., Robson, P., & Weber, B. (2004). Red blood cell metabolism shows anomalies in Rhinocerotidae and Equidae, suggesting a novel role in general antioxidant metabolism. *International Congress Series*, ***1275***, 334-340.

Hearne, J. W., & Swart, J. (1991). Optimal translocation strategies for saving the black rhino. *Ecological Modelling*, ***59***, 279-292.

Hebert, P. D., Stoeckle, M. Y., Zemlak, T. S., & Francis, C. M. (2004). Identification of birds through DNA Barcodes. *Plos Biology*, ***2***, e312.

Heilmann, L. C., de Jong, K., Lent, P. C., & de Boer, W. F. (2006). Will tree Euphorbia (*Euphorbia tetragonia* and *Euphorbia triangularies*) survive under the impact of black rhinoceros (*Bicornis diceros minor*) browsing in the Great Fish River Reserve, in South Africa. *African Journal of Ecology*, ***44***, 87-94.

Helary, S. (2007). Nutritional ecology of black rhinoceros. Doctoral thesis. University of Witwatersrand, Johannesburg, South Africa.

Henley, S. R., Smith, D. G., & Raats, J. Ğ. (2001). Evaluation of 3 techniques for determining diet composition. *Journal of Range Management*, ***54***, 582-588.

Hofreiter, M., Betancourt, J. L., de Sbriller, A. P., Markgraf, V., & McDonald, H. G. (2003). Phylogeny, diet, and habitat of an extinct ground sloth from Cuchilo Curá, Neuquén Province, Southwest Argentina. *Quaternary Research*, ***59***, 364-378.

Höss, M., Kohn, M., & Pääbo, S. (1992). Excrement analysis by PCR. *Nature*, ***359***, 199.

References

Huber, S., Bruns, U., & Arnold, W. (2002). Sex determination of red deer using Polymerase Chain reaction of DNA from feaces. *Wildlife Society Bulletin, **30**,* 208-212.

Huber, S., Bruns, U., & Arnold, W. (2003). Genotyping herbivore feces facilitating their further analysis. *Wildlife Society Bulletin*, ***31***, 692-697.

Hutchins, M., & Kreger, M. D. (2006). Rhinoceros behaviour; implications for captive management and conservation. *International Zoo Yearbook*, ***40***, 150-173.

Hutzler, P., Fiscbasch, R., Heller, W., Jungblut, T. P., Reuber, S., Schmitz, R., Veit, M., Weissenböck, G., & Schnitzler, J-P. (1998). Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *Journal of Experimental Botany*, ***49***, 953-965.

Ionita, P. (2005). Is DPPH stable free radical a good scavenger for oxygen active species? *Chemistry Paper*, ***59***, 11-16.

Iotti, M., & Zambonelli, A. (2006). A quick and precise technique for identifying ectomycorrhizas by PCR. *Mycological Research*, ***110***, 60-65.

Jarman, S. N., Gales, N. J., Tierney, M., Gill, P. C., & Elliot, N.G. (2002). A DNA-based method for identification of krill species and its application to analysing the diet of marine vertebrate predators. *Molecular Ecology*, ***11***, 2079-2690.

Joubert, E., & Eloff, F. C. (1971). Notes on feeding ecology and behaviour of the black rhinoceros (*Diceros bicornis*). *Madoqua, **1***, 5-53.

Kapli, P., Lymberakis, P., Poulakakis, N., Mantziou, G., Parmakelis, A., & Mylonas, M. (2008). Molecular phylogeny of three *Mesalina* (Reptalia; Lacertidae) species (*M. guttulata, M. brevirostris* and *M. bahaeldini*) from North Africa and Middle East: Another case of paraphyly? *Molecular Phylogenetics and Evolution*, ***49***, 102-110.

Karawita, R., Siriwardhana, N., Lee, K-W., Heo, M-S., Yeo, I-K., Lee, Y-D., & Jeon, Y-J. (2005). Reactive oxygen species scavenging, metal cheating, reducing

and lipid peroxidation inhibition properties of different solvent fractions from *Hizikia fusiformis*. *European Food Research Technology*, ***220***, 363-371.

Katalinic, V., Milos, M., Kulisic, T., & Jukic, M. (2006). Screening of 70 medicinal plants for antioxidant capacity and total phenols. *Food Chemistry*, ***94***, 550-557.

Kefalas, P., Kallithraka, S., Parejo, I., & Makris, D. P. (2003). Note: A comparative study on the in vitro antiradical activity and hydroxyl free radical scavenging activity in aged red wines. *Food Science Technology International*, ***9***, 383-387.

Kessler, W. B., Kasworm, W. F., & Bodie, W. L. (1981). Three methods compared for analysis of Pronghorn diets. *Journal of Wildlife Management*, ***45***, 612-619.

Kress, W. J., & Erickson, D. L. (2008). DNA barcodes. Genes, genomics and bioinformatics. *Proceedings of the National Academy of Science*, ***105***, 2761-2762.

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., & Jansen, D. H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Science*, ***102***, 8369-8374.

Kurose, N., Masuda, R., & Tamara, M. (2005). Fecal DNA for identifying species and sex of sympatric carnivores: a noninvasive method of conservation on the Tsushima Islands, Japan. *Journal of Heredity*, ***96***, 688-697.

Kwapena, N. (1984). Traditional conservation and utilization of wildlife in Papua New Guinea. *The Enviromentalist*, ***4***, 22-26.

Lahaye, R., van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Dithoit, S., Barraclough, T. G., & Savolainen, V. (2008). DNA barcoding the flora of biodiversity hotspots. *Proceedings of the National Academy of Science,* ***105***, 2923-2928.

Lee, G. J., & MacGregor, C. M. (2004). Comparison of a microhistological analysis and alkane concentration of faeces to estimate the botanical composition of the diet of grazing sheep. *Animal Production in Australia*, ***25***, 108-111.

References

Lima, M. J. R., Tóth, I., & Rangel, A. O. S. S. (2005). A new approach for the sequential injection spectrophotometric determination of the total antioxidant activity. *Talanta*, ***68***, 207-213.

Luximon-Ramma, A., Bahorun, T., Soobratee, M. A., & Aruoma, O. I. (2002). Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of *Cassia fistula*. *Journal of Agriculture and Food Chemistry*, ***50***, 5042-5047.

Mabinya, L. V., Brand, J. M., Raats, J. G., & Trollope, W. S. W. (2002). Estimation of grazing by herbivores from analysis of dung. *African Journal of Range and Forage Science*, ***19***, 175-176.

Mardis, E. R. (2008). The impact of next-generation sequencing technology on genetics. *Trends in Genetics*, ***24***, 133-141.

Martin, W., Deusch, O., Stawski, N., Grünheit, N., & Goremykin, V. (2005). Chloroplast genome phylogenetics: why we need independent approaches to plant molecular evolution. *Trends in Plant Science*, ***10***, 203-209.

Martínez-Cayuela, M. (1995). Oxygen free radicals and human disease. *Biochemistry*, *77*, 147-161.

Masood, M., Nishikawa, T., Fukuoka, S., Njenga, P. K., Tsudzuki, T., & Kadowaki, K. (2004). The complete nucleotide sequence of wild rice (*Oryza nivara)* chloroplast genome: first genome wide comparative sequence analysis of wild and cultivated rice. *Gene, **340***, 133-139.

Matheson, C. D., Muller, G. C., Junnila, A., Vernon, K., Hausmann, A., Miller, M. A., Greenbalt, C., & Schlein, Y. (2008). A PCR method for the detection of plant meals from the guts of insects. *Organisms, Diversity and Evolution*, *7*, 294-303.

References

Mavi, A., Terzi, Z., Ozoen, U., Yildirim, A., & Coskun, M. (2004). Antioxidant properties of some medicinal plants: *Prangos ferulacea* (Apiaceae), *Sedum sempervivoides* (Crassulaceae), *Malva neglecta* (Malvaceae), *Cruciata taurica* (Rubiaceae), *Rosa pimpinellifolia* (Rosaceae), *Galium verum subsp. verum* (Rubiaceae), *Urtica dioica* (Urticaceae). *Biology and Pharmacological Bulletin*, ***27***, 702-705.

Mcinnis M. L., Vavra, M., & Krueger W.C. (1983). A comparison of four methods used to determine the diet of large herbivores. *Journal of Range Management*, ***36***, 302-306.

Mclntire, P. W., & Carey, A. B. (1989). A microhistological technique for analysis of food habits of Mycophagous rodents. *U. S. Forest Research Paper*, Pacific Northwest Research Station-404,16p.

Miller, N. J., & Rice-Evans, C. A. (1997)a. Factors influencing the antioxidant activity determined by the $ABTS^{\cdot+}$ radical cation. *Free Radical*, ***26***, 195-199.

Miller, N. J., & Rice-Evans, C. A. (1997)b. Cinnamate and hydroxybenzoates in the diet: antioxidant activity assessed using the $ABTS^{\cdot+}$ radical cation. *British Food Journal*, ***99***, 57-62.

Moehlman, P. D., Amoto, G., & Runyoro, V. (1996). Genetic and demographic threats to the black rhino population in the Ngorongoro crater. *Conservation Biology*, ***10***, 1107-1114.

Mofareh, M. M., Beck, R. F., & Schneberger, A. G. (1997). Comparing technique for determining steer diets in northern Chihuahuan Desert. *Journal of Range Management*, ***50***, 27-32.

Mohammed, A. G., Pieper, R. D., Wallace, J. D., Holechek, J. L., & Murray, L. W. (1995). Comparison of fecal analysis and rumen evacuation techniques for sampling diet botanical composition of grazing cattle. *Journal of Range Management*, ***48***, 202-205.

References

Moritz, C., & Cicero, C. (2004). DNA barcording. Promise and pitfalls. *Blos Biology*, ***2***, 1529-1531.

Mosquera, O. M., Correa, Y. M., Buitrago, D. C., & Niño, J. (2007). Antioxidant activity of twenty plants from Colombian biodiversity. *Memórias do Instututo Oswaldo Cruz*, ***102***, 631-634.

Moure, A., Cruz, J. M., Franco, D., Dominquez, J. M., Sineiro, J., Dominquea, J., Nunez, M. J., & Parajó, J. C. (2001). Natural antioxidants from residual sources. *Food Chemistry*, ***72***, 145-171.

Munson, L., Koehler, J. W., Wilkinson, J. E., & Miller, R. E. (1998). Vesicular and ulcerative dermatopathy resembling superficial necrolytic dermatitis in captive black rhinoceros (*Diceros bicornis*). *Veterinary Pathology*, ***35***, 31-42.

Muya, S. M., & Oguge, N. O. (2000). Effects of browse availability and quality on black rhino (*Diceros bicornis michaeli* Groves 1967) diet in Nairobi National park, Kenya. East African Wildlife Society. *African Journal of Ecology*, ***38***, 62-71.

Ndondo, I. B., Wilhelmi, B. S., Mabinya, L. V., & Brand, J. M. (2004). Alpha-tocopherol and fatty acids of major browse plant species of black rhinoceros in the Great Fish River Reserve. *South African Journal of Wildlife Research, **34***, 87-93.

Nenaids, N., Wang, L-F., Tsimidou, M., & Zhang, H-Y. (2004). Estimation of scavenging activity of phenolic compounds using the $ABTS^{\cdot+}$ assay. *Journal of Food Chemistry*, ***52***, 4669-4674.

Nordberg, J., & Arnér, E. S. J. (2001). Reactive oxygen species, antioxidant, and the mammalian thioredoxin system. *Free Radical Biology and Medicine*, ***31***, 1287-1312.

O'Ryan, C. O., Fland, J. R. B., & Harley, E. H. (1994). Mitochondrial DNA variation in black rhinoceros (*Diceros bicornis*): Conservations Management Implications. *Conservation Biology*, ***8***, 495-500.

References

Oloo, T., Brett, R., & Young, T. P. (1994). Food plants of the black rhinoceros (*Diceros bicornis*) on Ol Ari Nyiro ranch, Laikipia, Kenya. *African Journal of Ecology*, ***32***, 142-157.

Paixão, N., Perestrelo, R., Margues, J. C., & Câmara, J. S. (2007). Relationship between antioxidant capacity and total phenolic content of red rosé and white wines. *Food Chemistry*, ***105***, 204-214.

Poinar, H. N., Hofreiter, M., Sapuling, W. W., Martin, P. S., Stankiewicz, B. A., Bland, H., Evershe, R. P., Possnert, G., & Pääbo, S. (1998). Molecular coproscopy: Dung and diet of the extinct ground sloth, *Nothrotheriops shastensis*. *Science*, ***281***, 402-406.

Poinar, H. N., Kuch, M., Sobolik, K. D., Stankiewicz, A. B., Kuder, T., Spaulding, W. G., Bryant, V. M., Cooper, A., & Pääbo, S. (2001). A molecular analysis of dietary diversity for three archaic Native Americans. *Proceedings of the National Academy of Science*, ***98***, 4317-4322.

Prakash, A. (2001). Antioxidant activity. *Analytical Progress*, ***19***, 1-6.

Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified Ferric Reducing/Antioxidant Power assay. *Journal of Agricultural and Food Chemistry*, ***48***, 3396-3402.

Race, H. L., Herman, R. G., & Martin, W. (1999). Why have organelles retained genomes? *Trends in Genetics*, ***15***, 364-370.

Rai, S., Wahile, A., Mukherjee, K., Saha, B. P., & Mukherjee, P. K. (2006). Antioxidant activity of *Nelumbo nucifera* (sacred lotus) seeds. *Journal of Ethnopharmacology*, ***104***, 322-327.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolourization assay. *Free Radical Biology and Medicine*, ***26***, 1231-1237.

References

Remya, R., Syamkumar, S., & Sasikumar, B. (2004). Isolation and amplification of DNA from turmeric powder. *British Food Journal*, ***106***, 673-678.

Samarth, R. M., Panwar, M., Kumar, M., Soni, A., Kumar, M., & Kumar, A. (2008). Evaluation of antioxidant and radical-scavenging activities of certain radioactive plant extracts. *Food Chemistry*, ***106***, 868-873.

Savolainen, V., & Chase, M. W. (2003). A decade of progress in plant molecular phylogenetics. *Trends in Genetics*, ***19***, 717-723.

Schulz, C. E., & Skonhoft, A. (1996). Wildlife management, land-use and conflicts, *Environment and Development Economics, **1***, 265–280.

Setsaas, H., Holmern , T., Mwakalebe, G., Stokke, S., & Røskaft, E. (2007). How does human exploitation affect impala populations in protected and partially protected areas? A case study from the Serengeti Ecosystem, Tanzania Trine. *Biological Conservation*, ***136***, 563-570.

Shimoi, K., Masuda, S., Shen, B., Furugori, B., & Kinae, N. (1996). Radioprotective effect of antioxidative plant flavonoids in mice. *Mutation Research*, ***350***, 153-161.

Sies, H. (1993). Strategies of antioxidant defense. *European Journal of Biochemistry*, ***215***, 213-219.

Silvia, M. M., Santos, M. R., Caroço, G., Rocha, R., Justino, G., & Mira, L. (2002). Structure-antioxidant activity relationships of flavonoids: a re-examination. *Free Radical Research*, ***36***, 1219-1227.

Singleton, V. L., & Rossie Jr, J. A. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, ***16***, 144-158.

Smith, W. P., & Zollner, P. A. (2005). Sustainable management of wildlife habitat and risk of extinction. *Biological Conservation*, ***125***, 287-295.

Stoeckle, M. Y., & Hebert, P. D. N. (2008). Barcode of Life. *Scientific American*, 82-88, 299.

References

Suddhuraju, P., & Becker, K. (2007). The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts. *Food Chemistry*, ***101***, 10-19.

Symondson, W. O. C. (2002). Molecular identification of prey in predator diets. *Molecular Ecology*, ***11***, 627-641.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Muquel, C., Valentini, A., Vermat, T. G., Brochmann, C., & Willerslev, E. (2006). Power and limitations of the chloroplast *trnL* (UAA) intron for plant DNA barcoding. *Nucleic Acids Research*, ***35***, 1-8.

Tanser, F., & Palmer, A. R. (2000). Vegetation mapping of the Great River Basin, South Africa. Integrating spatial and multi-spectral remote sensing techniques. *Applied Vegetation Science*, ***3***, 197-203.

Tawaha, K., Alali, F. Q., Gharaibeh, M., Mohammad, M., & E-Elimat, T. (2007). Antioxidant activity and total phenolic content of selected Jordanian plant species. *Food Chemistry*, ***104***, 1372-1378.

Terryn, N., Rouzé, P., & Van Montagu, M. (1999). Plant genomics. *Federation of European Biochemical Societies*, ***452***, 3-6.

Thompson, H. J. (2004). Free radicals: the pros and cons of antioxidants. DNA oxidation products, antioxidant status and anticancer prevention. *Journal of Nutrition*, ***134***, 3186S-3187S.

Tivy, J. (1985). Nature conservation in the Nordic countries: Consensus rather than conflict. *Geoforum*, ***16***, 239-255.

Tougard, D., Delefosse, T., Hänni, C., & Montgelard, C. (2001). Phylogenetic relationship of the five extant rhinoceros species (Rhinocerotidae, Perisodactyla) based on mitochondrial cytochrome b and 12S rRNA genes. *Molecular Phylogenetics and Evolution*, ***19***, 34-44.

References

Van Lieverloo, R., & Schuiling, B. (2004). The diet profile of the black rhinoceros in the Great Fish River Reserve, South Africa. *A thesis in Resource Ecology*.Wageningen University and University of Fort Hare. *Forest and Nature Conservation*, (pp 1-46).

Velioglu, Y. S., Mazza, G., Gao, Y. L., & Oomah, B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *Journal of Agriculture and Food Chemistry*, ***46***, 4113–4117.

Villaño, D., Fernández-Pachón, M. S., Moyá, M. L., Troncoso, A. M., & Garcia-Parrilla, M. C. (2007). Radical scavenging ability of phenolic compounds towards DPPH free radical. *Talanta*, ***71***, 230-235.

Villaño, D., Fernández-Pachón, M. S., Troncoso, A. M., & Garcia-Parrilla, M. C. (2004). The antioxidant activity of wine determined by the $ABTS^{\cdot+}$ method: influence of sample dilution and time. *Talanta,* ***64,*** 501-509.

Villaño, D., Fernández-Pachón., Troncoso, A. M., & Garcia-Parrilla, M. C. (2005). Comparison of antioxidant activity of wine phenolic compounds and metabolites. *Analytica Chimica Acta*, ***538***, 391-398.

Walpole, M. J., Morgan-Davies, M., Millede, S., Bett, P., & Leader-Williams, N. (2001). Population dynamics and future conservation of a free ranging black rhinoceros (*Diceros bicornis*) population in Kenya. *Biological Conservation,* ***99***, 237-243.

Western, D. (1987). Africa's Elephants and Rhinoceroses: Flagships in Crisis. *Trends in Ecology and Evolution,* ***2***, 343-346.

Wong, C-C., Li, H-B., Cheng, K-W., & Chen F. (2006). A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chemistry*, ***97***, 705-711.

Zhang, X-Z., Tapia, M., Webb, J. B., Huang, Y-H., & Miao, S. (2008). Molecular signatures of two cattail species, *Typiha domingensis* and *Typha latifolia* (Typhaceae), in South Florida. *Molecular Phylogenetics and Evolution*, ***49***, 368-276.

Online access

IJdema, H., & de Boer, F. (2008). Competition between black rhinoceros (*Diceros bicornis)* and greater kudu (*Tragelaphus strepsiceros*) in the Great Fish River Reserve, South Africa. *Resource Ecology Group*, Wageningen University. Nertherlands *Colloquium*. http://www.reg.wur.nl/UK/newsagenda/archieve/ agenda/2008/Colloquium_5_presenations.htm. Date accessed: 15.09.2008.

Liang, H. (1997). The phylogenetic reconstruction of the grass family (Poaceae) using *matK* gene sequences. *Doctoral thesis*. Virginia Polytechnic Institute and State University, Blurgburg, Virginia. http://scholar.lib.vt.edu/thesis/available/etd-11597-103132/unrestricted/four.pdf. Date accessed: 20.12.2008.

Oliver, S. Z. (2007). Small-scale feeding and habitat preferences of herbivore game species in the grassland of the central Free State. *Master of Science thesis*. University of Free State, South Africa, (pp 1-45).

Stahl, E. (2003). The secondary metabolism of plants: secondary defence compounds. *Botany Online*. (Pp 1-8). http://www.biologie.uni-hamburg.de/b-online/e20/20.htm. Date accessed: 09.10.2008.

Waterhouse, A. L. (no date). Folin-Ciocalteau micro method for total phenol in wine. http://waterhouse.ucddavis.edu/phenol/folinmicro.htm. Date accessed: 21.06. 2008.

http://www.adventurezone.co.za. Date accessed: 23.05.2008.

http://www.ebi.ac.uk/Tools/emboss/align/index. 10.09.2008.

References

http://www.ecparks.co.za/parks-reserves/great-fish-river/index.html. Date accessed: 18.04.2007.

http://www.ipni.org/ipni/plantnamesearchpage.do. Date accessed: 10.05.2007.

http://www.kws.org/rhino.html. Date accessed: 27.05.2007.

http://www.ncbi.nlm.nih.gov. Date accessed: 12.09.2008.

APPENDICES

Appendix A: Isolation of genomic DNA from plant tissue using the DNeasy Plant Mini Kit

Plant material (0.1 g) was ground to a fine powder with liquid nitrogen. The material was placed into a safe lock microcentrifuge tube containing 400 µl AP1 buffer, followed by the addition of 4 µl RNase A. The mixture was incubated for 10 min at 65°C, followed by mixing (x 3) between incubation by inversion. Thereafter, 130 µl AP2 buffer was added to the lysate, followed by incubation for 5 min on ice. The lysate was applied to the QIAshredder Mini Spin Column which was placed in a 2 ml collection tube, and centrifuged (15 500 x g, 2 min at room temperature). The fraction collected was transferred to a new microcentrifuge tube without disturbing the pelleted cells. A 1.5 volume of AP/3 buffer added to the recovered lysate and was mixed immediately by pipetting. A 650 µl of the lysate was pippeted into the DNeasy Mini Spin Column set in a collection tube. The mixture was centrifuged (6 000 x g, for 1 min at room temperature) and the recovered eluent was discarded. The column was placed in a new 2 ml collection tube and 500 µl AW buffer was added. The tube was centrifuged (6 000 x g, 1 min at room temperature). The eluent was discarded and the collection tube was reused. A further 500 µl AW buffer was added to the DNeasy Mini Spin Column, followed by centrifugation (15 500 x g, 2 min at room temperature). The column was centrifuged (15 500 x g, 5 min at room temperature) to dry the membrane. The column was transferred to a 1.5 ml microcentrifuge tube and 100 µl ddH_2O was added directly to the DNeasy membrane. The tubes were incubated for 5 min at room temperature and centrifuged for 1 min at 6 000 x g to elute genomic DNA. The eluent was stored at –20°C.

Appendix B: Composition of buffers and solutions

Appendix B1: Tris (1 M), pH 8 per litre

Tris base salt (121.2 g) was added to 700 ml $dddH_2O$ and the pH of 8 was adjusted with HCl. The solution was made to a litre with $dddH_2O$ and autoclaved for 20 min.

Appendix B2: 0.5 M EDTA, pH 8.0 per 200 ml

EDTA salt (37.2 g) was added in 100 ml $dddH_2O$ and the pH was adjusted with 5 M NaOH. The solution was made up to 200 ml with $dddH_2O$ and autoclaved prior to use.

Appendix B3: 50 X TAE (Tris-Acetate-EDTA) buffer, pH 8, per litre

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA, pH 8.0	100 ml

Appendix B4: CTAB extraction buffer

2% CTAB

1.4 M NaCl

20 mM EDTA

100 mM Tris buffer, pH 8

2% PVP

2% BSA

Appendix C: PCR mixture for the amplification of the *rbc*L (802 bp) gene in a total of 15 µl

Reagents	Final Concentration	Quantity (vol)
Triple deionized water		Variable
5 X *Taq* Buffer	1X	3 µl
10 mM dNTP mix	200 µM each	0.3 µl
25 mM $MgCl_2$	1.4 mM	0.84 µl
Genomic DNA template	200 ng DNA/15 µl	X (Variable)
Forward primer *rbc*L 10 µM	0.8 µM	1.2 µl
Reverse Primer *rbc*L 10 µM	0.8 µM	1.2 µl
Taq Polymerase 5U/ µl	1.0 U/µl	0.2 µl
BSA 20 mg/ml	2.4 – 2.7 mg/ml	1.8 - 3 µl

Appendix D: BSA addition to the extraction of DNA of the listed plants

Plant species	**BSA (µg)**
Azima tetracantha *Euclea undulata*	39
Phyllanthus verrucosus *Mayetenus nemorosa*	69

Appendix E: Preparations of chemically competent cells, JM 109 strain

To maintain lab stock of highly efficient low background *Escherichia coli* strain, JM 109 competent cells for plasmid transformations was prepared as follows:

Appendix E1: Time required for preparing competent cells

Day 1: Cells were plated and incubated at 37°C overnight.

Day 2: Colonies were selected and cultured for 4 hours at 37°C until the OD reached between 0.6 and 0.8.

Appendix E2: Preparations of reagents

The KCl of 3 M was prepared in 50 ml; 1M MnCl in 50 ml; 1M CaCl in 50 ml. The CH_3COOK 30 mM, pH 5.8, 10 mM MOPS, pH 6.8 and 15% m/v glycerol was prepared in 500 ml using $dddH_2O$.

Appendix E3: Buffer 1: RF1, pH 5.8

The 90 mM KCl, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 30 mM CH_3COOK, pH 5.8; 15% m/v glycerol per 100 ml total volume.

Approximately, 30 mM CH_3COOK and 15 % m/v glycerol were mixed and the pH was adjusted to 5.8 with HCl and autoclaved prior to addition of 90 mM KCl, 50 mM $MnCl_2$ and 10 mM $CaCl_2$, respectively. The mixture was then stored at 4°C until required.

Appendix E4: Buffer 2: RF2, pH 6.8

MOPS 10 mM, pH 6.8; 15 % m/v Glycerol; 10 mM KCl; 75 mM $CaCl_2$ per 100 ml. Approximately, 10 mM MOPS, 15 % m/v glycerol were mixed in a 100 ml clean reagent bottle and the pH was adjusted to 6.8 with KOH and autoclaved prior to addition of 10 mM KCl and 75 mM $CaCl_2$ respectively. The solution was stored in 4°C until required.

Appendix E5: Procedure for preparing competent cells

Step 1: *E. coli* JM 109 strain cells, stored at -80°C were streaked on a LB agar plates (without any selective agent) and incubated at 37°C for 16 h to isolate colonies.

Step 2: Using a sterile inoculating loop, a single colony was scraped from the agar surface, inoculated and grown in a sterile 5 ml LB broth medium (without a selective antibiotic since these cells do not contain plasmid) and incubated at 37°C with vigorous shaking at 180 rpm for 12 h using a Multishaker PSU 20, BOECO (Germany)

Step 3: Cells were diluted 1:200 in sterile LB broth and were grown at 37°C on a 180 rpm shaker until they reach an OD between 0.6 and 0.8 (approximately 4 h). The cells were quantified using Biowave spectrophotometer at 600 nm by making 1:5 dilutions in 1 ml cuvette.

Step 4: The cells were cooled for 10 min in an ice bath prior to processing. The cells were pelleted into four separated pre-chilled 25 ml sterile centrifuge bottles and were centrifuged for 10 min at 2 300 x g at 4°C using the Beckman Avanti centrifuge (JA-20 rotor).

Step 5: The supernatant was decanted and the bacterial pellets were gently resuspended in 1/200-culture volume of ice-cold RF1 solution on ice, making sure that less than 5 min was taken for this procedure. These suspensions were kept for 20 min on ice prior to centrifugation at 2 300 x g at 4°C for 10 min.

Step 6: The supernatant was decanted and the bacterial pellets were gently resuspended in 1/400 culture volume of ice-cold RF2 solution on ice and were dispensed in multiple 100 µL aliquots using pre-chilled eppendorf tubes. The chemically competent cells were then stored at -70°C freezer until required.

Appendix F: Media and plates

Appendix F1: Luria Bertani (LB) plates with Ampicillin

The LB medium prepared was supplemented with the agar powder (15 g) to a litre using $dddH_2O$. The medium was autoclaved and allowed to cool to 50°C before adding 100 µg/ml ampicillin. Ready-made *E. coli* FastMedia LB Agar IPTG/X-Gal was also used as alternative in the preparations of plates.

Appendix F2: LB plates with Ampicillin/IPTG/X-Gal

The LB plates were was supplemented with 0.5 mM IPTG and 80 µg/ml X-Gal. Alternatively, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal was spread over the surface of the LB-ampicillin plates and was allowed to absorb for 30 minutes at 37°C prior to use. Alternatively, the ready-made *E. coli* FastMedia LB Liquid Amp was used for preparation of plates.

Appendix F3: LB medium per litre or Ready made LB medium

Bacto®-Tryptone (10 g), 5 g Bacto®- Yeast Extract and 5 g NaCl were dissolved in a litre of $dddH_2O$. The pH was adjusted to 7.0. Ready-made LB medium was also used as an efficient media for bacterial growth.

Appendix F4: SOC (Super Optimized Culture) medium in 100 ml

The Bacto®-Tryptone (2 g), 0.5 g Bacto®-Yeast Extract, 1 ml of 1 M NaCl and 0.25 ml 1 M KCl were dissolved in 97 ml $dddH_2O$ and autoclaved. The medium was allowed to cool at room temperature and later, 1 ml of 2 M Mg^{2+} and glucose stock solutions were each added to a final concentration of 20 mM, followed by the addition of 1 ml $dddH_2O$. The pH of the solution was adjusted to 7.0 and was kept at 4°C until used. This media was used for growth and recovery of *E. coli* cells after transformation.

Appendix G: Transformations

High efficiency JM109 competent cells prepared and stored frozen as described in Appendix E were thawed for 5 min in an ice bath. In brief, 50 µl of the cells were carefully transferred into 1.5 ml microcentrifuge tubes containing 2 µl of ligation reaction mixture. The tubes were gently agitated and incubated on ice for 20 min for binding. The transformation mixture was heat-shocked for 35s in a heating block at 42°C for permeation of the plasmid DNA into the cells. The tubes were immediately placed on ice and incubated for 2 min. Super Optimized Culture (SOC) medium at room temperature was added (950 µl) to both tubes containing transformants for recovery and were further incubated for 45 min at 37°C with vigorous shaking at 180 rpm. The tubes were microcentrifuged at 6 000 x g for 1 min to collect the cells. Each transformation culture of 100 µl was plated separately into a selective antibiotic plate as prepared in Appendix F1 and F2. The plates were incubated for 12 h at 37°C for colony growth.

Appendix H: Plasmid "Easy" Preparation

Preparations of easy buffer in 50 ml total volume are listed below

1 M Tris-Cl Stock pH 8	0.5 ml
0.5 M EDTA stock pH 8	0.1 ml
15% w/v Sucrose (weigh out fresh)	7.5 g
10 mg/ml RNAse A stock (DNAse free)	1 ml
100 mg/ml lysozyme	1 ml

The buffer was filter sterilized and stored at –20°C.

Procedure

Overnight cultures of approximately 1.5 ml were microcentrifuged in 1.5 ml sterilized centrifuge tubes for 2 min at 7 400 x g using a benchtop spectrafuge 24D (Labnet International, Inc). The supernatants were decanted and 50 ul of easy buffer prepared was added to the pelleted bacterial cells for resuspension. The cells were incubated at 37°C for 30 min and boiled for 1 min at 100°C. The tubes were immediately incubated on ice for 5 min and microfuged for 10 min at 15 500 x g. The supernatants withdrawn were used in PCR for screening of the *rbc*L insert.

Appendix I: Purification of plasmid DNA from *E. coli* cultures using a QIAprep Spin Miniprep Kit

Overnight cultures of *E. coli* in LB medium were pelleted for 2 min at 7 400 x g. The recovered medium was discarded and the pelleted bacterial cells were resuspended in 250 µl of P1 buffer. 250 µl of P2 buffer was added to the bacterial mixtures and the tubes were gently inverted two to three times. After addition of 350 µl N3 buffer, the tubes were immediately mixed two to three times by inversion. The tubes were centrifuged for 10 min at 15 500 x g in a table-top microcentrifuge. The supernatant recovered from each tube was applied to the QIAprep Spin Column by adding 0.5 ml PB buffer and centrifuged for 1 min at 15 500 x g. The eluate was discarded and the columns were placed in new microcentrifuged tubes. The columns were washed by adding 0.75 ml PE buffer and centruged for 1 min at 6 000 x g. The eluate was discarded and additional centrifugations were carried out to all tubes to remove the residual buffer. The columns were further placed in new sterile 1.5 ml microcentrifuge tubes, followed by addition of 50 µl sterile ddH_2O. The tubes were left to stand at room temperature for 1 min before elution of the plasmid DNA by centrifugation for 1 min at 6 000 x g. The plasmid DNA was stored at –20°C required.

Appendix J: Plasmid polymerase chain reaction mixture for the screening of *rbc*L gene inserts using M13 primers

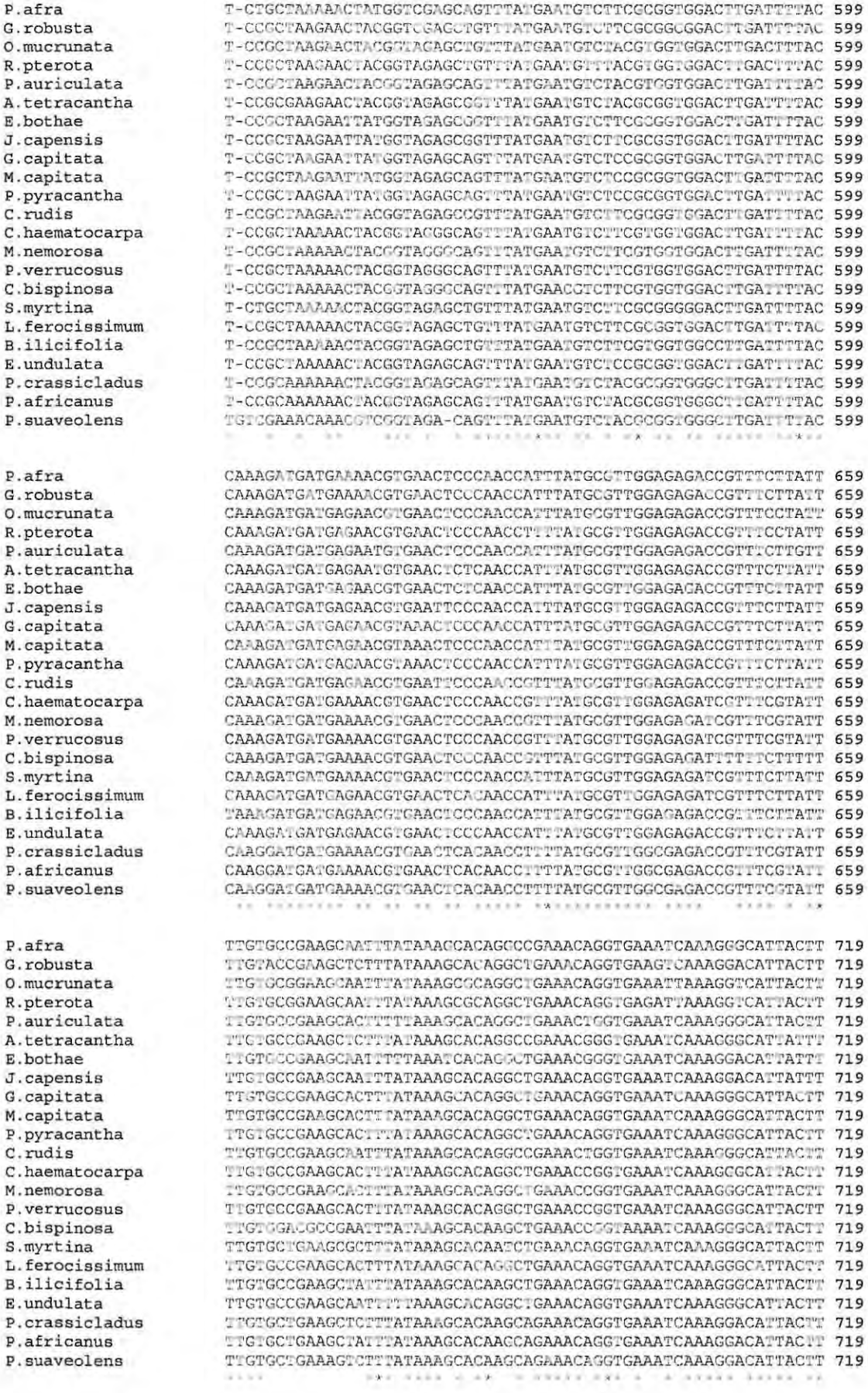
Reagents	Final Concentration	Quantity in 15 µL
Triple deionized water		Variable
5 X *Taq* Buffer	1X	3 µl
10 mM dNTP mix	200 µM each	0.3 µl
25 mM $MgCl_2$	1.4 mM	0.84 µl
Plasmid DNA	200 ng/15 µl	1.2 µl
Forward primer M13 10 µM	0.8 µM	1.2 µl
Reverse Primer M13 10 µM	0.8 µM	1.2 µl
Taq Polymerase 5U/µl	1.0 U/µl	0.2 µl

Appendix K: ClustalW 2.0.8 multiple sequence alignment of plants from the GFRR

```
P.afra              ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCAGGTGTTAAAGATTAC 60
G.robusta           ATGTCACCACAAACAGAGACTAAAGCTTTTGTTGGATTCAAAGCTGGTGTTAAACATTAC 60
O.mucrunata         ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGCGTTAAAGACTAT 60
R.pterota           ATGTCACCACAAACAGAGACTAAAGCACATGTTGGATTCAAAGCCGGCGTTAAAGACTAT 60
P.auriculata        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAT 60
A.tetracantha       ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTAT 60
E.bothae            ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
J.capensis          ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
G.capitata          ATGTCACCACAAACAGAGACTAAAGCGAGTGTTGGATTCAAGGCTGGCGTTAAAGATTAT 60
M.capitata          ATGTCACCACAAACAGAGACTAAAGCGAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
P.pyracantha        ATGTCACCACAAACAGAGACTAAAGCGAGTGTTGGATTCAAGGCTGGCGTTAAAGATTAT 60
C.rudis             ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAGGCTGGTGTTAAAGATTAT 60
C.haematocarpa      ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
M.nemorosa          ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
P.verrucosus        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
C.bispinosa         ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTAC 60
S.myrtina           ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGAGTAC 60
L.ferocissimum      ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGAGTAC 60
B.ilicifolia        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTAT 60
E.undulata          ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTAC 60
P.crassicladus      ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCTGGTGTTAAAGATTAC 60
P.africanus         ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCTGGTGTTAAAGATTAC 60
P.suaveolens        ATGTCACCACAAACAGAGACTAAAGCAAGTGTTGGATTTAAAGCTGGTGTTAAAGATTAC 60
                    **************************   ******** ** ** ** ****** * ** 

P.afra              AAATTGACTTATTATACTCCTGAATATCAACCTCAGGATACTGATATCTTGGCAGCATTC 120
G.robusta           AAATTGACTTATTATACTCCTGATTATCAAACCCTAGATACTGATATCTTGGCAGCATTT 120
O.mucrunata         AAATTGACTTATTATACTCCTGACTATATAACCAAAGATACTGATATCTTGGCAGCATTC 120
R.pterota           AAATTGACTTATTATACTCCTGACTATATAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.auriculata        AAATTGACTTATTATACTCCTGAGTATCAAGTCAAAGATACTGATATCTTGGCGGCCTTC 120
A.tetracantha       AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATTTTGGCAGCATTC 120
E.bothae            AAATTGACTTATTATACTCCTGAATATCAAACCAAAGATACTGATATCTTGGCAGCATTC 120
J.capensis          AAATTGACTTATTATACTCCTCAGTATCAAACCAAAGATACTGATATCTTGGCAGCATTC 120
G.capitata          AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
M.capitata          AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.pyracantha        AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
C.rudis             AAATTGACTTATTACACTCCTGACTATGAAACCAAAGATACCGATATCTTGGCAGCATTT 120
C.haematocarpa      AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
M.nemorosa          AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
P.verrucosus        AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
C.bispinosa         AAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTC 120
S.myrtina           AAATTGACTTATTATACTCCTGAATACGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
L.ferocissimum      AAATTGACTTATTATACTCCTGAGTACCAAACCAAGGATACTGATATATTGGCAGCATTC 120
B.ilicifolia        AAATTGACTTATTATACTCCTGACTATCAAACCAAGGATACTGATATCTTGGCAGCATTT 120
E.undulata          AAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.crassicladus      AGATTGACTTATTATACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.africanus         AGATTGACTTATTATACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
P.suaveolens        AGATTGACTTATTATACTCCTGATTACGAAACCAAAGATACTGATATCTTGGCAGCATTC 120
                    * ************ ******  * **   * *    ***** ***** ***** ** ** 

P.afra              CGAGTAACTCCTCAACCTGGAGTTCCGTCAGAAGAAGCAGGGGCCGCAGTAGCTGCCGAA 180
G.robusta           CGAGTAACTGCTCAACCTGGAGTTCCACCAGAGGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
O.mucrunata         CGAGTAACTCCTCAACCTGGAGTTCCACCCGAGGAAGCAGGGGCTGCGGTAGCTGCGGAA 180
R.pterota           CGAGTAACTCCTCAACCTGGAGTTCCACCCGAGGAAGCAGGGGCTGCGGTAGCTGCGGAA 180
P.auriculata        CGAGTAACTCCTCAACCCGGAGTTCCGCCTGAGGAAGCAGGAGCCGCGGTAGCTGCTGAA 180
A.tetracantha       CGAGTAACTCCTCAACCCGGAGTTCCACCTGAAGAGGCAGGGGCTGCGGTAGCTGCTGAA 180
E.bothae            CGAGTAAGTCCTCAACCTGGAGTTCCACCTGACGAAGCAGGAGCTGCGGTAGCTGCTGAA 180
J.capensis          CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAGGAAGCAGGAGCTGCGGTAGCTGCTGAA 180
G.capitata          CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCGGCGGTAGCTGCTGAA 180
M.capitata          CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCGGCGGTAGCTGCTGAA 180
P.pyracantha        CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCGGCGGTAGCTGCTGAA 180
C.rudis             CGAGTAACTCCTCAACCCGGAGTTCCACCTGAGGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
C.haematocarpa      CGAGTAACTCCTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
M.nemorosa          CGAGTAACTCCTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
P.verrucosus        CGAGTAACTCCTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
C.bispinosa         CGAGTAACTCCTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
S.myrtina           CGAGTAACTCCTCAACCGGGAGTTCCACCTGAAGAAGCGGGGGCCGCGGTAGCTGCCGAA 180
L.ferocissimum      CGAGTAACTCCTCAACCTGGAGTTCCACCTGAAGAAGCAGGGGCCGCGGTAGCTGCGGAA 180
B.ilicifolia        CGAGTAACTCCTCAACCTGGAGTTCCGCCTGAAGAAGCAGGGGCCGCAGTAGCTGCCGAA 180
E.undulata          CGAGTAACTCCTCAACCTGGAGTTCCACCGGAAGAAGCAGGGGCCGCGGTAGCTGCCGAA 180
```

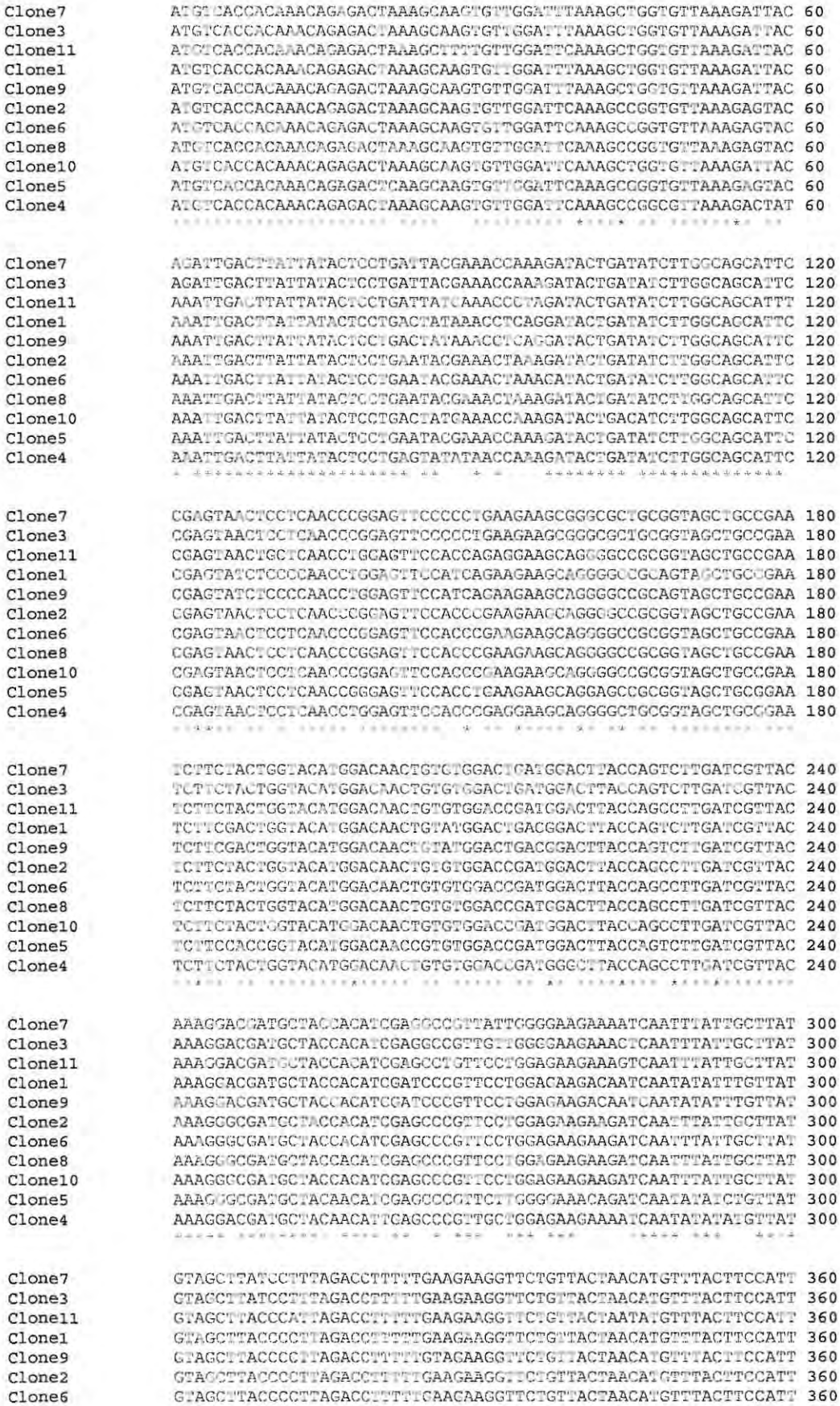
```
P.crassicladus      CGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGAAGCGGGCGCTGCGGTAGCTGCCGAA 180
P.africanus         CGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGAAGCGGGCGCTGCGGTAGCTGCCGAA 180
P.suaveolens        CGAGTAACTCCTCAACCCGGAGTTCCCCCTGAAGAAGCGGGCCCTGCGGTAGCTGCCGAA 180
                    ******* * ******* ******** * ** ** ** ** ** ** ******** ***

P.afra              TCTTCTACTGGTACATGGACAACTGTATGGACCGACGGACTTACCAGTCTTGATCGTTAC 240
G.robusta           TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
O.mucrunata         TCTTCTACTGGTACATCGACAACTGTGTGGACCGACGGGCTTACCAGCCTTGATCGTTAC 240
R.pterota           TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTAC 240
P.auriculata        TCTTCTACTGGTACATGGACAACCGTGTGGACCGATGGGCTTACCAGCCTTGGTCGTTAC 240
A.tetracantha       TCTTCTACTGGTACATGGACAAGTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTAC 240
E.bothae            TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAT 240
J.capensis          TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAT 240
G.capitata          TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAC 240
M.capitata          TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAC 240
P.pyracantha        TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTAC 240
C.rudis             TCTTCTACTCGTACATGGACAACTGTATGGACTGACGGGCTTACCAGTCTTGATCGTTAC 240
C.haematocarpa      TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
M.nemorosa          TCTTCTACTGGTACATCGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
P.verrucosus        TCTTCTACTGGTACATCGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
C.bispinosa         TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
S.myrtina           TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTAC 240
L.ferocissimum      TCTTCTACTGGTACATGGACAACTGTATGGACCGATGGACTTACCAGCCTTGATCGTTAC 240
B.ilicifolia        TCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGACTTACGAGCCTTGATCGTTAC 240
E.undulata          TCTTCTACTGGTACATGGACAGCTGTGTGGACCGATGGACTTACTAGTCTTGATCGTTAC 240
P.crassicladus      TCTTCTACTGGTACATGGACAACTGTGTGGACTGATGGACTTACCAGTCTTGATCGTTAC 240
P.africanus         TCTTCTACTGGTACATGGACAACTGTGTGGACTGATGGACTTACCAGTCTTGATCGTTAC 240
P.suaveolens        TCTTCTACTGGTACATGGACAACTGTGTGGACTGATGGACTTACCAGTCTTGATCGTTAC 240
                    ********************** ** ***** ** ** ***** ** **** ******

P.afra              AAAGGACGATGCTACCACATTGATGCCGTTCCTGGAGAAGACAATCAATATATTTGTTAT 300
G.robusta           AAAGGACGATGCTACCACATCGAGCCTGTTCCTGGAGAAGAAAGTCAATTTATTGCTTAT 300
O.mucrunata         AAACGACGATGCTACAACATTGAGCCCGTTGCTGGAGAAGAAAATCAATATATATGTTAT 300
R.pterota           AAAGGACCATGCTACAACATTGAGCCCGTTGCTGGAGAAGAAAATCAATATATATGTTAT 300
P.auriculata        AAAGGGCGATGCTACAACATCGAGCCTGTTGCTGGAGAAGAAAATCAATATATATGTTAT 300
A.tetracantha       AAAGGACGATGCTACCACATTGAGCCTGTTCTTGGAGAAGAAAATCAATATATTGCTTAT 300
E.bothae            AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATATATTGCTTAT 300
J.capensis          AAAGGACGATGCTACGACATCGAGCCCGTTGCTGGAGAACAAAATCAATATATTGCTTAT 300
G.capitata          AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATTTATTGCTTAT 300
M.capitata          AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAGTCAATTTATTGCTTAT 300
P.pyracantha        AAAGGACGATGCTACCACATCGAGCCCGTTGCTGGACAGAAAAATCAATTTATTGCTTAT 300
C.rudis             AAAGGTCGATGCTACCACATCCAGCCCGTTGCTGGAGAAGAAACTCAATTTATTGCTTAT 300
C.haematocarpa      AAAGGGCGATGCTACCACATCGAGCCCGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
M.nemorosa          AAAGGGCGATGCTACCACATCGAGCCCGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
P.verrucosus        AAAGGGCGATGCTACCACATCGAGCCCGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
C.bispinosa         AAAGGGCGATGCTACCACATCGAGCCCGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
S.myrtina           AAAGGTCGATGCTATCACATCGAGCCAGTTCCTGGAGAAGAAGATCAATTTATTGCTTAT 300
L.ferocissimum      AAAGGGCGATGCTACCGCATCGAGCCGTGTTGTTGGAGAAAAAGATCAGTATATTGCTTAT 300
B.ilicifolia        AAACGCCCATGCTATGGAATCGAGCCCGTTCCTGGAGAAGAAAATCAATATATTGCTTAT 300
E.undulata          AAAGGGCGATGCTACCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATATATTGCTTAT 300
P.crassicladus      AAAGGACGATGCTACCACATCGAGGCCGTTATTGGGGAAGAAAATCAATTTATTGCTTAT 300
P.africanus         AAAGGACGATGCTACCACATCGAGCCCGTTGTTGGGGAAGAAAGTCAATTTATTGCTTAT 300
P.suaveolens        AAAGCACGATGCTACCACATCGAGGCCGTTGTTGGGGAAGAAACTCAATTTATTGCTTAT 300
                    ***** ********    ** **    *** *** ** *  *** * ***   ****

P.afra              GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATT 360
G.robusta           GTAGCTTACCCATTAGACCTTTTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATT 360
O.mucrunata         GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
R.pterota           GTAGCTTACCCTTTAGACCTTTTTCAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
P.auriculata        GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
A.tetracantha       GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
E.bothae            GTAGCTTACCCCTTAGACCTTTTTGAAGAACGTTCTGTTACTAACATGTTTACCTCCATT 360
J.capensis          GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
G.capitata          GTAGCTTATCCTTTAGACCTTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
M.capitata          GTAGCTTATCCTTTAGACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
P.pyracantha        GTAGCTTATCCTTTAGACCTTTTCGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
C.rudis             GTAGCTTACCCCTTAGACCTTTTTGAAGAGGGTTCTGTTACTAACATGTTTACTTCCATT 360
C.haematocarpa      GTAGCTTACCCCTTAGACCTTTTTGAAGAACGTTCTGTTACTAACATGTTTACTTCCATT 360
M.nemorosa          GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
P.verrucosus        GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
C.bispinosa         GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
S.myrtina           GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
L.ferocissimum      GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACCAACATGTTTACTTCCATT 360
B.ilicifolia        GTAGCTTACCCATTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
E.undulata          GTAGCTTATCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
P.crassicladus      GTAGCTTATCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
P.africanus         GTAGCTTATCCTTTAGACCTTTTTGAAGAGGGTTCTGTTACTAACATGTTTACTTCCATT 360
```

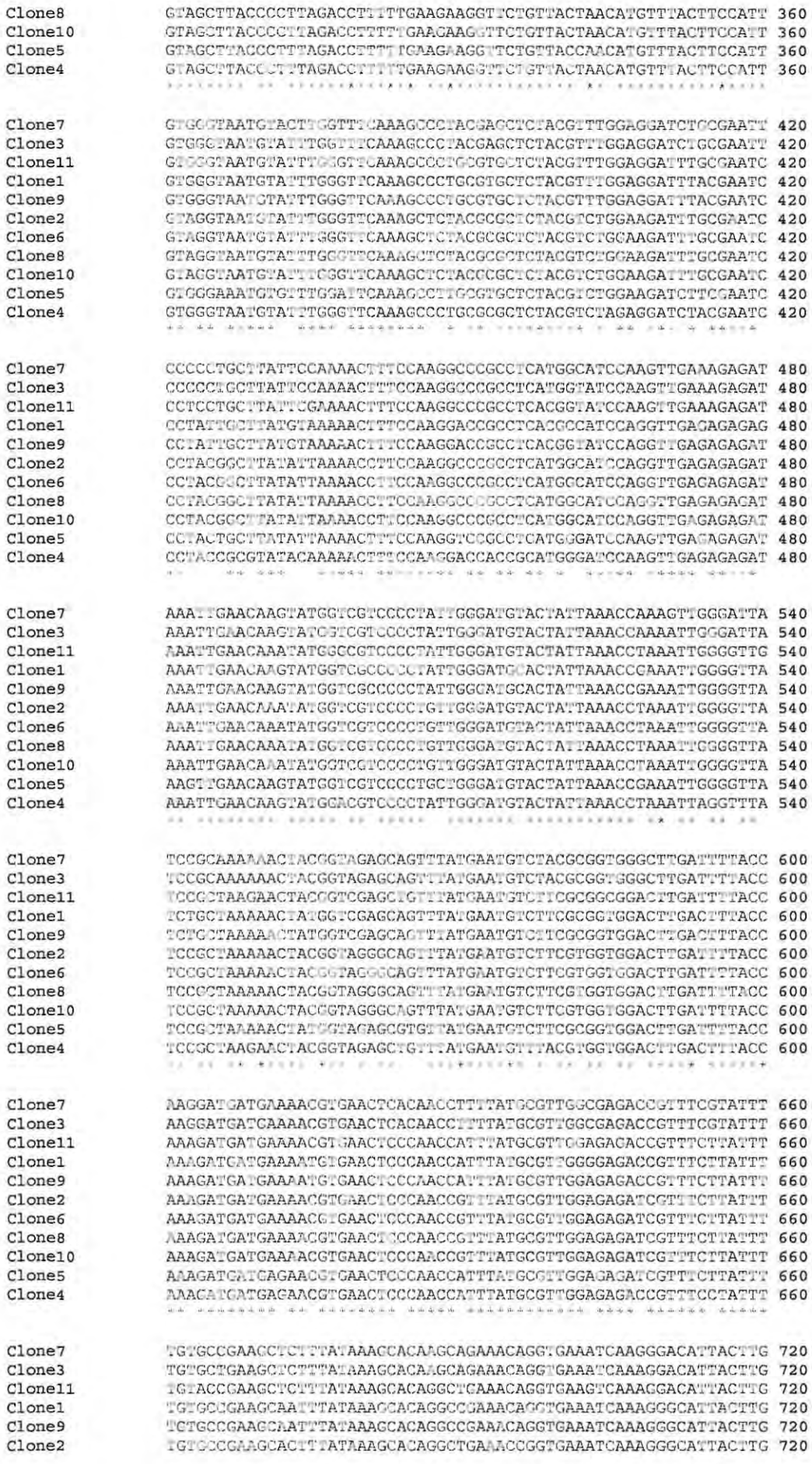
```
P.suaveolens        GTACCTTATCCTTTAGACCTTTTTGAACAAGGTTCTGTTACTAACATGTTTACTTCCATT 360

P.afra              GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGTGCTCTACGTTTGGAGGATTTGCGAATC 420
G.robusta           GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGTGCTCTACGTTTGGAGGATTTGCGAATC 420
O.mucrunata         GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATC 420
R.pterota           GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATC 420
P.auriculata        GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTGCGAATC 420
A.tetracantha       GTGGCTAATGTATTTGGTTTCAAAGCCCTGCGCGCTCTACGCCTAGAGGATTTGCGAATC 420
E.bothae            GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCGCTACGTCTGGAGGATTTGCGAATC 420
J.capensis          GTGGGTAATGTATTTGGGTTCAAAGCCCTACGCGCCCTACGTCTGGAGGATTTGCGAATC 420
G.capitata          GTGGGTAATGTCTTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAGGATTTGCGAATC 420
M.capitata          GTGGGTAATGTCTTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAGGATTTGCGAATC 420
P.pyracantha        GTGCGTAATGTATTTGGGTTCAAAGCCCTACCGCCTCTACGTCTGGAAGATTTGCGAATC 420
C.rudis             GTGGGTAATGTATTTGCGTTCAAGGCCCTGCGCGCTCTACGTCTGGAGGATTTGCGAATC 420
C.haematocarpa      GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGGAAGATTTGCGAATC 420
M.nemorosa          GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGGAAGATTTGCGAATC 420
P.verrucosus        GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGGAAGATTTGCGAATC 420
C.bispinosa         GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGGAAGATTTGCGAATC 420
S.myrtina           GTAGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAAGATTTGCGAATC 420
L.ferocissimum      GTAGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAAGATCTGCGAATC 420
B.ilicifolia        GTAGGTAATGTATTTGGGTTCAAAGCCCTGCGTGCTCTACGTCTGGAAGATTTGCGAATC 420
E.undulata          GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTGGAAGATTTGCGAATC 420
P.crassicladus      GTGGGTAATGTATTTGGTTTCAAAGCCCTACGAGCTCTACGTTTGGAGGATCTGCGAATT 420
P.africanus         GTGGGTAATGTATTTGGTTTCAAAGCCCTACGAGCTCTACGTTTGGAGGATCTGCGAATT 420
P.suaveolens        GTGGGTAATGTATTTGGTTTCAAAGCCCTACGAGCTCTACGTTTGGAGGATCTGCGAATT 420

P.afra              CCTGTTGCTTATATAAAAACTTTCCAAGGCCCGCCTCACGGTATCCAAGTTGAGAGAGAT 480
G.robusta           CCTCCTGCTTATTCGAAAACTTTCCAAGGCCCGCCTCACGGTATCCAAGTTGAAAGAGAT 480
O.mucrunata         CCTACCGCGTATACAAAAACTTTCCAAGGACCACCGCATGGGATCCAAGTTGAGAGAGAT 480
R.pterota           CCTACCGCGTATACAAAAACTTTCCAAGGACCACCGCATGGGATCCAAGTTGAGAGAGAT 480
P.auriculata        CCTCCTGCTTATATTAAAACTTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAAAGAGAT 480
A.tetracantha       CCTCCTCCTTATAGTAAAACTTTCATGGGACCACCTCATGGTATCCAAGTTGAAAGAGAT 480
E.bothae            CCTACTTCTTATACTAAAACTTTCCAAGGGCCACCTCATGGAATCCAAGTTGAGAGAGAT 480
J.capensis          CCTACTGCTTATACTAAAACTTTCCAAGGGCCGCCTCATGGTATCCAAGTTGAGAGAGAT 480
G.capitata          CCCCCTGCTTATTCTAAAACTTTCCAAGGCCCGCCGCATGGTATCCAAGTTGACAGAGAT 480
M.capitata          CCCCCTGCTTATTCTAAAACTTTCCAAGGCCCGCCGCATGGTATCCAAGTTGAGAGAGAT 480
P.pyracantha        CCCCCTGCTTATTCTAAAACTTTCCAAGGCCCGCCGCATGGTATCCAAGTTGAGAGAGAT 480
C.rudis             CCCCCTGCTTATACTAAAACTTTCCAAGGCCCGCCTCATGGCATCCAAGTTGAGAGAGAT 480
C.haematocarpa      CCTACGGCTTATGTTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAGAGAGAT 480
M.nemorosa          CCTACGGCTTATGTTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAGAGAGAT 480
P.verrucosus        CCTACGGCTTATGTTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGCTTGAGAGAGAT 480
C.bispinosa         CCTACGGCTTATATTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAGAGAGAT 480
S.myrtina           CCTACTTCTTATATTAAAACCTTCCAAGGGCCGCCCCATGGCATCCAAGTTGAGAGAGAT 480
L.ferocissimum      CCTACTGCTTATGTTAAAACTTTCCAAGGTCCGCCTCATGGGATCCAAGTTGAAAGAGAT 480
B.ilicifolia        CCTACTGCGTATGTTAAAACTTTCCAAGGTCCGCCTCACGGCATCCAAGTTGAGAGAGAT 480
E.undulata          CCTACTTCGTATTCTAAAACTTTCCAAGGACCACCTCATGGTATCCAAGTTGAAAGAGAT 480
P.crassicladus      CCCCCTGCTTATTCCAAAACTTTCCAAGGCCCGCCTCATGGTATCCAAGTTGAAAGAGAT 480
P.africanus         CCCCCTGCTTATTCCAAAACTTTCCAAGGCCCGCCTCATGGTATCCAAGTTGAAAGAGAT 480
P.suaveolens        CCCCCTGCTTATTCCAAAACTTTCCAAGGCCCGCCTCATGGTATCCAAGTTGAAAGAGAT 480

P.afra              AAATTGAACAAGTATGGCCGTCCTCTATTGGGATGCACTATTAAACCGAAATTGGGGTTA 540
G.robusta           AAATTGAACAAATATGGGCGTCCCCTATTGGGATGTACTATTAAACCTAAATTGGGGTTG 540
O.mucrunata         AAATTGAACAAGTATGGACGTCCCCTATTGGGATGTACTATTAAACCTAAATTAGGTTTA 540
R.pterota           AAATTGAACAAGTATGGACGTCCCCTATTGGGATGTACTATTAAACCTAAATTAGGTTTA 540
P.auriculata        AAATTGAACAAGTACGGTCGTCCCCTATTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
A.tetracantha       AAATTGAACAAGTATGCTCGTCCCCTATTAGGATGTACTATTAAACCTAAATTGGGGTTA 540
E.bothae            AAATTGAACAAATATGGTCGCCCTCTATTGGGTTGTACTATTAAACCAAAATTGGGGCTA 540
J.capensis          AAATTGAACAAGTATGGTCGCCCCCTATTGGGTTGTACTATTAAACCTAAATTGGGGCTA 540
G.capitata          AAATTGAACAAGTATGGACGCCCTCTATTGGGTGTACTATTAAACCTAAATTGGGATTA 540
M.capitata          AAATTGAACAAGTATGGACGCCCTCTATTGGGGTGTACTATTAAACCTAAATTGGGATTA 540
P.pyracantha        AAATTGAACAAGTATGGACGCCCTCTATTGGCGTGTACTATTAAACCTAAATTGGGATTA 540
C.rudis             AAGTTGAACAAGTATGGCCGCCCCCTATTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
C.haematocarpa      AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
M.nemorosa          AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
P.verrucosus        AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
C.bispinosa         AAATTCAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
S.myrtina           AAATTGAACAAGTATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTAGGTTTA 540
L.ferocissimum      AAATTGAACAAGTATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
B.ilicifolia        AAATTGAACAAGTATGGTCGTCCCCTGTTGCGATGTACTATTAAACCTAAATTGGGGTTA 540
E.undulata          AAATTGAACAAGTATGGTCGTCCCCTGTTGGGATGTACTATTAAACCGAAATTGGGGTTA 540
P.crassicladus      AAATTGAACAAGTATGGTCGTCCCCTATTGGGATGTACTATTAAACCAAAATTGGGATTA 540
P.africanus         AAATTGAACAAGTATGGTCGTCCCCTATTGGGATGTACTATTAAACCAAAATTGGGATTA 540
P.suaveolens        AAATTGAACAAGTATGGTCGTCCCCTATTGGGCTGTGCTATTAAACCAAAATTGATTTAG 540
```


P.afra T-CTGCTAAAAACTATGGTCGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGATTTTAC 599
G.robusta T-CCGCTAAGAACTACGGTCGAGCTGTTTATGAATGTCTTCGCGGCGGACTTGATTTTAC 599
O.mucrunata T-CCGCTAAGAACTACGGTAGAGCTGTTTATGAATGTCTACGTGGTGGACTTGACTTTAC 599
R.pterota T-CCCCTAAGAACTACGGTAGAGCTGTTTATGAATGTTTACGTGGTGGACTTGACTTTAC 599
P.auriculata T-CCGCTAAGAACTACGGTAGAGCAGTTTATGAATGTCTACGTGGTGGACTTGATTTTAC 599
A.tetracantha T-CCGCGAAGAACTACGGTAGAGCCGTTTATGAATGTCTACGCGGTGGACTTGATTTTAC 599
E.bothae T-CCGCTAAGAATTATGGTAGAGCGGTTTATGAATGTCTTCGCGGTGGACTTGATTTTAC 599
J.capensis T-CCCCTAAGAATTATGGTAGAGCGGTTTATGAATGTCTTCGCGGTGGACTTGATTTTAC 599
G.capitata T-CCGCTAAGAATTATGGTAGAGCAGTTTATGAATGTCTCCGCGGTGGACTTGATTTTAC 599
M.capitata T-CCGCTAAGAATTATGGTAGAGCAGTTTATGAATGTCTCCGCGGTGGACTTGATTTTAC 599
P.pyracantha T-CCGCTAAGAATTATGGTAGAGCAGTTTATGAATGTCTCCGCGGTGGACTTGATTTTAC 599
C.rudis T-CCGCTAAGAATTACGGTAGAGCCGTTTATGAATGTCTTCGCGGTGGACTTGATTTTAC 599
C.haematocarpa T-CCGCTAAAAACTACGGTACGGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTAC 599
M.nemorosa T-CCGCTAAAAACTACGGTAGGGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTAC 599
P.verrucosus T-CCGCTAAAAACTACGGTAGGGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTAC 599
C.bispinosa T-CCGCTAAAAACTACGGTAGGGCAGTTTATGAACGTCTTCGTGGTGGACTTGATTTTAC 599
S.myrtina T-CTGCTAAAAACTACGGTAGAGCTGTTTATGAATGTCTTCGCGGGGGACTTGATTTTAC 599
L.ferocissimum T-CCGCTAAAAACTACGGTAGAGCTGTTTATGAATGTCTTCGCGGTGGACTTGATTTTAC 599
B.ilicifolia T-CCGCTAAAAACTACGGTAGAGCTGTTTATGAATGTCTTCGTGGTGGCCTTGATTTTAC 599
E.undulata T-CCGCTAAAAACTACGGTAGAGCAGTTTATGAATGTCTCCGCGGTGGACTTGATTTTAC 599
P.crassicladus T-CCGCAAAAAACTACGGTAGAGCAGTTTATGAATGTCTACGCGGTGGGCTTGATTTTAC 599
P.africanus T-CCGCAAAAAACTACGGTAGAGCAGTTTATGAATGTCTACGCGGTGGGCTTCATTTTAC 599
P.suaveolens TGTCGAAACAAACGTCGGTAGA-CAGTTTATGAATGTCTACGCGGTGGGCTTGATTTTAC 599
P.afra CAAAGATGATGAAAACGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
G.robusta CAAAGATGATGAAAACGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
O.mucrunata CAAAGATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCCTATT 659
R.pterota CAAAGATGATGAGAACGTGAACTCCCAACCTTTTATGCGTTGGAGAGACCGTTTCCTATT 659
P.auriculata CAAAGATGATGAGAATGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTGTT 659
A.tetracantha CAAAGATGATGAGAATGTGAACTCTCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
E.bothae CAAAGATGATGAGAACGTGAACTCTCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
J.capensis CAAACATGATGAGAACGTGAATTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
G.capitata CAAAGATGATGAGAACGTAAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
M.capitata CAAAGATGATGAGAACGTAAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
P.pyracantha CAAAGATGATGAGAACGTAAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
C.rudis CAAAGATGATGAGAACGTGAATTCCCAACCGTTTATGCGTTGGAGAGACCGTTTCTTATT 659
C.haematocarpa CAAACATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTTTCGTATT 659
M.nemorosa CAAAGATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTTTCGTATT 659
P.verrucosus CAAAGATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTTTCGTATT 659
C.bispinosa CAAAGATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATTTTTTCTTTTT 659
S.myrtina CAAAGATGATGAAAACGTGAACTCCCAACCATTTATGCGTTGGAGAGATCGTTTCTTATT 659
L.ferocissimum CAAACATGATGAGAACGTGAACTCACAACCATTTATGCGTTGGAGAGATCGTTTCTTATT 659
B.ilicifolia TAAAGATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
E.undulata CAAAGATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATT 659
P.crassicladus CAAGGATGATGAAAACGTGAACTCACAACCTTTTATGCGTTGGCGAGACCGTTTCGTATT 659
P.africanus CAAGGATGATGAAAACGTGAACTCACAACCTTTTATGCGTTGGCGAGACCGTTTCGTATT 659
P.suaveolens CAAGGATGATGAAAACGTGAACTCACAACCTTTTATGCGTTGGCGAGACCGTTTCGTATT 659
P.afra TTGTGCCGAAGCAATTTATAAAGCACAGCCCGAAACAGGTGAAATCAAAGGGCATTACTT 719
G.robusta TTGTACCGAAGCTCTTTATAAAGCACAGGCTGAAACAGGTGAAGTCAAAGGACATTACTT 719
O.mucrunata TTGTGCGGAAGCAATTTATAAAGCGCAGGCTGAAACAGGTGAAATTAAAGGTCATTACTT 719
R.pterota TTGTGCGGAAGCAATTTATAAAGCGCAGGCTGAAACAGGTGAGATTAAAGGTCATTACTT 719
P.auriculata TTGTGCCGAAGCACTTTTTAAAGCACAGGCTGAAACTGGTGAAATCAAAGGGCATTACTT 719
A.tetracantha TTGTGCCGAAGCTCTTTATAAAGCACAGGCCGAAACGGGTGAAATCAAACGGCATTATTT 719
E.bothae TTGTGCCGAAGCAATTTTTAAATCACAGGCTGAAACGGGTGAAATCAAAGGACATTATTT 719
J.capensis TTGTGCCGAAGCAATTTATAAAGCACAGGCTGAAACAGGTGAAATCAAAGGACATTATTT 719
G.capitata TTGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACAGGTGAAATCAAAGGGCATTACTT 719
M.capitata TTGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACAGGTGAAATCAAAGGGCATTACTT 719
P.pyracantha TTGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACAGGTGAAATCAAAGGGCATTACTT 719
C.rudis TTGTGCCGAAGCAATTTATAAAGCACAGGCCGAAACTGGTGAAATCAAAGGGCATTACTT 719
C.haematocarpa TTGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACCGGTGAAATCAAAGCGCATTACTT 719
M.nemorosa TTGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACCGGTGAAATCAAAGGGCATTACTT 719
P.verrucosus TTGTCCCGAAGCACTTTATAAAGCACAGGCTGAAACCGGTGAAATCAAAGGGCATTACTT 719
C.bispinosa TTGTGGACGCCGAATTTATAAAGCACAAGCTGAAACCGGTAAAATCAAAGGGCATTACTT 719
S.myrtina TTGTGCTGAAGCGCTTTATAAAGCACAATCTGAAACAGGTGAAATCAAAGGGCATTACTT 719
L.ferocissimum TTGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACAGGTGAAATCAAAGGGCATTACTT 719
B.ilicifolia TTGTGCCGAAGCTATTTATAAAGCACAAGCTGAAACAGGTGAAATCAAAGGGCATTACTT 719
E.undulata TTGTGCCGAAGCAATTTTTAAAGCACAGGCTGAAACAGGTGAAATCAAAGGGCATTACTT 719
P.crassicladus TTGTGCTGAAGCTCTTTATAAAGCACAAGCAGAAACAGGTGAAATCAAAGGACATTACTT 719
P.africanus TTGTGCTGAAGCTATTTATAAAGCACAACCAGAAACAGGTGAAATCAAAGGACATTACTT 719
P.suaveolens TTGTGCTGAAAGTCTTTATAAAGCACAAGCAGAAACAGGTGAAATCAAAGGACATTACTT 719

```
P.afra              GAATGCTACCGCAGGTACATGCGAAGAAATGATAAAAAGGGCTGTATTTGCCAGAGAATT 779
G.robusta           GAATGCTACTGCAGGTACATCCGAAGAAATGATAAAAAGAGCTTCATGTGCCAGAGAATT 779
O.mucrunata         GAATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGGGCTGTATTTGCAAGAGAGTT 779
R.pterota           GAATGCTACTGCAGGTACATGCGAAGACATGCTAAAAAGGGCTGTATTTGCAAGAGAGTT 779
P.auriculata        GAATGCTACTGCGGGTACATGTGAAGACATGATGAAAAGGGCCGTATGTGCCAGAGAATT 779
A.tetracantha       GAATGCTACTGCGGGTACATGCGAAGAAATGATGAAAAGAGCTGTATTTGCCAGAGAATT 779
E.bothae            GAATGCTACTGCAGGTACATCCGAACAAATGATCAAAAGGGCTGTATTTGCCAGGGAATT 779
J.capensis          GAATGCTACTGCAGGTACATGTGAAGAAATGATCAAAAGGGCTGTATTTGCCAGAGAATT 779
G.capitata          GAATGCTACTGCCGGTACATGCGAAGAAATGATCAAAAGGGCTGTATTTGCTAGAGAACT 779
M.capitata          GAATGCTACTGCCGGTACATGCGAAGAAATGATCAAAAGGGCTGTATTTGCTAGAGAACT 779
P.pyracantha        GAATGCTACTGCCGGTACATGCGAAGAAATGATCAAAAGGGCTGTATTTGCTAGAGAACT 779
C.rudis             GAATGCTACGGCAGGTACATGGGAAGATATGCTCAAAAGGGCTGTATGTGCCAGAGAATT 779
C.haematocarpa      GAATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGAGCTGCATTTGCTAGAGAATT 779
M.nemorosa          GAATGCTACTGCAGGTACATCCGAAGAAATGATGAAAAGAGCTGCATTTGCTAGAGAATT 779
P.verrucosus        GAATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGAGCTGCATTTGCTAGAGAATT 779
C.bispinosa         GAATGCTACTGCAGGTACATCCGAAGAAATGATGAAAAGAGCTGGAGACGCCAGAGAATT 779
S.myrtina           GAATGCTACTGCAGGTACATGCGAAGAAATGATCAAAAGAGCTGTATTTGCTAGAGAATT 779
L.ferocissimum      GAATGCTACTGCAGGTACATCCGAAGAGATGATGAAAAGAGCTATATTTGCTAGAGAATT 779
B.ilicifolia        GAATGCTACTGCGGGTACATGCGAAGAAATGATGAAAAGGGCTGTATTTGCCAGAGAATT 779
E.undulata          GAATGCTACTGCAGGTACATGTGAAGAAATGATAAAAAGGGCTGTATTTGCCAGAGAATT 779
P.crassicladus      GAATGCAACTGCAGGTACATGTGAAGAAATGATGAAAAGGGCCGTATTTGCCAGAGAATT 779
P.africanus         GAATGCAACTGCAGGTACATGTGAAGAAATGATTAAAAGGGCCGTATTTGCCAGAGAATT 779
P.suaveolens        GAATGCAACTGCAGGTACATGTGAAGAAATCATGAAAAGGGCCGCATTTGCCAGAGAATT 779
                    ****** ** ** *******  ***** *** * ****** **   *  ** ** ** *

P.afra              GGGAGTTCCTATCGTAATGCATG 802
G.robusta           GGGAGTTCCTATCGTAATGCATG 802
O.mucrunata         GGGAGTTCCTATCGTAATGCATG 802
R.pterota           GGGAGTTCCTATCGTAATGCATG 802
P.auriculata        GGGAGTTCCTATCGTAATGCATG 802
A.tetracantha       GGGAGTTCCTATCGTAATGCATG 802
E.bothae            GGGAGTTCCTATCGTAATGCATG 802
J.capensis          AGGAGTTCCTATCGTAATGCATG 802
G.capitata          GGGAGTTCCTATCGTAATCCATG 802
M.capitata          GGGAGTTCCTATCGTAATGCATG 802
P.pyracantha        GGGAGTTCCTATCGTAATGCATC 802
C.rudis             GGGAGTTCCTATCGTAATGCATG 802
C.haematocarpa      GGGAGTTCCTATCGTAATGCATG 802
M.nemorosa          GGGAGTTCCTATCGTAATGCATG 802
P.verrucosus        GGGAGTTCCTATCGTAATGCATG 802
C.bispinosa         GGGAGTTCCTATCGTAATGCATG 802
S.myrtina           GGGAGTTCCAATCGTAATGCATG 802
L.ferocissimum      GGGAGTTCCTATCGTAATGCATG 802
B.ilicifolia        GGGAGTTCCTATCGTAATGCATG 802
E.undulata          AGGAGTTCCTATCGTAATGCATG 802
P.crassicladus      GGGAGTTCCTATCGTAATGCATG 802
P.africanus         GGGAGTTCCTATCGTAATGCATG 802
P.suaveolens        GGGAGTTCCTATCGTAATGCATG 802
                     ******** *************
```

Appendix L: ClustalW 2.0.8 multiple sequence alignment of clones




Clone8 GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
Clone10 GTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
Clone5 GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACCAACATGTTTACTTCCATT 360
Clone4 GTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATT 360
Clone7 GTGGGTAATGTACTTGGTTTCAAAGCCCTACGAGCTCTACGTTTGGAGGATCTGCGAATT 420
Clone3 GTGGGTAATGTATTTGGTTTCAAAGCCCTACGAGCTCTACGTTTGGAGGATCTGCGAATT 420
Clone11 GTGGGTAATGTATTTGGGTTCAAACCCCTGCGTGCTCTACGTTTGGAGGATTTGCGAATC 420
Clone1 GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGTGCTCTACGTTTGGAGGATTTACGAATC 420
Clone9 GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGTGCTCTACCTTTGGAGGATTTACGAATC 420
Clone2 GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGGAAGATTTGCGAATC 420
Clone6 GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGCAAGATTTGCGAATC 420
Clone8 GTAGGTAATGTATTTGGGTTCAAAGCTCTACGCGCTCTACGTCTGGAAGATTTGCGAATC 420
Clone10 GTACGTAATGTATTTCGGTTCAAAGCTCTACCCGCTCTACGTCTGGAAGATTTGCGAATC 420
Clone5 GTGGGAAATGTGTTTGGATTCAAAGCCTTGCGTGCTCTACGTCTGGAAGATCTTCGAATC 420
Clone4 GTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATC 420
Clone7 CCCCCTGCTTATTCCAAAACTTTCCAAGGCCCGCCTCATGGCATCCAAGTTCAAAGAGAT 480
Clone3 CCCCCTGCTTATTCCAAAACTTTCCAAGGCCCGCCTCATGGTATCCAAGTTGAAAGAGAT 480
Clone11 CCTCCTGCTTATTCGAAAACTTTCCAAGGCCCGCCTCACGGTATCCAAGTTGAAAGAGAT 480
Clone1 CCTATTGCTTATGTAAAAACTTTCCAAGGACCGCCTCACGCCATCCAGGTTGAGAGAGAG 480
Clone9 CCTATTGCTTATGTAAAAACTTTCCAAGGACCGCCTCACGGTATCCAGGTTGAGAGAGAT 480
Clone2 CCTACGGCTTATATTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAGAGAGAT 480
Clone6 CCTACGGCTTATATTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGGTTGACAGAGAT 480
Clone8 CCTACGGCTTATATTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAGAGAGAT 480
Clone10 CCTACGGCTTATATTAAAACCTTCCAAGGCCCGCCTCATGGCATCCAGGTTGAGAGAGAT 480
Clone5 CCTACTGCTTATATTAAAACTTTCCAAGGTCCGCCTCATGGGATCCAAGTTGAGAGAGAT 480
Clone4 CCTACCGCGTATACAAAAACTTTCCAAGGACCACCGCATGGGATCCAAGTTGAGAGAGAT 480
Clone7 AAATTGAACAAGTATGGTCGTCCCCTATTGGGATGTACTATTAAACCAAAGTTGGGATTA 540
Clone3 AAATTGAACAAGTATGGTCGTCCCCTATTGGGATGTACTATTAAACCAAAATTGGGATTA 540
Clone11 AAATTGAACAAATATGGCCGTCCCCTATTGGGATGTACTATTAAACCTAAATTGGGGTTG 540
Clone1 AAATTGAACAAGTATGGTCGCCCCCTATTGGGATGCACTATTAAACCCAAATTGGGGTTA 540
Clone9 AAATTGAACAAGTATGGTCGCCCCCTATTGGCATGCACTATTAAACCGAAATTGGGGTTA 540
Clone2 AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
Clone6 AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
Clone8 AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
Clone10 AAATTGAACAAATATGGTCGTCCCCTGTTGGGATGTACTATTAAACCTAAATTGGGGTTA 540
Clone5 AAGTTGAACAAGTATGGTCGTCCCCTGCTGGGATGTACTATTAAACCGAAATTGGGGTTA 540
Clone4 AAATTGAACAAGTATGGACGTCCCCTATTGGGATGTACTATTAAACCTAAATTAGGTTTA 540
Clone7 TCCGCAAAAAACTACGGTAGAGCAGTTTATGAATGTCTACGCGGTGGGCTTGATTTTACC 600
Clone3 TCCGCAAAAAACTACGGTAGAGCAGTTTATGAATGTCTACGCGGTGGGCTTGATTTTACC 600
Clone11 TCCGCTAAGAACTACGGTCGAGCTGTTTATGAATGTCTTCGCGGCGGACTTGATTTTACC 600
Clone1 TCTGCTAAAAACTATGGTCGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGACTTTACC 600
Clone9 TCTGCTAAAAACTATGGTCGAGCAGTTTATGAATGTCTTCGCGGTGGACTTGACTTTACC 600
Clone2 TCCGCTAAAAACTACGGTAGGGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTACC 600
Clone6 TCCGCTAAAAACTACGGTAGGGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTACC 600
Clone8 TCCGCTAAAAACTACGGTAGGGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTACC 600
Clone10 TCCGCTAAAAACTACGGTAGGGCAGTTTATGAATGTCTTCGTGGTGGACTTGATTTTACC 600
Clone5 TCCGCTAAAAACTATGGTAGAGCGTGTTATGAATGTCTTCGCGGTGGACTTGATTTTACC 600
Clone4 TCCGCTAAGAACTACGGTAGAGCTGTTTATGAATGTTTACGTGGTGGACTTGACTTTACC 600
Clone7 AAGGATGATGAAAACGTGAACTCACAACCTTTTATGCGTTGGCGAGACCGTTTCGTATTT 660
Clone3 AAGGATGATGAAAACGTGAACTCACAACCTTTTATGCGTTGGCGAGACCGTTTCGTATTT 660
Clone11 AAAGATGATGAAAACGTGAACTCCCAACCATTTATGCGTTGGAGACACCGTTTCTTATTT 660
Clone1 AAAGATGATGAAAATGTGAACTCCCAACCATTTATGCGTTGGGGAGACCGTTTCTTATTT 660
Clone9 AAAGATGATGAAAATGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCTTATTT 660
Clone2 AAAGATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTTTCTTATTT 660
Clone6 AAAGATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTTTCTTATTT 660
Clone8 AAAGATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTTTCTTATTT 660
Clone10 AAAGATGATGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTTTCTTATTT 660
Clone5 AAAGATGATCAGAACGTGAACTCCCAACCATTTATGCGTTGGAGAGATCGTTTCTTATTT 660
Clone4 AAACATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCCTATTT 660
Clone7 TGTGCCGAACCTCTTTATAAACCACAAGCAGAAACAGGTGAAATCAAGGGACATTACTTG 720
Clone3 TGTGCTGAAGCTCTTTATAAAGCACAAGCAGAAACAGGTGAAATCAAAGGACATTACTTG 720
Clone11 TGTACCGAAGCTCTTTATAAAGCACAGGCTCAAACAGGTGAAGTCAAAGGACATTACTTG 720
Clone1 TGTGCCGAAGCAATTTATAAAGCACAGGCCGAAACAGGTGAAATCAAAGGGCATTACTTG 720
Clone9 TCTGCCGAAGCAATTTATAAAGCACAGGCCGAAACAGGTGAAATCAAAGGGCATTACTTG 720
Clone2 TGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACCGGTGAAATCAAAGGGCATTACTTG 720

```
Clone6          TGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACCGGTGAAATCAAAGGGCATTACTTG 720
Clone8          TGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACCGGTGAAATCAAAGGGCATTACTTG 720
Clone10         TGTGCCGAAGCACTTTATAAAGCACAGGCTGAAACCGGTGAAATCAAAGGGCATTACTTG 720
Clone5          TGTGCTGAAGCAATTTATAAATCACAAGCTGAAACAGGCGAAATCAAAGGGCATTACTTG 720
Clone4          TGTCCGGAAGCAATTTATAAAGCGCAGGCTGAAACAGGTGAAACTAAAGGTCATTACTTG 720
                *** * ***** ***** ** ** ** ***** ** **** ** ** **********

Clone7          AATCCAACTGCAGGTACATGTGAAGAAATGATGAAAAGGGCCGTATTTGCCAGAGAATTG 780
Clone3          AATGCAACTGCAGGTACATGTGAAGAAATGATGAAAAGGGCCGCATTTCCCAGAGAATTG 780
Clone11         AATGCTACTGCAGGTACATCCGAAGAAATGATAAAAAGAGCTTCATGTGCCAGAGAATTG 780
Clone1          AATGCTACCGCGGGTACATGCGAACAAATGATAAAAAGGGCTGTATTTGCCAGAGAATTG 780
Clone9          AATGCTACCGCGGGTACATGCGAAGAAATGATAAAAAGGGCTGTATTTGCCAGAGAATTG 780
Clone2          AATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGAGCTGTATTTGCTAGAGAATTG 780
Clone6          AATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGAGCTGTATTTGCTAGAGAATTG 780
Clone8          AATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGAGCTGTATTTGCTAGAGAATTG 780
Clone10         AATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGAGCTGTATTTGCTAGAGAATTG 780
Clone5          AATGCTACTGCGGGTACATGCGAAGAAATGATCAAAAGAGCTGTATTTGCTAGGGAGTTG 780
Clone4          AATGCTACTGCAGGTACATGCGAAGACATGCTAAAAAGGGCTGTATGTGCCAGAGAGTTG 780
                ***** ** ** ******* ***** *** * ***** ** ** ** *** ** ** ***

Clone7          GGAGTTCCTATCGTAATGCATG 802
Clone3          GGAGTTCCTATCGTAATGCATG 802
Clone11         GGAGTTCCTATCGTAATGCATG 802
Clone1          GGAGTTCCTATCGTAATGCATG 802
Clone9          GGAGTTCCTATCGTAATGCATG 802
Clone2          GGAGTTCCTATCGTAATGCATG 802
Clone6          GGAGTTCCTATCGTAATGCATG 802
Clone8          GGAGTTCCTATCGTAATGCATG 802
Clone10         GGAGTTCCTATCGTAATGCATG 802
Clone5          GGAGTTCCTATCGTAATGCATG 802
Clone4          GGAGTTCCTATCGTAATGCATG 802
                ***********************
```

Appendix M: Comparison of eleven clone sequences to the GFRR plant sequence database

	Clone number and percent identity										
Plants	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
P. auriculata	91.4	92.5	91.6	90.9	89.4	92.5	91.1	92.5	91.8	92.9	**100.0**
E. undulata	92.8	94.4	92.9	92.5	92.5	94.4	92.6	94.4	93.2	94.9	93.0
B. illicifolia	92.7	94.1	90.9	91.8	92.2	94.1	91.0	94.1	92.8	94.6	93.0
P. afra	**96.8**	92.5	90.8	90.9	90.9	92.5	90.6	92.5	**97.1**	92.4	92.9
P. verucosus	91.6	**99.6**	91.9	91.3	92.5	**99.6**	91.8	**99.6**	91.8	**98.9**	92.5
C. haematocarpa	91.6	**99.6**	91.9	91.1	92.3	**99.6**	91.8	**99.6**	91.8	**98.9**	92.5
M. nemorosa	91.6	**99.6**	91.9	91.1	92.3	**99.6**	91.8	**99.6**	91.8	**98.9**	92.5
M. capitata	91.2	92.8	92.4	92.2	91.1	92.8	92.0	92.8	91.5	93.3	92.5
L. ferocissimum	91.2	95.0	91.4	91.8	**93.8**	95.0	91.3	95.0	91.4	94.9	92.4
J. capensis	91.7	92.9	91.8	92.9	91.4	92.9	91.5	92.9	92.0	93.3	92.4
G capitata	91.2	92.8	92.3	92.4	90.9	92.8	92.0	92.8	91.5	93.3	92.3
S. myrtina	91.0	96.0	91.4	91.4	93.0	96.0	91.3	96.0	91.2	95.8	92.1
C. rudis	91.7	92.9	91.4	91.8	90.2	92.9	91.1	92.9	91.8	93.4	92.1
A. tetracantha	90.9	92.6	91.9	92.2	90.7	92.6	91.8	92.6	91.3	93.1	92.1
P. pyracantha	90.5	92.7	91.9	91.8	90.7	92.7	91.6	92.7	90.9	93.2	91.6
E. bothae	91.0	92.5	90.8	92.2	91.4	92.5	90.6	92.5	91.3	92.8	91.6
P. crassicladus	90.2	91.9	**99.6**	89.8	90.2	91.9	**99.4**	91.9	90.4	92.0	91.5
G. robusta	90.7	93.3	90.8	93.0	91.0	93.3	90.8	93.3	90.8	93.2	91.4
P. africanus	90.0	91.4	99.4	89.8	90.2	91.4	98.8	91.4	90.3	91.5	91.4
C. bispinosa	90.2	97.6	90.3	90.0	91.2	97.6	90.1	97.6	90.3	96.9	90.8
O. mucrunata	90.9	92.0	90.0	98.9	90.8	92.0	89.8	92.0	91.1	92.1	90.8
R. pterota	90.0	91.4	89.5	**99.1**	90.2	91.4	89.3	91.4	90.3	91.4	90.5
P. sauveolens	87.7	89.5	97.9	88.1	87.7	89.5	96.9	89.5	87.9	89.6	90.0

Appendix N: Comparison of the *rbc*L FLX generated sequences from black rhinoceros dung samples against the GFRR *rbc*L plant sequence database

Table N1: Summer dung sample

No. of Sequences	Family of match	Genus	Species	% Match
Twenty-seven	**Apocynaceae**	*Carissa*	*bispinosa*	98.8
One	**Ebenaceae**	*Euclea*	*undulata*	96.0
Three	**Portulacaceae**	*Portulacaria*	*afra*	96.3 to 96.6

Table N2: Autumn dung sample

No. of sequences	Family of match	Genus	Species	% Match
Twenty-five	**Apocynaceae**	*Carissa*	*bispinosa*	94.7 to 98.4
Five	**Asteraceae**	*Brachylaena*	*ilicifolia*	91.0 to 95.8
Five	**Celastraceae**	*Gymnosporia, Puttterlickia*	*capitata pyracantha*	80.1 to 98.9
One	**Euphorbiaceae**	*Jatropha*	*capensis*	96
Nine	**Portulacaceae**	*Portulacaria*	*afra*	92.8 to 98.4
Two	**Solanaceae**	*Lycium*	*feroscissimum*	93.9

Table N3: Winter dung sample

No. of sequences	Family of match	Genus	Species	% Match
Thirty-four	**Apocynaceae**	*Carissa*	*bispinosa*	99.2 to 98.4
One	**Anacardiaceae**	*Rhus*	*pterota*	95.4 to 95.9
Eleven	**Portulacaceae**	*Portulacaria*	*afra*	95.5 to 98.8
Two	**Rhamnaceae**	*Scutia*	*myrtina*	92.7 to 95.9

Table N4: Spring dung sample

No. of sequences	Family of match	Genus	Species	% Match
One	**Asteraceae**	*Brachylaena*	*ilicifolia*	96.2
Eleven	**Apocynaceae**	*Carissa*	*bispinosa*	91.2 to 96.9
Five	**Ebenaceae**	*Euclea*	*undulata*	95.0

Appendix O: Reagents for antioxidant assays

Appendix O1: Acetate buffer: 300 mM, pH 3.6 in 1 litre

3.1 g sodium acetate.$3H_2O$

16 ml glacial acetic acid

ddH_2O was added to a litre and stored at 4°C

Appendix O2: PBS, pH 7.4 in 1 litre

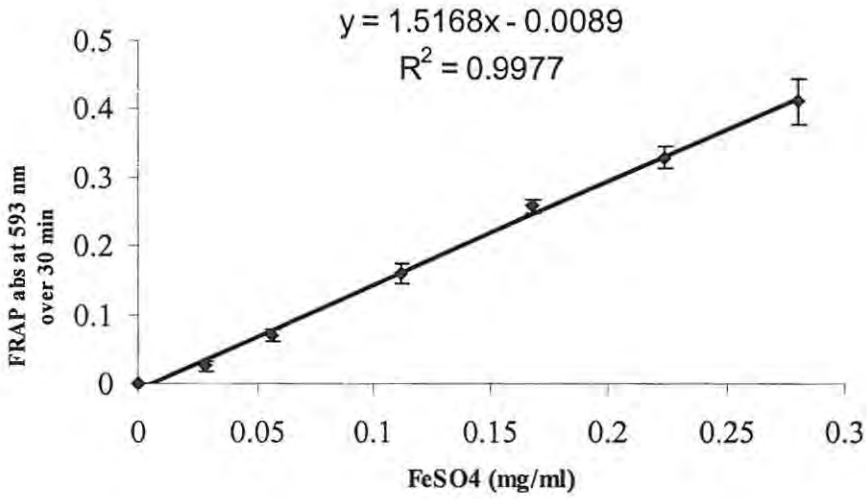
137 mM NaCl

2.7 mM KCl

10 mM $NaHPO_4$

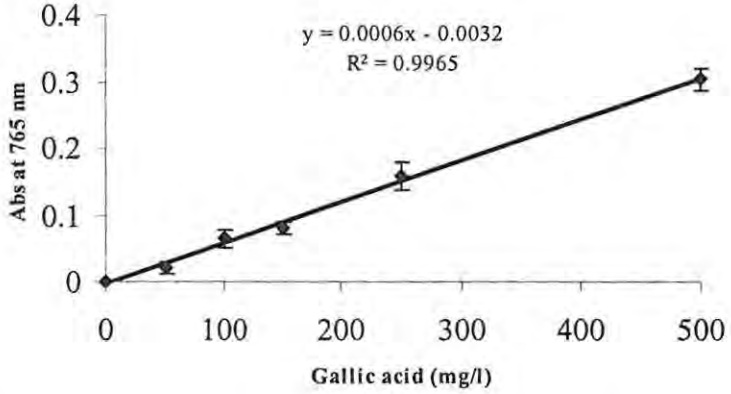
2 mM KH_2PO_4

Appendix P: Ferrous sulfate calibration curve



Concentration response curve for ferrous sulfate standard with the absorbance measured at 593 nm. The experiment was run in triplicate ± SD.

Appendix Q: Gallic acid calibration curve



Concentration response curve for gallic acid standard with the absorbance measured at 765 nm. All data are shown as means ± SD. The experiment was run in triplicate.

Appendix R: Total recovery of plant extracts

Phenolic compounds were extracted from twenty-five different plants representing fourteen families, twenty-one genus and twenty-four species for using 80% methanol

Plant family	Plant species	mg dry weight recovered/ml
Euphorbiaceae	*Jatropha capensis*	246.67
Ebenaceae	*Euclea undulata*	226.62
Celastraceae	*Maytenus heterophylla*	188.33
Boraginaceae	*Ehretia rigida*	180.00
Euphorbiaceae	*Euphorbia bothae*	176.67
Asteraceae	*Brachylaena ilicifolia*	176.67
Asparagaceae	*Protasparagus suaveolens*	170.00
Apocynaceae	*Carissa haematocarpa*	149.50
Portulacaceae	*Portulacaria afra*	146.67
Fabaceae	*Schortia afra*	137.58
Solanaceae	*Lycium ferocissimum*	134.30
Celastraceae	*Maytenus capitata*	133.33
Asparagaceae	*Protasparagus crassicladus*	133.33
Bignoniaceae	*Rhygozum obovatum*	133.33
Capparaceae	*Capparis sepiaria*	133.33
Asparagaceae	*Protasparagus africanus*	133.33
Salvadoraceae	*Azima tetracantha*	133.33
Apocynaceae	*Carissa bispinosa*	130.33
Celastraceae	*Putterlickia pyracantha*	125.00
Anacardiaceae	*Ozoroa mucrunata*	103.33
Anacardiaceae	*Rhus pterota*	100.00
Asteraceae	*Tarchonanthus camphoratus*	97.33
Euphorbiaceae	*Phyllanthus verrucosus*	63.33
Plumbaginaceae	*Plumbago auriculata*	56.67
Tiliaceae	*Grewia robusta*	50.67

Values are the total amount of dry plant material recovered/amount of the initial volume used for extraction. Working solutions of 1 mg/ml of each plant extract were prepared and analyzed for antioxidant activities and total phenolic content.