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

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DECLARATION

This work is the original work produced by Siyavuya I. Bulani, submitted to Rhodes University in January 2007, for a Master of Science degree in Biochemistry.

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ABSTRACT

The black rhinoceros remains one of the world's extremely endangered species despite a variety of policies to protect it. The black rhinoceros population at the Great Fish River Reserve (GFRR) in the Eastern Cape in South Africa has increased steadily since their re-introduction in 1986. This megaherbivore is a browser, with a diet obtained largely from the short and medium succulent thicket of the GFRR. Knowledge of the preferential diet of the black rhinoceros on the reserve is an important factor for the effective management of the land and the herbivores that compete for its resources. The dietary preferences of the black rhinoceros at the reserve have been established using backtracking methods. In this study the *rbc*L gene was used to establish an *rbc*L gene database of the plants from the GFRR and determine the botanical composition of the black rhinoceros dung from the GFRR. Due to the limited number of *rbc*L gene plant sequences from the GFRR deposited in the GenBank database, 18 plant species from the GFRR were sequenced. Sequence analyses between the partial *rbc*L gene sequences generated were able to distinguish between plants down to species level. Plant species from the family Euphorbiaceae and Fabaceae showed sequence variation at intra-specific level compared to those of Tiliaceae which were more conserved. The generated *rbc*L gene sequences from seasonal dung samples were compared to the *rbc*L gene sequenced from 18 plant species obtained from the GFRR and those from the GenBank database. A wide range of plant species were identified from the dung samples. There were no major differences in botanical composition between the dung samples, except that *Grewia spp.* were found to dominate in almost all seasons.

The results obtained on the free radical scavenging activity of the extracts against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) increased in the order of methanol > ethyl acetate > chloroform. The DPPH free radical scavenging activity of the methanol plant extracts increased in the order *Brachylaena elliptica* > *Plumbago auriculata* > *Grewia robusta* > *Azima tetracantha*. Methanol extracts on the TLC plate sprayed with Fe^{3+}-2,4,6-Tri-2-pyridyl-*s*-triazine (TPTZ) showed that the compounds present in the extracts react differently to ferric ion, with most compounds unable to reduce ferric ion. Furthermore the methanol extracts were able to exhibit reduction potentials vs. Ag/AgCl at low concentrations. The compounds in the extracts were shown to be phenolic acids and flavonoid glycosides.

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LITERATURE REVIEW

1.1 Introduction

Protection of wildlife has been of concern to many different conservation organizations for many years. The establishment of the International Union for the Conservation of Nature (IUCN) in 1948 made nature conservation a universally accepted concept with the basic idea of conserving wildlife (Tivy and O'Hare, 1981). Wildlife is threatened by many factors, such as habitat encroachment, which alone is believed to threaten one-third of the endangered mammals. The World Conservation Monitoring Center documented 486 extinctions since 1600, and of those, 80 are a result of hunting and 98 as a result of habitat destruction (Bulte and Horan, 2003). For this reason, many countries have enforced laws to protect wildlife species. In most African countries, laws on the protection of wildlife date back to the colonial era, where the primary objective was to protect wild animals and their natural habitats using restricted wildlife areas (Johannesen and Skonhoft, 2005). These laws, to some extent, have managed to minimize extinction of the critically endangered plants and animals. First and second world countries have managed with difficulty to formulate programs to conserve the plants and animals that are slowly becoming extinct. Countries that have taken the responsibility of protecting wildlife have used zoos, sanctuaries, game reserves and national parks to preserve and breed animals (Dasmann, 1964). These areas have long been recognized as the single most important method of conserving wildlife and preserving biological diversity (Johannesen and Skonhoft, 2005).

In South Africa, rangelands form an important part of the ecosystem as more than 70% of the land is too arid for crop production. Therefore, most of the land is used for either commercial livestock ranching, communal livestock ranching, or game ranching. These three systems have different management strategies as a result of the combinations of the animals in each system (Smet and Ward, 2006). In South Africa, private land allocated to wildlife has increased and this has increased the population of ungulates in game reserves in the past thirty years (Dekker, 1997; Bulte and Horan, 2003).

Game reserves and national parks have been the preferred areas for conservation of wildlife due to their size. The size of a game reserve depends largely on the type of habitat. The required minimum size of a game reserve in the Lowveld is 2000 hectares and 10 000 hectares in the arid areas in order to sustain population size. Zoos and sanctuaries are small and require more intensive management, whereas nature reserves require less management practices due to their large size (Bothma, 2002). However, range management, in particular, the management of elephant and buffalo numbers, is necessary even in large areas such as the Kruger National Park which is 2 million hectares and still not large enough to allow a completely natural equilibrium to develop. This challenges range managers to understand the ecological carrying capacity of the land since game reserves have different animals that compete for resources. Ecological carrying capacity is very complex as it is dependant on many factors such as plant diversity, climate, land encroachment and previous land use by owners (Bothma, 2002).

The type of habitat and purpose for which the game reserve is used determines the optimal carrying capacity for ungulates. Thorough range management practices and other methods of habitat manipulation can be used to increase the optimal carrying capacity of an area for a combination of grazers and browsers (Bothma, 1996). To supply optimal forage among different animal species it is necessary to maintain or improve the conditions of these ranges and this requires a thorough knowledge of the natural diet of all animals inhabiting a particular rangeland. Compared to domestic animals, little is known about the nutrient requirements of wild animals and knowledge of the animal's diet is important for their successful conservation and propagation (Slifka *et al.*, 1999). Published data on the feeding habits of herbivores, particularly ungulates, has largely been on direct observation and fistula methods (Vavra *et al.*, 1978; Mofareh *et al.*, 1997; Henley *et al.*, 2001). Although these methods have generated much of the data that is used today, generally these techniques are time consuming, particularly fistula methods, are limited to small domestic animals and cannot be used on large ungulates (Vavra *et al.*, 1978).

In the past few years research on diet composition of ungulates has included analyses of nutrients such as vitamins, tannins, proteins and other important diet components (Graffam *et al.*, 1997). Due to advances in current technology, new methods have been used to reveal the diet composition

of herbivores using molecular methods. These methods have been used largely on ancient dung, where they have determined the diet of extinct animals (Poinar *et al.*, 1998; Hofreiter *et al.*, 2000).

1.2 The black rhinoceros

The black rhinoceros belongs to the family *Rhinocerotidae,* which has five living species, three found in Asia and two in Africa. The two African species are the black rhinoceros (*Diceros bicornis*) and the white rhinoceros (*Ceratotherium simum*). The Asian species are the Indian rhinoceros (*Rhinoceros unicornis*), the Javan rhinoceros (*Rhinoceros sondaicus*), and the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) (Emslie and Brooks, 1999; Tougard *et al.*, 2001). All these five species have been listed as critically endangered by the Convention on International Trade in Endangered Species (CITES) (Kapur *et al.*, 2003). With the exception of the white rhinoceros, all species are on the verge of extinction (Cunningham and Berger, 1997). Although the species are morphologically well defined, classification and evolutionary relationships among the species remain debated. The questions are whether the two African rhinos are closely related to the Sumatran rhinoceros, or whether the three Asian species are sister taxa (Tougard *et al.*, 2001). Phylogenetic studies by Tougard *et al.* (2001) using the sequences of the mitochondrial 12S rRNA and cytochrome *b* genes have revealed divergence between the African and Asian species, suggesting that the three Asian genera are sister taxa.

1.2.1 The black rhinoceros subspecies

The black rhinoceros (Fig. 1.2) has two horns, weighs up to 1350 kg and stands about 1.4-1.7 m tall at the shoulder and remains one of Africa's extremely endangered species despite a variety of policies to protect it (Cunningham and Berger, 1997). It has four recognized subspecies distributed in central and southern Africa: Western (*D. bicornis longipes*); Eastern (*D. bicornis michaeli*); South-western (*D. bicornis bicornis*); and South-central (*D. bicornis minor*). Of the four subspecies, *D. bicornis longipes* is the rarest and most endangered subspecies of black rhinoceros. Their numbers are continuing to decline and it is threatened with extinction in the near future. Most of the remaining animals live in small groups that are widely scattered and may not be in breeding contact. The *D. bicornis minor* is the most numerous of the black rhinoceros subspecies,

and today its strong hold is South Africa (Emslie and Brooks, 1999). Fig. 1.1 shows the distribution of these four black rhinoceros subspecies.

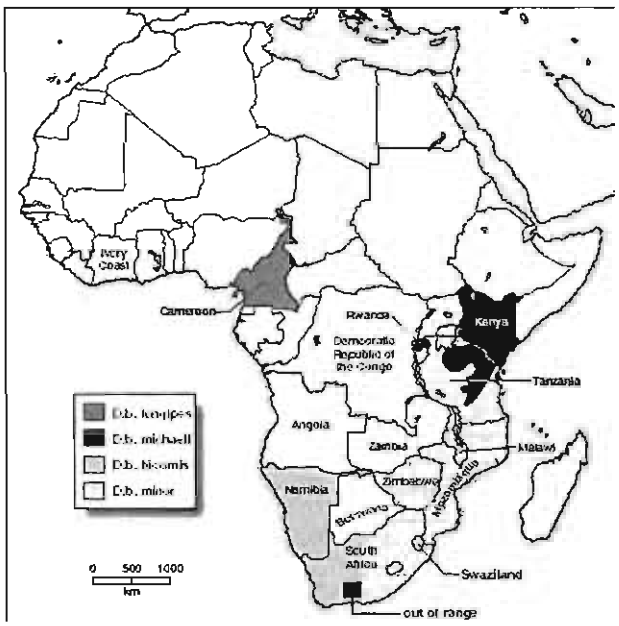

Fig. 1.1. Distribution of the four black rhinoceros subspecies in Africa (Emslie and Brooks, 1999).

1.2.2 Decline of black rhinoceros population

Black rhinoceros are distributed in central and southern Africa and in the 1960's there were about 100,000 black rhinoceros in this region, but a 97% decline between 1970 and 1992 decreased the population to 2600 in 1997 (Emslie and Brooks, 1999; Save the rhino international, 25-02-05). Habitat encroachment and widespread poaching are reported as the main reasons for this dramatic decline of the black rhinoceros population (Muya and Oguge, 2000; Cunningham *et al.*, 2001; Kapur *et al.*, 2003). These factors have caused extinction of the animals in most parts of their previous range (Cunningham *et al.*, 2001). Smithers (1983) as cited by O'Ryan *et al.* (1994) states that the decline of *D. bicornis* and *C. simum* populations in the sub-Saharan region was largely due to the massive invasion of their rangeland by humans. When habitats are destroyed or reduced, the remaining habitat is often too small to maintain viable populations of all species. Consequently, habitat reduction leads to extinction (Pimm and Askins, 1995).

Between 1970 and 1987 Zimbabwe, South Africa and Namibia recorded stable populations (Muya and Oguge, 2000). South Africa and Namibia were the only countries in Africa to have a net increase in black rhinoceros numbers between 1980 and 1987 (Emslie and Brooks, 1999), which was a result of re-introducing the wild rhinos into private and government areas, and by intensifying anti-poaching efforts (Walpole *et al.*, 2001). Management techniques to effectively prevent poaching and continued habitat encroachment that contribute to the decline in black rhinoceros have not yet been developed. Therefore, much effort has been directed towards maintenance of black rhinoceros in protected situations such as zoos and game farms (Grant *et al.*, 2002). Protection of the black rhinoceros population outside fenced reserves has been shown to be ineffective against poaching. In addition, black rhinoceros face threats to their genetic and demographic health due to their small population size and isolation (Moehlman *et al.*, 1996).

Fig. 1.2. Black rhinoceros from the Great Fish River Reserve (Picture by B. Fike).

1.2.3 Black rhinoceros and predators

Unlike other free-ranging herbivores, adult black rhinoceros do not have natural predators (Schenkel and Schenkel-Hullinger, 1969; Cunningham and Berger, 1997), but isolated cases where black rhinoceros were attacked and killed by lions have been observed. The reported killings were of sick black rhinoceros and unprotected calves, which are vulnerable to attacks both by lions or hyenas (Schenkel and Schenkel-Hullinger, 1969).

In the past fifteen years African range managers have been faced with a different form of black rhinoceros deaths; killings by elephants (*Loxodonta africana*). Between 1991 and 2001 five black rhinoceros and fifty-eight white rhinoceros have been killed by elephants in the Hluhluwe-Umfolozi Park. Further deaths have been recorded in the Pilanesberg National Park, where fifty white rhinoceros were killed by elephants in just five years between 1992 and 1997. The deaths were predominantly adult rhinoceros (86%), with a higher number of males killed (Slotow *et al.*, 2001). The killings were caused by young elephant males (17-25 years old) that were entering a state of musth. The condition is a result of heightened aggression from elevated hormones associated with reproductive competition. The problem was solved by introducing older male elephants, up to 35 years of age, which suppressed the musth of the young males (Slotow *et al.*, 2001).

1.2.4 Free-ranging black rhinoceros

Free-ranging wild black rhinoceros live in areas greater than 10 km^2 in their historical range, at natural densities and spacing, without routine food supplementation (Emslie and Brooks, 1999). The natural habitat of the black rhinoceros provides its ideal diet, which is mostly composed of woody shrubs, herbs and succulent plants. The prehensile upper lip makes the black rhinoceros very well adapted to browsing. The diet of this browsing non-ruminant megaherbivore covers a wide variety of often more than 100 species of herbs, succulents, and woody plants, browsed throughout the year (Schenkel and Schenkel-Hullinger, 1969; Graffam *et al.*, 1997).

Although the black rhinoceros consume a wide variety of plants, they are strongly selective for shrubs and herbs. Herbs, which are green and succulent, are preferred throughout the year. During some periods of the year the black rhinoceros is highly selective and not all parts of a particular plant species are browsed. Black rhinoceros have been observed eating grass, predominantly during wet seasons. However, it constitutes a relatively small proportion of the diet and is usually rejected. Black rhinoceros also browse plants that are considered to be toxic such as *Euphorbia bothae* (Goddard, 1968; Brown *et al,* 2003).

Diet selection is very complex since it is influenced by many factors. Schenkel and Schenkel-Hullinger (1969) formulated three categories of plants browsed by black rhinoceros, and they are: (1) those plants that occur frequently in the area and are eaten preferably, (2) those plants that occur only occasionally and are eaten extensively, and (3) those plants that occur only occasionally and are eaten occasionally.

1.2.5 Captive black rhinoceros

Captive populations of black rhinoceros often occur in small areas of less than 1 km^2, either in or out of the historical range of the taxon. They have a compressed density and spacing, and require partial or full food supplementation (Emslie and Brooks, 1999). Because of the small size of the land occupied by captive animals, more intense programs are required to manage them compared to larger areas that require less management (Bothma, 1996).

Due to the limited natural browse, captive black rhinoceros are sustained on a diet consisting of hay (grass, alfalfa or mixed), herbivore pellets, produce and occasional browse. The Association of Zoos and Aquariums (AZA) Rhino Taxonomic Advisory Group (TAG) dietary recommendations for browsing black rhinoceros are mixed grass: legume hay and/or a mixture of legume hay and less digestible browse as the forage source(s), with salt blocks available at all times (Graffam *et al.*, 1997; Dierenfeld *et al.*, 2000). Although a lot of effort is made to maintain the black rhinoceros in captive places such as zoos, these areas are faced with many challenges such as running expenses and availability of food resources required for captive black rhinoceros. This becomes costly in areas where the black rhinoceros are not in their natural habitat and their natural browse

has to be imported in huge quantities (>20 000 kg/ year/ rhinoceros), or their new range is not suitable for growing their natural browse. In some zoos, black rhinoceros are fed a diet similar to that given to white rhinoceros in captivity and this becomes a big problem for black rhinoceros as they have different foraging habits compared to the white rhinoceros (Grant *et al.*, 2002). White rhinoceros are selective grazers that prefer the more palatable broad-leaved grasses (Bothma, 2002). Captive black rhinoceros from United States have been reported to have an iron overload which is likely due to changes in their natural browse (Harley *et al.*, 2004). Unlike other members of the family Rhinocerotidae, captive black rhinoceros have been shown to suffer from various diseases. Some diseases have been linked to deficiencies such as fatty acids (Grant *et al.*, 2002) and vitamin E in the diet supplied in captivity (Dierenfeld *et. al.*, 1988). Studies by Munson *et al.* (1998) showed that metabolic changes and stress response from maladaptation or nutritional inadequacy of captive diets contribute to the development of vesicular and ulcerative dermathophathy in black rhinoceros. Hemolytic anaemia remains one of the most common causes of death in captive black rhinoceros (Harley *et al.*, 2004).

1.3 Great Fish River Reserve

The Great Fish River Reserve (GFRR) is situated in the Eastern Cape of South Africa (Fig. 1.3). It was established in phases between 1976 and 1989. The reserve is medium sized and comprises a total area of about 45 000 hectares that incorporates Double drift, Sam Knott, and Andries Vosloo reserves (Ausland and Sviepe, 2000; Amendola, 2003). The area is semi-arid and receives approximately 250-500 mm rainfall annually, with peaks in February and October. The area consists primarily of valley bushveld habitat and is surrounded by both communal (tribal) and commercial game (or mixed) farms (Ausland and Sviepe, 2000; Amendola, 2003).

The valley bushveld has the vegetation type with the highest conservation value in the Eastern Cape. It has a number of rare and endangered plant species, as well as at least 206 endemic plants, most of which are succulents. With its fertile soils and dense bush, the valley bushveld offers an ideal habitat for browsing herbivores (Novellie *et al.*, 1996).

Of the seven biomes distinguished in South Africa, the GFRR has the Thicket biome that was previously classified under Savanna biome. The vegetation type is mostly succulent thorny shrub about 2-3 m high and species richness is relatively high (Evans *et al.*, 1997; Ausland and Sviepe, 2000; Amendola, 2003).

The vegetation is very dense with many succulent species such as *Portulacaria afra* (spekboom), *Euphorbia species* and *Aloe species* being conspicuous (Fig. 1.4) (Bothma, 1996). This type of vegetation has not been studied extensively and only 10% of this vegetation is known to be conserved (Evans *et al.*, 1997). This is of great concern as the conservation of representative communities within such a major ecosystem is necessary to preserve the natural heritage and maintain genetic diversity given the demand for land by humans and animals (Taggard, 1994).

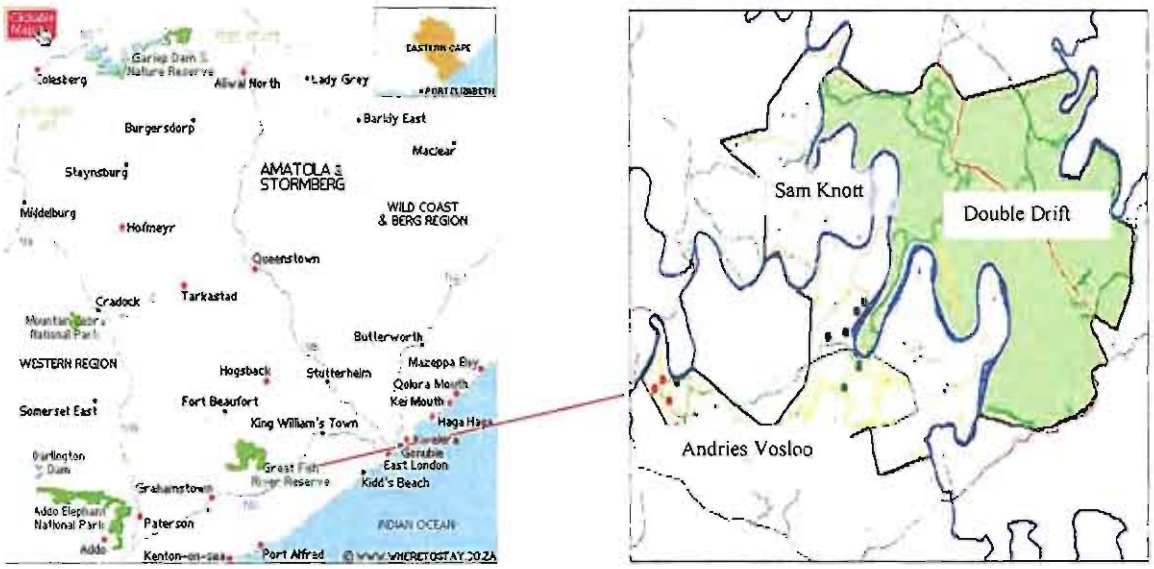

Fig. 1. 3. The research area: (left) Eastern Cape, detail; (right) the Great Fish River Reserve covering an area of 45 000 hectares (www.wheretostay.co.za).

Fig. 1. 4. Vegetation of the GFRR is very complex. The vegetation is very dense with succulent species such as *Euphorbia spp.* (Picture by B. Wilhelmi).

1.3.1 Diet of the Black rhinoceros in the GFRR

The wildlife populations on the reserve include eland (*Taurotragus oryx*), kudu (*Tragelaphus strepsiceros*), red hartebeest (*Alceplaphus buselaphus*), steenbok (*Raphicerus campestris*), springbok (*Antidorcas marsupialis*), duiker (*Sylvicapra grimmia*), cape buffalo (*Syncerus caffer*), black rhinoceros (*Diceros bicornis*), hippopotomus (*Hippopotamus amphibius*), warthog (*Phacochoerus aethiopicus*), leopard (*Panthera pardus*), African rock python, flightless dung beetle and red-billed oxpecker (Fabricius *et al.*, 1996).

The Double Drift Nature Reserve includes additional species such as white rhinoceros (*Ceratotherium simum*), giraffe (*Giraffa camelopardis*), zebra (*Equus zebra*), bontebok (*Damaliscus dorcas*), waterbuck (*Kobus ellipsiprymnus*), gemsbok (*Oryx gazella*), blue wildebeest (*Connochaetes taurinus*), impala (*Aepyceros melampus*), nyala (*Tragelaphus angassi*), southern reedbuck (*Redunca fulvorufula*) and elephant (*Loxodonta africana*) (Fabricius *et al.*, 1996).

Forty six black rhinoceros of both sexes and of different ages were introduced into the Andries Vosloo Kudu Reserve between 1986 and 1997 from the KwaZulu Natal region. Since their re-

introduction the black rhinoceros population in the GFRR has increased steadily as a result of inhabiting an area rich in xeric succulent thicket and variety of highly palatable plant species

(Brown *et al.*, 2003). Current data on diet composition of the black rhinoceros is based on direct observation and has been studied at the GFRR using a backtracking technique. Preliminary studies conducted by Brown *et al.* (2003) from two vegetation types, *Euphorbia* and *Portulacaria* dominated vegetation regions, have given 10 top plant species browsed from each region (Table 1.1 and 1.2).

Table 1.1. Most preferred browse by black rhinoceros in a *Euphorbia* dominated region in the GFRR, given as the percentage of bites recorded throughout the year (Brown *et al.*, 2003).

Plant species	**% bites**
Euphorbia bothae	**41**
Grewia robusta	**16**
Euclea undulata	**8**
Azima tetracantha	**5**
Asparagus species	**4**
Lycium species	**4**
Maytenas capitata	**3**
Ozoroa mucronata	**3**
Brachylaena ilicifolia	**3**
Rhigozum obovatum	**2**
Other species	**11**

Table 1.2. Most preferred browse by black rhinos in a *Portulacaria* dominated region in the GFRR, given as the percentage of bites recorded throughout the year (Brown *et al.*, 2003).

Plant species	% bites
Rhigozum obovatum	22
Grewia robusta	13
Euclea undulata	12
Ozoroa mucronata	8
Lycium species	7
Brachylaena ilicifolia	6
Asparagus species	4
Schotia afra	3
Azima tetracantha	3
Phylobolus sp.	3
Other species	19

The black rhinoceros consumes a wide spectrum of plant species. This megaherbivore competes with other animals such as kudu, elephant and other browsers for food resources, with kudu being a major competitor (Venter and Venter, 2005). Black rhinoceros and elephants, which have overlapping food preferences especially when the food is scarce, tend to focus on the same nutritional plants located close to water areas. An additional problem for black rhinoceros is associated with the destruction of tree and bush vegetation by elephants (Schenkel and Schenkel-Hullinger, 1966). Competition for browse between black and white rhinoceros does not exist as the two rhinoceros have different foraging habits (Bothma, 2002).

1.4 Methods used to study the diet composition of herbivores

Studies on the diet composition of free-ranging herbivores, particularly ungulates, have become increasingly important for range management (Mohammad *et al.*, 1995; Volesky and Coleman, 1996). As game farms have different animals that compete for resources, it is very important for range managers to know the quality of forage material available, and to establish dietary requirements of these animals to sustain animal population growth without adversely affecting long-term forage production (Daugherty *et al.*, 1982).

Holechek *et al.* (1998) as cited by Fuhlendorf and Engle (2001) stated that rangelands form 70% of the entire land surface in the world and consist primarily of native plant communities managed, typically, for animal production. To supply optimal forage to different animal species it is necessary to maintain or improve the conditions of these ranges. This requires a thorough knowledge of the animals' food habits (Malechek and Leinweber, 1972). Information from these studies allows range managers to estimate the carrying capacity of the land (McInnis *et al.*, 1983). This is essential especially when range managers want to understand the extent various species, particularly those that are usually considered to be browsers, are actually grazing during the various seasons in different vegetation systems (Mabinya *et al.*, 2002).

Considerable data has been generated from studies on diet composition of herbivores based on various methods since the 1950s in order to understand foraging preferences for efficient range management (Malechek and Leinweber, 1972; Holecheck *et al.*, 1982). Malechek and Leinweber (1972) have pointed out that this information is required for: (a) the effective design of grazing systems, (b) evaluation of the effects of grazing on plant communities and, (c) formulation of economical supplementation programmes on nutritional deficient ranges. Widely used methods to evaluate the diet composition of herbivores include direct observation of the animal, fistula techniques, and fecal analysis (Vavra *et al.*, 1978; Mofareh *et al.*, 1997; Henley *et al.*, 2001).

1.4.1 Direct observation

Direct observation of grazing/browsing animals has been used in studies of herbivore diet composition (Holechek *et al.,* 1982). The unending use of this method stems from its simplicity, minor equipment requirements and its ease of use. This traditional method uses a manually operated data logger or video recorder to record information (Lebopa, 2000). For quantitative analysis this method has relied on bite-counts and feeding minute estimates. When the feeding minutes approach is employed, time spent grazing each plant species is quantified and assumed to be proportional to the importance of the species in the diet. The bite count records the number of hites taken from each species, rather than the length of grazing time. The difficulties faced by direct observation are in species identification, particularly when evaluating complex communities, and quantification of foraged plant species. The problems are further extended to wild animals in that these animals are often difficult to locate and approach closely enough for accurate observation. In addition it may be difficult to differentiate between mere nibbling and active grazing/browsing (Holechek *et al.*, 1982).

Studies conducted by Henley *et al.* (2001) on direct observation of grazing goats using the bite-count method clearly showed that the method is time consuming and tedious, and the presence of a human observer can alter the behaviour of even tame animals (Gordon, 1995). In addition, the method can put the observer into danger when observing dangerous wild animals such as black rhinoceros.

1.4.2 Fistula methods

Holechek *et al.* (1982) describe the advantages of oesophageal fistula technique over other methods such as direct observation and fecal analysis. Samples analyzed by this method have been accepted as more indicative of the true diet of grazing and browsing animals. Goats with oesophageal fistulas have been used in a study to determine their browsing preferences (Lebopa, 2000). However, in a follow-up to this study, conducted by Mabinya and Brand (unpublished data) using Thin Layer Chromatography to analyse phenolic compounds on the same samples used by Lebopa (2000), they showed that results obtained by this method are not always accurate and can be misleading. The use of fistula methods has been in question since the 1950's, as doubts on these

methods are based on whether the fistula collects a representative sample of the diet and whether the presence of the fistula interferes with the normal foraging behaviour of the animal (Crocker, 1959). Studies conducted by Vavra *et al.* (1978) have shown that the use of the oesophageal fistula technique is limited to small domestic animals. Its use has not been applied to large wild herbivores (McInnis *et al.*, 1983). Problems associated with the use of this technique include contamination by rumen contents, incomplete recoveries, high costs, and low sampling precision for individual species in the diet. Samples contaminated by rumen contents cannot be used for botanical analysis (Holechek *et al.*, 1982).

1.4.3 Fecal analysis using microhistology

The microhistological technique was developed by Baumgartner and Martin (1939), and later refined by Sparks and Malechek (1968) as cited by Holechek *et al.* (1982). Baumgartner and Martin (1939) first used a microhistological method for contents of squirrel stomachs and pioneered the technique for food habit determination (Mohammad *et al.*, 1995). Fecal analysis, using microhistology, has received greater use for evaluating range herbivore food habits than any other procedure. This method has several unique advantages that account for its popularity as a research tool. These advantages include no interference with the normal habits of the animals and permitting practically unlimited sampling. Actual sampling requires very little equipment and no restriction on animal movement. It has particular value where animals range over mixed communities, and it can be used to compare the diets of two or more animals at the same time. It is the only feasible procedure to use when studying secretive and/or endangered species (Crocker, 1959; Anthony and Smith, 1974; Holechek *et al.*, 1982).

Microscopic analysis of fecal material has been a popular method for determining herbivore diets and its focus has been on the remains of identifiable plant cuticles (Anthony and Smith, 1974; Vavra *et al.*, 1978; McInnis *et al.*, 1983; Holecheck, 1982). Each plant species has its own unique cuticular characteristics, and most plant cuticles are not digested in ruminant digestive processes, which allow foraged plants to be identified microscopically in fecal samples of grazing or browsing herbivores (Anthony and Smith, 1974).

1.5 Project objectives

The diet of the black rhinoceros has been determined at the GFRR using traditional methods such as direct observation and backtracking methods (Ausland and Sviepe, 2002; Brown *et al.*, 2003). These studies have provided insight on the diet profile of the black rhinoceros and also looked at forage quality of the browse by analyzing vitamin E (Ndondo *et al.*, 2004) and compounds such as sodium, calcium and magnesium (van Lieverloo and Schuiling, 2004). Because of recent advances in technology, diet composition of extinct herbivores has been evaluated using molecular markers such as the *rbc*L gene. Determination of the diet composition of the black rhinoceros at the GFRR has not been analysed using molecular methods. The browse of the black rhinoceros at the reserve has also not been analysed for its antioxidant and its total phenolic content.

The aim of this research was to determine the diet composition of black rhinoceros from dung at the GFRR by using the *rbc*L gene as a molecular marker. This objective was based on the hypothesis that DNA sequences of the *rbc*L gene can be used to distinguish between the preferred browse of black rhinoceros. The objectives set were as follows:

1.5.1 Develop a DNA database from selected plant species obtained from the GFRR by sequencing a portion of the *rbc*L gene.

1.5.2 Amplify the same portion of the *rbc*L gene from black rhinoceros dung.

1.5.3 Determine the botanical composition of the black rhinoceros by comparing the *rbc*L gene sequences of the created DNA database with those from dung samples and identify the plant species in the dung.

1.5.4 Assay for antioxidant capacity and phenolic compounds of selected plant species browsed by the black rhinoceros.

CHAPTER TWO
VARIATION OF THE *rbc*L GENE BETWEEN SELECTED PLANT SPECIES

2.1 Introduction

In addition to the genetic information contained in the nucleus, plants also possess DNA in the mitochondrion and chloroplast. The DNA contained in the mitochondrion and chloroplast resembles that of bacteria and not eukaryotic nuclear DNA in its organization. The DNA contained in these plastids does not have nucleoprotein, a characteristic of eukaryotic nuclear DNA, but several genes have introns, and encode some of the information necessary to ensure growth and replication of the chloroplast and mitochondrion (Schuler and Zielinski, 1989). Due to developments in recombinant DNA technology, a region of the *rbc*L gene from chloroplast DNA (cpDNA) was chosen for sequencing. The first physical map of cpDNA was reported for maize (*Zea mays*) in 1976 (Sugiura, 2003) and this was followed by the construction of a gene map of cpDNA for tobacco (*Nicotiana tabacum*) in 1986 (Sugiura, 2003). The subsequent rapid development in molecular techniques has contributed enormously to the current knowledge about genome organization, gene content and gene structure (Clark, 1997; Sugiura, 2003).

With few notable exceptions, the chloroplast genome is highly conserved in size and gene arrangement, with different regions evolving at different rates (Palmer, 1990). For these reasons the chloroplast genome has become a major focus for studies on plant phylogenetics (Curtis and Clegg, 1984). More recently, the chloroplast DNA has been used as a genetic marker for studies focused on intraspecific evolution, particularly estimates of population size (Hamilton *et al.*, 2003). The use of chloroplast DNA as an intraspecific genetic marker has been based on the complete sequence of the chloroplast genome, and insertion/deletion (indel) polymorphism. In addition, the chloroplast DNA intergenic regions exhibit substantial intraspecific indel polymorphism within and among plant populations (Hamilton *et al.,* 2003). As a result, a wide range of possibilities exist for resolving relationships using data from the chloroplast genome, from the level of species and genus to family and even higher levels (Soltis *et al.*, 1998). Restriction-site analysis of chloroplast DNA has been shown to be a powerful tool for phylogenetic reconstruction at both inter- and intra-species levels (Lumaret *et al.*, 2000).

2.1.1 Size, base composition and genomic structure

The size of the maize chloroplast genome is 85 x 10^6 dalton (Coen *et al.*, 1977) and represents approximately 30% of the total DNA in a mature leaf cell (Schuler and Zielinski, 1989). The chloroplast of higher plants possesses small, self-replicating DNA molecules varying in size from 120 to 220 kb with highly conserved gene content across species (Lilly e*t al.*, 2001), and with the genes generally occurring in the same order (Grivet e*t al.*, 2001). The chloroplast genome (Fig. 2.1) is present as a large inverted repeat (IR) sequence of approximately 10-25 kb in length separating a large single copy (LSC) and a small single copy (SSC) of approximately 80 kb and 20 kb, respectively. The LSC region is slightly less conserved in sequence than the rest of the chloroplast genome, and hence potentially more useful for studies at low taxonomic levels and consensus primers have been developed in this region (Palmer, 1982; Chiang *et al.*, 1998; Grivet e*t al.*, 2001).

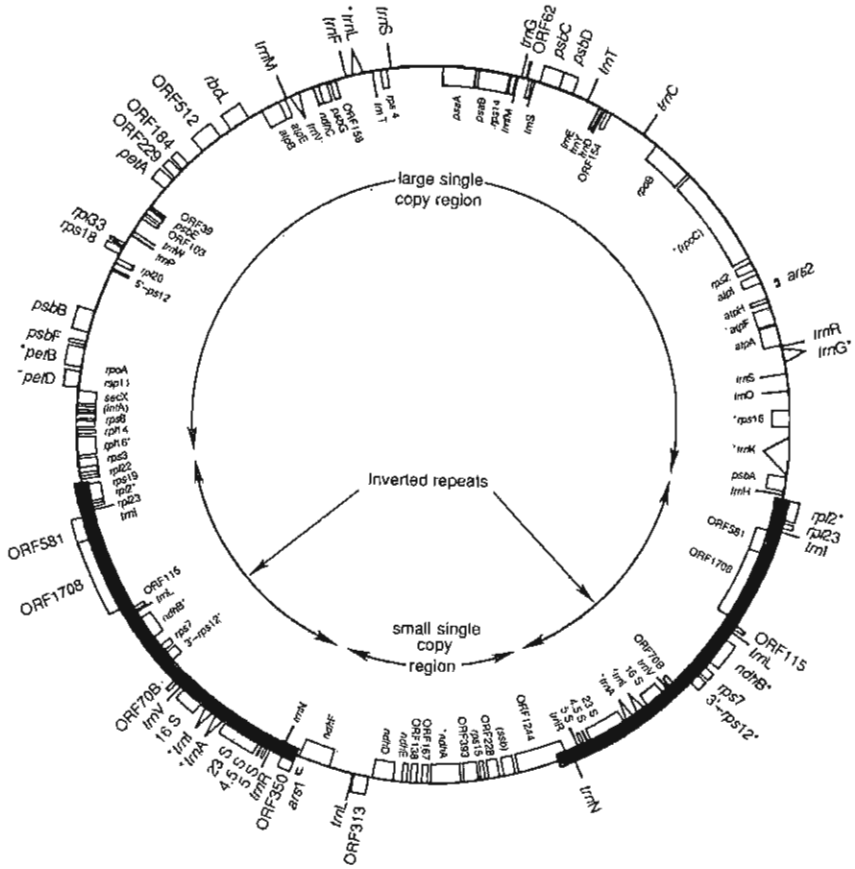

Fig. 2. 1. Genetic map of the 155,844-bp circular chloroplast DNA of *Nicotiana tabacum*. The large and small single copy regions and the inverted repeat regions are indicated (Schuler and Zielinski, 1989).

The IR sequences present in the genomes of cpDNA play an important role in the physical organization of cpDNA. Although the IR structure arose early in plant evolution, it appears that the chloroplast can function without it. The absence of the IR region is associated with relatively high frequencies of rearrangements, an observation that has led to the hypothesis that it stabilizes the chloroplast genome (Selander *et al.*, 1991).

2.1.2 Genes and coding capacity

The cpDNA has been completely sequenced in the species *Marchantia polymorpha* (liverworts), *N. tabacum* (tobacco) and *Oryza sativa* (rice) (Clark, 1997). The sequence data has shown that the chloroplast genome is made up of 80 open reading frames (ORFs), 30 tRNA genes, 4 rRNA genes and the *rrn* operon found within the IR region, which includes the 16S, 23S, 4.5S, and 5S sequences. Approximately 27 ORFs have not been assigned a coding function (Selander *et al.*, 1991). Even though higher plant cpDNA, in general, is smaller than mitochondrial DNA, the chloroplast genome contains a larger number of genes than the mitochondrial genome. Higher plant cpDNA contains 120 different genes, of which 80 code for proteins. Gene content is relatively stable in spite of differences in size, and the same linear order of genes has been found among most vascular species investigated (Clark, 1997).

2.2 Ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene

Studies on *Z. mays* have shown that the *rbc*L gene is 1431 nucleotides long, with the length of the coding region varying slightly among flowering plants (Clegg, 1993), where indels are sometimes found in the 3' end of the gene (Calie and Manhart, 1994). Unlike some chloroplast genes that are interrupted by introns, the *rbc*L gene contains no introns. Owing to its abundance in nature and early studies on the chloroplast genome, the molecular characterization of the *rbc*L gene was a major goal of plant molecular biology in the 1970's (Clegg, 1993). The holoenzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), which is responsible for carbon dioxide fixation in the Calvin cycle (Clegg, 1993), is comprised of 8 large subunits encoded by the *rbc*L gene and 8 small subunits encoded in the nucleus (Zurawski *et al.*, 1981; Halliwel, 1984; Hudson *et al.*, 1990).

Coen *et al.* (1977) presented direct physical evidence that the large subunit of the enzyme RUBISCO from *Z. mays* is encoded by the cpDNA, in particular, the *rbc*L gene. Although a wide range of chloroplast genes have been employed in constructing phylogenetic relationships between plants, the *rbc*L gene has emerged as the preferred gene for constructing higher-level phylogenetic relationships. The reason for this preference includes the slow rate of evolution of the *rbc*L gene and its widely available plant sequences that show the gene to be reliable for phylogenetic analysis at higher taxonomic levels. The large size of the *rbc*L gene (>1400 base pairs) provides numerous sequence variation suitable for phylogenetic studies (Soltis *et al.*, 1990). The *rbc*L gene sequences have been employed largely to reveal detailed phylogeny of the seed plants and angiosperms (Clegg, 1993). Phylogenetics based on *rbc*L sequences were found to be successful at the family level and also at higher levels, but limited to interordinal or intrafamilial level in some orders such as Zingiberales. Phylogenetic relationships using *rbc*L sequences have also been inferred at lower taxonomic levels (inter- and intrageneric) in some families indicating that the *rbc*L can be used at generic level. However, the *rbc*L gene is sometimes too conserved to clarify relationships between closely related genera (Gielly and Taberlet, 1994).

This part of the study employed the *rbc*L gene to compare sequence variation between selected plant species obtained from the GFRR.

2.3 Materials and methods

Agarose, bovine serum albumin (BSA) and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Aldrich, and ampicillin from Roche. Isopropyl-β-thiogalactosidase (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and the 10 kb Mass Ruler DNA ladder were purchased from Fermentas Life Sciences, South Africa. The Qiagen plant DNA extraction kit, Polymerase Chain Reaction (PCR) kit, pGem-T Easy kit, Qiagen spin miniprep kit and the Wizard SV Gel and PCR clean up kit were purchased from Promega. Ethanol was purchased from Merck, South Africa, and the primers (T7 and SP6), BigDye terminator v3.1 cycle sequencing kit (Applied Biosystematics) from Inqaba Biotechnical Industries, South Africa.

2.3.1 Sample collection

Leaf samples of 18 plant species from 14 different families were collected at the GFRR (Table 2.1). Selection of plants was based on previous studies in the same area that used backtracking and observational methods to study the foraging habits of the black rhinoceros. Twigs of plants and stems of *E. bothae* and *E. fimbriata* were cut off, placed in separate sealed plastic bags containing silica gel, and transported to the laboratory for analysis. Molecular studies were started on arrival and samples given to the Selmar Schonland Herbarium for identification and voucher specimens were lodged with the herbarium.

Table 2.1. Plant species collected from the GFRR*.

Family	Genus	Species
Portulacaceae	*Portulacaria*	*afra*
Fabaceae	*Acacia*	*karroo*
Fabaceae	*Schotia*	*afra*
Euphorbiaceae	*Jatropha*	*capensis*
Euphorbiaceae	*Euphorbia*	*bothae*
Euphorbiaceae	*Euphorbia*	*fimbriata*
Celastraceae	*Putterlickia*	*pyracantha*
Tiliaceae	*Grewia*	*robusta*
Tiliaceae	*Grewia*	*occidentalis*
Plumbaginaceae	*Plumbago*	*auriculata*
Ebenaceae	*Euclea*	*undulata*
Salvadoraceae	*Azima*	*tetracantha*
Boraginaceae	*Ehretia*	*rigida*
Solanaceae	*Lycium*	*cinereum*
Asteraceae	*Brachylaena*	*elliptica*
Anacardiaceae	*Rhus*	*lucida*
Apocynaceae	*Carrisa*	*bispinosa*
Bignoniaceae	*Rhigozum*	*obovatum*

* The plant sample photographs are shown in Appendix A, with the exception of *G. occidentalis* and *L. cinereum*.

2.4 Genomic DNA extraction from plants

Genomic DNA was extracted by grinding 100 mg of leaf tissue in liquid nitrogen and using an autoclaved mortar and pestle. DNA from *E. bothae* and *E. fimbriata* was extracted from the green epidermis of the plant, as it does not have leaves. A Qiagen DNeasy plant DNA extraction kit was used according to the manufacturer's instructions. Genomic DNA and a 10 kb Mass Ruler DNA ladder were run on a 1% agarose gel containing 0.15 μg/ml ethidium bromide and the bands visualized using a chemiluminescene and fluorescence documentation system (UviproChem, UK). All agarose gels were made in 0.5% TAE buffer (the preparation of the buffer is described in Appendix B) and electrophoresed at 120 V for 45 min.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 Primers

The reverse primer was designed by identifying, conserved sequences from multiple alignments. The complete sequenced *rbc*L genes of *A. tetracantha, P. auriculata,* and the partial *rbc*L gene fragment of *Coddia rudis,* accession number U36782, M77701 and AJ286685 (NCBI), respectively were used for designing the reverse primer. Conserved sequences flanking the regions to be amplified were identified with the computer software BLAST (Basic Local Alignment Search Tool), provided by the NCBI (National Center for Biotechnology Information) from the GenBank database (Fig. 2.2). The exact position and length of the primers was chosen according to their thermodynamic parameters using the OLIGO Primer Analysis software. The forward primer (1For) is composed of the first 20 bases of *rbc*L, and the reverse primer (*rbc*L Rev 646), a complementary 22-mer, beginning at position 646 of the *rbc*L sequence of the three plants.

The primers (Fig. 2.2) were 1For: 5'-ATGTCACCACAAACAGAGAC-3' and *rbc*L Rev 646: 5'-CTCTCCAACGCATAAATGGTTG-3'. The forward primer is reported to be used for amplification and sequencing of the *rbc*L gene (Wanntorp *et al.*, 2001; Sulaiman *et al.*, 2003).

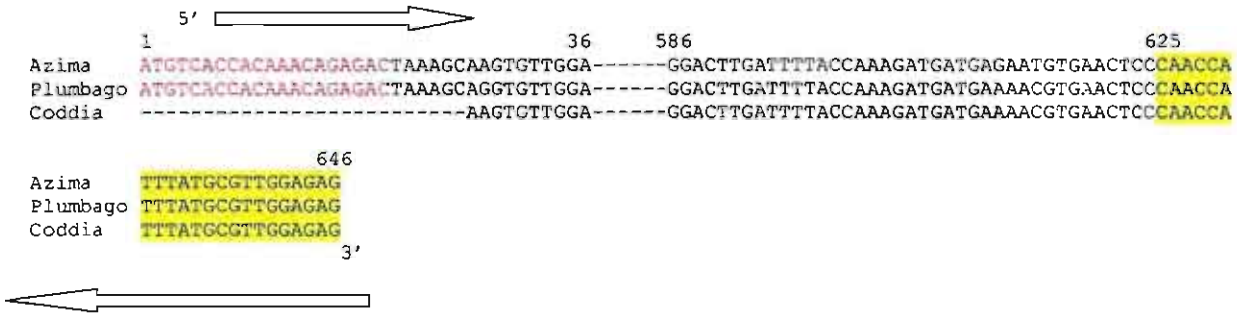

Fig. 2.2. *rbc*L Sequences of *A. tetracantha*, *P. auriculata*, and *C. rudis*. The region highlighted in red shows the forward primer sequence and the region highlighted in yellow shows the conserved region used to design the reverse primer.

2.5.2 Amplification of the *rbc*L gene from plants

The double-stranded *rbc*L gene was amplified from genomic DNA of all plant species listed in Table 2.1 using PCR. The PCR reaction contained 300 ng of DNA template, 10 X thermophilic buffer, 25 mM $MgCl_2$, 10 mM dNTP, 5 U/µl *Taq* DNA polymerase, and 1.5 mM of each primer, to a total volume of 35 µl made up with triple distilled water. The volumes used for the PCR reaction mix are shown in Appendix C. For *E. rigida* and *B. elliptica* 3 µl of BSA (3 µg/µl) was added to the PCR mix. The amplifications were performed in a Labnet Multi Gene thermocycler.

The thermal cycling parameters were an initial denaturation step for 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min (Hamilton *et al.*, 2003), with a final extension step of 72°C for 5 min and hold at 4°C. The PCR products were quantified on a 1% agarose gel, stained with 0.15 µg/ml ethidium bromide, using a Mass Ruler DNA ladder. The remaining PCR products were cleaned up using the Promega Wizard SV Gel and PCR clean up kit following the manufacturer's instructions and quantified spectrophotometrically at 260 nm.

2.6 Cloning

The amplified *rbc*L fragments were made blunt-ended by treatment with T4-DNA polymerase and cloned into pGem-T Easy vector. The ligation reaction mixture consisted of 2 μl of the clean PCR products, 1 μl of pGem-T vector, 1 μl of T4 DNA ligase, 5 μl of 2 X rapid ligation buffer to a total volume of 10 μl with nuclease free water. The reaction mixture was incubated at 4°C overnight to produce a maximum number of transformants. Aliquots of the ligation mixture were transformed into highly competent JM109 *E. coli* cells (the cells were prepared as described in Appendix D). Colony transformation was carried out using the Promega pGem-T Easy kit following the manufacturer's instructions (Appendix E). This was followed by picking white colonies from LB/ Ampicillin/ IPTG/ X-Gal plates and inoculating in a 5 ml test tube containing TYP/ Ampicillin broth (preparations of plates and broth are described in Appendix F) and incubated overnight at 37°C.

2.6.1 Plasmid isolation

Plasmids were isolated using a Qiagen spin miniprep kit following the manufacturer's instructions. The presence of an insert was confirmed by restriction digest and PCR using the isolated plasmid as a template. A restriction digest was performed by adding 2 μl of restriction buffer, 1 μl EcoR1 and 5 μl of plasmid DNA, made up to a final volume of 20 μl with triple distilled water. The restriction digest mixture was incubated at 37°C for 3 h, and fragments were visualized on a 1% agarose gel, stained with ethidium bromide. For PCR, the same parameters used for the amplification of the *rbc*L gene were used for amplification of the insert DNA fragment, except that T7 and SP6 vector primers were used. The plasmid-PCR products were quantified on a 1% agarose gel stained with ethidium bromide.

2.7 Cycle sequencing

BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used to perform fluorescence-based cycle reaction on the cloned PCR fragments. For sequencing reactions, both strands were sequenced by using the pGem-T Easy vector primers T7 and SP6. Each sequencing reaction contained 300 ng of plasmid DNA, 3.2 ρMole primer, 2 µl buffer, 4 µl BigDye and the volume made up to a final volume of 20 µl with nuclease free water. Cycle sequencing was carried out on a GeneAmp PCR system 9700 thermocycler version 3.05 (25 cycles: 10 sec of denaturation at 96°C, 5 sec of annealing at 50°C, and 4 min of extension at 60°C).

The cycle sequencing products were cleaned-up using ethanol/ EDTA precipitation. The products were briefly centrifuged, 5 µl of 125 mM EDTA and 60 µl absolute ethanol added and the tubes incubated at room temperature for 15 min. The tubes were centrifuged at 14000 x g for 30 min and the supernatant discarded. To each tube 60 µl of ice cold ethanol (70%) was added and the tubes centrifuged at 14000 x g for 15 min at 4°C. The supernatant was removed and the samples dried at 95°C for 5 min on the thermocycler following the manufacturer's instructions. Pellets were resuspended in a template suppression reagent and the nucleotide extension products separated by capillary electrophoresis using an ABI Prism 3100 Genetic Analyser (Hitachi, Applied Biosystems) and POP6 polymer in a 50 cm capillary. The sequences were sequenced either at Rhodes University or at Inqaba Biotechnical Industries, South Africa.

2.8 Results

2.8.1 DNA recovery

High molecular weight DNA was extracted from the plant species. The presence of BSA in the PCR reaction mixture increased DNA amplification efficiency from *B. elliptica* and *E. rigida*. All amplification products yielded *rbc*L gene fragments (646 bp) that were visualized in ethidium-stained agarose gels (Fig. 2.3).

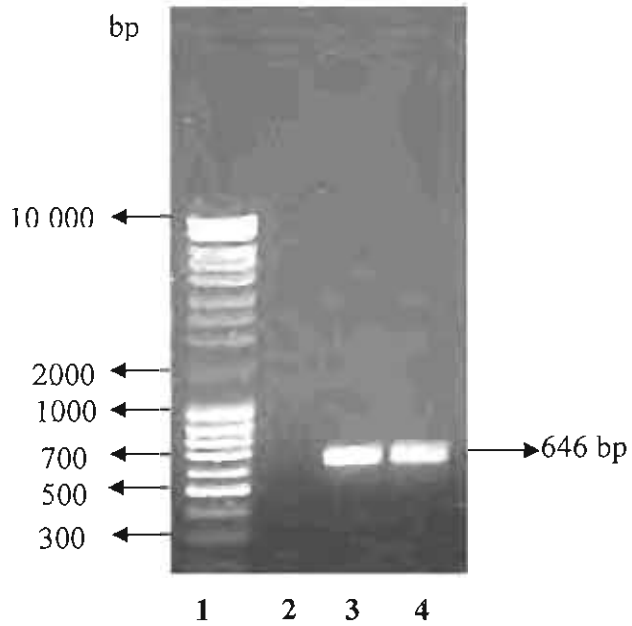

Fig. 2.3. Agarose gel (1%) electrophoresis of a 646 bp chloroplast amplification for the *rbc*L gene. Lane 1: 10 kb Mass Ruler ladder, Lane 2: negative control (no template added), Lane 3: *E. bothae* and Lane 4: *P. auriculata*.

Amplification of the *rbcL gene* from the plastid using the vector primers confirmed the presence of the inserts. Fig. 2.4 shows the *rbcL* gene PCR products.

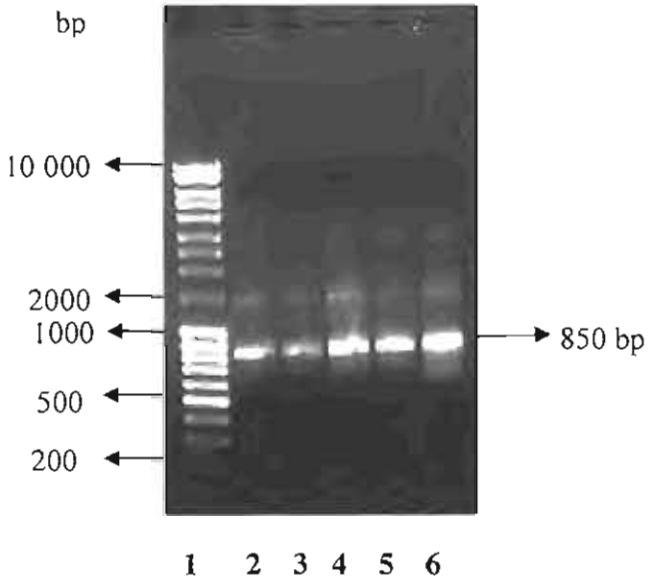

Fig. 2.4. Plasmid PCR products from plant clones electrophoresed on a 1% agarose gel. Lane 1: 10 kb Mass Ruler ladder, Lane 2: *J. capensis*, Lane 3: *P. pyracantha*, Lane 4: *S. afra*, Lane 5: *B. elliptica*, and Lane 6: *A. tetracantha.*

2.8.2 *rbcL* Gene sequence alignments

To assess the efficiency with which the taxonomic affiliation of *rbcL* gene sequences can be determined, the *rbcL* gene sequences from the GFRR investigated, for which the correct taxonomic affiliations are known, were compared to *rbcL* sequences in GenBank database by means of the BLAST program. The number of mismatches to the most similar sequence in the database was noted. For each plant species sequence, the family, genus and species that gave the closest nucleotide composition was noted. An identification was classified as 'correct' only when the correct family, genus and species of plant was found, as 'ambiguous' when several families or orders were found and as 'incorrect' when only one but incorrect family or order was found (Table 2.2). At the genus level the identification was correct in 5 of the 18 plant species, correct in 4 at species level, ambiguous in 1, and incorrect in 8. At the family level, identifications were correct in 11 cases, ambiguous in 1 case and incorrect in 6 cases.

Table 2.2. Comparison of the plant sequences with those from the GenBank using BLAST search.

Family	Genus	Species	Match from GenBank	Family of match	% Identity	No. of variable Nucleotides
Portulacaceae	*Portulacaria*	*afra* *	*P. afra*	**Portulacaceae**	99	4
Fabaceae	*Acacia*	*karroo*	*A. farnesiana*	**Leguminosae**	99	4
Fabaceae	*Schotia*	*afra*	*Brownea spp.*	**Caesalpiniaceae**	98	7
Euphorbiaceae	*Jatropha*	*capensis*	*J. integerrima*	**Euphorbiaceae**	99	2
Euphorbiaceae	*Euphorbia*	*bothae*	*E. abyssinica*	**Euphorbiaceae**	99	4
Euphorbiaceae	*Euphorbia*	*fimbriata*	*Cubanthus umbelliformis*	**Euphorbiaceae**	98	10
Celastraceae	*Putterlickia*	*pyracantha*	*Maytenus arbutifolia*	**Celastraceae**	99	4
Tiliaceae	*Grewia*	*robusta*	*G. occidentalis*	**Tiliaceae**	99	1
Tiliaceae	*Grewia*	*occidentalis*	*G. occidentalis*	**Tiliaceae**	99	3
Plumbaginaceae	*Plumbago*	*auriculata* *	*Rheum x cultorum* *P. capensis*	**Polygonaceae** **Plumbaginaceae**	100 100	0 0
Ebenaceae	*Euclea*	*undulata*	*Encephalartos natalensis*	**Zamiaceae**	99	4
Salvadoraceae	*Azima*	*tetracantha* *	*A. tetracantha*	**Salvadoraceae**	99	5
Boraginaceae	*Ehretia*	*rigida*	*E. anacua*	**Boraginaceae**	98	7
Solanaceae	*Lycium*	*cinereum*	*Comoranthus minor*	**Oleaceae**	97	18
Asteraceae	*Brachylaena*	*elliptica*	*Abies procera*	**Pinaeeae**	98	11
Anacardiaceae	*Rhus*	*lucida*	*Comoranthus minor*	**Oleaceae**	97	18
Apocynaceae	*Carrisa*	*bispinosa* *	*C. bispinosa*	**Apocynaceae**	99	2
Bignoniaceae	*Rhigozum*	*obovatum*	*Comoranthus-minor*	**Oleaceae**	97	11

Plant species positively identified in the GenBank database*

The *rbc*L gene sequences from the GenBank that gave close matches to *E. bothae, E. undulata, E. fimbriata, P. pyracantha, G. occidentalis* and *G. robusta*, excluded the forward primer sequence and alignments were based on 626 nucleotides. Some alignments were based on less than 620 nucleotides, as the sequences from the GenBank in addition to the forward primer sequence, excluded some of the gene sequence bases. For example, the sequence (*Acacia farnesiana*) that gave 99 % identity to *A. karroo*, excluded 26 bases in addition to the primer sequence. Other sequences were *P. capensis* (a synonym of *P. auriculata*) which excluded 24 bases and *Comoranthus minor* which excluded 30 bases. *P. afra* sequence alignment was based on 581 nucleotides as the GenBank sequence excluded 65 bases inclusive of the forward primer sequence. The matches to *A. tetracantha, B. elliptica,* and *L. cinereum* were based on the 646 nucleotides. The GenBank *A. tetracantha* sequence has two unidentified nucleotides, which affects percentage

similarity to sequenced plant species. Column 7 in Table 2.2 shows the number of mismatches for each plant sequence compared to the sequences in the GenBank (NCBI).

The families that had more than one plant species in the plants investigated were Euphorbiaceae (*E. bothae, E. fimbriata,* and *J. capensis*), Fabaceae (*A. karroo* and *S. afra*), and Tiliaceae (*G. robusta* and *G. occidentalis*). The *rbc*L gene sequences of the plants in the families Euphorbia and Fabaceae were assembled separately and aligned by using ClustalW program version 1.83. The position of mismatches was noted manually. There were no insertions or deletions. This was done to show the degree of sequence variation between the selected plant species from the same family. There was 1 mismatch between *G. robusta* and *G. occidentalis* (Appendix G). Alignment of the *rbc*L gene sequences from *A. karoo* and *S. afra* indicated high variation between these two species with 26 nucleotide differences observed (Fig. 2.5). The gaps show the positions where the mismatches occur between the two sequences.

```
A. karroo    CTCTCCAACGCATAAATGGTTGGGAATTCACATTTTCATCATCTTTGGTAAAATCAAGTC 60
S. afra      CTCTCCAACGCATAAATGGTTGGGAATTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
             ************************************ ** ************************

A. karroo    CACCACGAAGACATTCATAAACCGCTCTACCGTAATTCTTAGCGGATAATCCCAATTTTG 120
S. afra      TACCGCGGAGAACTTCATAAACCGCTCTACCGTAATTCTTAGCGGATAACCCCAATTTAG 120
              *** ** ***  ************************************** ******** *

A. karroo    GTTTAATAGTACATCCCAATAGGGGACGGCCGTACTTGTTCAATTTATCTCTCTCAACTT 180
S. afra      GTTTAATAGTACATCCCAATAGGGGACGGCCATACTTGTTCAATTTATCTCTCTCAACTT 180
             ********************************** ************************

A. karroo    GGATGCCGTGAGGCGGACCTTGGAAAGTTTTAGAATAAGAAGTAGGGATTCGCAAATCCT 240
S. afra      GGATACCGTGAGGCGGACCCTGGAAAGTTTTAATATAAGCAGTAGGGATTCGCAAATCCT 240
             **** ************** ***********  ***** ********************

A. karroo    CCAGACGTAGAGCGCGCAGAGCCTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
S. afra      CCAGACGTAGAGCGCGCAGGGCCTTGAACCCAAAGACATTACCCACAATAGAAGTAAACA 300
             ****************** ************** ********** ********** *****

A. karroo    TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGATAAGCTACATAAGCAATAAATT 360
S. afra      TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGATAAGCTACATAAGCAATAAATT 360
             ************************************************************

A. karroo    GATTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTGTAACGATCAAGAC 420
S. afra      GAGTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTGTAACGATCAAGGC 420
             ** ********************************************************* *

A. karroo    TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
S. afra      TGGTAAGCCCGTCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACTG 480
             ********** ************************************************ *

A. karroo    CGGCACCTGCTTCTTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
S. afra      CGGCACCTGCTTCTTCGGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
             ***************** ************************************************

A. karroo    TATCACTATCTTTGGTTTCATAGTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
S. afra      TATCAGTATCCTTGGTTTCATAGTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
             ***** **** ************************************************

A. karroo    CAGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
S. afra      CAGCTTTGAACCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
             ********** ***********************************
```

Fig. 2.5. Alignment of *rbc*L gene sequences of *A. karroo* and *S. afra* from the Fabaceae family. The gaps between the asterisks indicate nucleotide base differences.

Figure 2.6 shows multiple alignments of the Euphorbiaceae plant *rbc*L gene sequences. A high number of different nucleotides between the sequences was observed.

```
E. bothae      CTCTCCAACGCATAAATGGTTGAGAGTTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
E. fimbriata   CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTCCCATCATCTTTGGTAAAATCAAGTC 60
J. capensis    CTCTCCAACGCATAAATGGTTGGGAATTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
               ************************ ** ********* ************************

E. bothae      CCCCGCGAAGACATTCATAAACCGCTCTACCATAATTCTTAGCGGATAGCCCCAATTTTG 120
E. fimbriata   CATCGCGAAGACATTCATAAACCGCTCTACCATAATTCTTAGTGGATAGCCCCAATTTTG 120
J. capensis    CACCGCGAAGACATTCATAAACCGCTCTACCATAATTCTTAGCGGATAGCCCCAATTTAG 120
               *  ************************************ ***************** *

E. bothae      GTTTAATAGTACAACCCCATAGAGGGCGACCATATTTGTTCAATTTATCTCTCTCAACTT 180
E. fimbriata   GTTTAATAGTACAACCCAATAGAGGGCGACCATACTTGTTCAATTTATCTCTCTCAACTT 180
J. capensis    GTTTAATAGTACAACCCAATAGGGGGCGACCATACTTGTTCAATTTATCTCTCTCAACTT 180
               ****************** **** *********** ************************

E. bothae      GGATTCCATGAGGTGGCCCTTGGAAAGTTTCAGTATAAGAAGTAGGGATTCGCAAATCCT 240
E. fimbriata   GGATGCCATGAGGTGGCCCTTGGAAAGTTTTAGTATAAGAAGGAGGGATTCGCAAATCCT 240
J. capensis    GGATACCATGAGGCGGCCCTTGGAAAGTTTTAGTATAAGCAGTAGGGATTCGCAAATCCT 240
               **** ******** ********************** ******** ** ************

E. bothae      CCAGACGTAGCGCGCGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAGGTAAACA 300
E. fimbriata   CCAGACGTAGAGCGCGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAGGTAAACA 300
J. capensis    CCAGACGTAGGGCGCGTAGGGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
               ********** ****** ********************************** *******

E. bothae      TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTGAGGGGTAAGCTACATAAGCAATATATT 360
E. fimbriata   TGTTAGTAACAGACCCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAAGCAATATATT 360
J. capensis    TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAAGCAATATATT 360
               ************ ****************** ****************************

E. bothae      GATTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTATAACGATCAAGAC 420
E. fimbriata   GATTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTATAACGATCAAGAC 420
J. capensis    GATTTTCTTCTCCAGCAACGGGCTCGATGTCGTAGCATCGTCCTTTATAACGATCAAGAC 420
               ********************************** ****************************

E. bothae      TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
E. fimbriata   TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
J. capensis    TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGTTTCAGCAGCTACCG 480
               ************************************************ ************

E. bothae      CAGCTCCTGCTTCCTCAGGTGGAACTCCAGGTTGAGGACTTACTCGGAATGCTGCCAAGA 540
E. fimbriata   CAGCTCCTGCTTCCTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
J. capensis    CAGCTCCTGCTTCCTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
               ******************* ******************* ***********************

E. bothae      TATCAGTATCTTTGGTTTCATATTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
E. fimbriata   TATCAGTATCTTTGGTTTCATATTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
J. capensis    TATCAGTATCTTTGGTTTGATACTCAGGAGTATAATAAGTCAATTTATAATCTTGAACAC 600
               ******************* *** ********************************* *****

E. bothae      CAGCCTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
E. fimbriata   CAGCCTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
J. capensis    CAGCCTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
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Fig. 2.6. Alignment of the *rbc*L sequences from three plant species from the family Euphorbiaceae. The aligned plants were *E. bothae*, *E. fimbriata*, *J. capensis*. The position and base differences are shown by gaps between asterisks.

Sequences of all the *rbc*L gene fragments were assembled and aligned by using ClustalW program version 1.83 (Appendix G).

Classification of organisms by morphological similarities has formed the backbone of taxonomy. This approach judges taxanomic affinities on the basis of measurable similarities and differencesand is termed numerical taxonomy or phenetics. In contrast, cladistics classifies organisms according to the historical order in which branches arise along a phylogenetic tree. This method of classification is ideally suited to molecular data, particularly DNA sequence divergence. A phytogenetic tree constructed in this fashion is called a cladogram and shows the order of evolutionary descent, and can also show the degree of divergence. Taxonomy today uses information from both phenetics and cladistics.

The Tables 2.3 to 2.20 numerical values are given based on the numerical taxonomy, as shown by Germishuizen and Meyer (2003), and also give the extent of DNA sequence divergence based on a 646 bp fragment of the *rbc*L gene of the plants studied. In each table a particular species is listed first and the other 17 plants compared to it by their percentage similarity as well as by the number of variable nucleotides. The closer the genus number of a species is to that of another species implies that taxonomically, based on morphological characters, the closer the species are in relation to the each other.

Table 2.3. Comparison of *J. capensis* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
44331	**Euphorbiaceae**	***Jatropha***	***capensis***		
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	97	19
44926	Euphorbiaceae	*Euphorbia*	*bothae*	96	22
344923	Fabaceae	*Acacia*	*karroo*	94	33
35061	Fabaceae	*Schotia*	*afra*	94	36
46281	Celastraceae	*Putterlickia*	*pyracantha*	94	37
496619	Tiliaceae	*Grewia*	*robusta*	93	42
496617	Tiliaceae	*Grewia*	*occidentalis*	93	41
640416	Ebenaceae	*Euclea*	*undulata*	93	43
64441	Salvadoraceae	*Azima*	*tetracantha*	93	45
70432	Boraginaceae	*Ehretia*	*rigida*	92	49
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	50
65592	Apocynaceae	*Carissa*	*bispinosa*	92	50
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	54
459442	Anacardiaceae	*Rhus*	*lucida*	91	54
73794	Solanaceae	*Lycium*	*cinereum*	91	55
89362	Asteraceae	*Brachylaena*	*elliptica*	91	58
24191	Portulacaceae	*Portulacaria*	*afra*	91	58

Table 2.4. Comparison of *E. bothae* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
44926	**Euphorbiaceae**	***Euphorbia***	***bothae***		
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	97	15
44331	Euphorbiaceae	*Jatropha*	*capensis*	96	22
344923	Fabaceae	*Acacia*	*karroo*	94	38
46281	Celastraceae	*Putterlickia*	*pyracantha*	93	44
640416	Ebenaceae	*Euclea*	*undulata*	93	44
35061	Fabaceae	*Schotia*	*afra*	92	47
64441	Salvadoraceae	*Azima*	*tetracantha*	92	49
496619	Tiliaceae	*Grewia*	*robusta*	92	51
496617	Tiliaceae	*Grewia*	*occidentalis*	92	50
65592	Apocynaceae	*Carissa*	*bispinosa*	91	54
63431	Plumbaginaceae	*Plumbago*	*auriculata*	91	55
70432	Boraginaceae	*Ehretia*	*rigida*	91	56
73794	Solanaceae	*Lycium*	*cinereum*	90	60
77222	Bignoniaceae	*Rhigozum*	*obovatum*	90	60
459442	Anacardiacea	*Rhus*	*lucida*	90	60
89362	Asteraceae	*Brachylaena*	*elliptica*	90	64
24191	Portulacaceae	*Portulacaria*	*afra*	90	64

Table 2.5. Comparison of *A. karroo* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
344923	**Fabaceae**	***Acacia***	***karroo***		
35061	Fabaceae	*Schotia*	*afra*	95	26
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	94	33
44331	Euphorbiaceae	*Jatropha*	*capensis*	94	33
640416	Ebenaceae	*Euclea*	*undulata*	94	37
46281	Celastraceae	*Putterlickia*	*pyracantha*	94	38
44926	Euphorbiaceae	*Euphorbia*	*bothae*	94	38
496617	Tiliaceae	*Grewia*	*occidentalis*	93	41
496619	Tiliaceae	*Grewia*	*robusta*	93	42
65592	Apocynaceae	*Carissa*	*bispinosa*	93	43
70432	Boraginaceae	*Ehretia*	*rigida*	93	44
64441	Salvadoraceae	*Azima*	*tetracantha*	92	46
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	47
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	56
459442	Anacardiacea	*Rhus*	*lucida*	91	56
73794	Solanaceae	*Lycium*	*cinereum*	91	57
89362	Asteraceae	*Brachylaena*	*elliptica*	91	57
24191	Portulacaceae	*Portulacaria*	*afra*	91	57

Table 2.6. Comparison of *S. afra* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
35061	**Fabaceae**	***Schotia***	***afra***		
344923	Fabaceae	*Acacia*	*karroo*	95	26
44331	Euphorbiaceae	*Jatropha*	*capensis*	94	37
46281	Celastraceae	*Putterlickia*	*pyracantha*	93	39
640416	Ebenaceae	*Euclea*	*undulata*	93	43
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	93	43
64441	Salvadoraceae	*Azima*	*tetracantha*	92	46
44926	Euphorbiaceae	*Euphorbia*	*bothae*	92	47
70432	Boraginaceae	*Ehretia*	*rigida*	92	47
496617	Tiliaceae	*Grewia*	*occidentalis*	92	48
496619	Tiliaceae	*Grewia*	*robusta*	92	49
65592	Apocynaceae	*Carissa*	*bispinosa*	92	50
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	51
24191	Portulacaceae	*Portulacaria*	*afra*	91	55
89362	Asteraceae	*Brachylaena*	*elliptica*	91	55
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	55
459442	Anacardiacea	*Rhus*	*lucida*	91	55
73794	Solanaceae	*Lycium*	*cinereum*	91	56

Table 2.7. Comparison of *G. robusta* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
496619	**Tiliaceae**	***Grewia***	***robusta***		
496617	Tiliaceae	*Grewia*	*occidentalis*	99	1
44331	Euphorbiaceae	*Jatropha*	*capensis*	93	42
344923	Fabaceae	*Acacia*	*karroo*	93	42
64441	Salvadoraceae	*Azima*	*tetracantha*	93	43
65592	Apocynaceae	*Carissa*	*bispinosa*	93	43
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	93	44
73794	Solanaceae	*Lycium*	*cinereum*	93	44
77222	Bignoniaceae	*Rhigozum*	*obovatum*	93	45
459442	Anacardiacea	*Rhus*	*lucida*	93	47
70432	Boraginaceae	*Ehretia*	*rigida*	92	47
459442	Anacardiacea	*Rhus*	*lucida*	93	47
35061	Fabaceae	*Schotia*	*afra*	92	49
640416	Ebenaceae	*Euclea*	*undulata*	92	49
44926	Euphorbiaceae	*Euphorbia*	*bothae*	92	51
63431	Plumbaginaceae	*Plumbago*	*auriculata*	91	53
46281	Celastraceae	*Putterlickia*	*pyracantha*	91	53
89362	Asteraceae	*Brachylaena*	*elliptica*	91	58
24191	Portulacaceae	*Portulacaria*	*afra*	90	60

Table 2.8. Comparison of *G. occidentalis* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
496617	**Tiliaceae**	***Grewia***	***occidentalis***		
496619	Tiliaceae	*Grewia*	*robusta*	99	1
44331	Euphorbiaceae	*Jatropha*	*capensis*	93	41
344923	Fabaceae	*Acacia*	*karroo*	93	41
64441	Salvadoraceae	*Azima*	*tetracantha*	93	42
65592	Apocynaceae	*Carissa*	*bispinosa*	93	42
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	93	43
73794	Solanaceae	*Lycium*	*cinereum*	93	43
459442	Anacardiacea	*Rhus*	*lucida*	93	44
77222	Bignoniaceae	*Rhigozum*	*obovatum*	93	44
70432	Boraginaceae	*Ehretia*	*rigida*	93	46
459442	Anacardiacea	*Rhus*	*lucida*	93	46
35061	Fabaceae	*Schotia*	*afra*	92	48
640416	Ebenaceae	*Euclea*	*undulata*	92	48
44926	Euphorbiaceae	*Euphorbia*	*bothae*	92	50
63431	Plumbaginaceae	*Plumbago*	*auriculata*	91	52
46281	Celastraceae	*Putterlickia*	*pyracantha*	91	52
89362	Asteraceae	*Brachylaena*	*elliptica*	91	57
24191	Portulacaceae	*Portulacaria*	*afra*	90	59

Table 2.9. Comparison of *P. pyracantha* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
46281	**Celastraceae**	***Putterlickia***	***pyracantha***		
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	94	36
44331	Euphorbiaceae	*Jatropha*	*capensis*	94	36
344923	Fabaceae	*Acacia*	*karroo*	94	38
35061	Fabaceae	*Schotia*	*afra*	93	41
640416	Ebenaceae	*Euclea*	*undulata*	93	42
44926	Euphorbiaceae	*Euphorbia*	*bothae*	93	44
70432	Boraginaceae	*Ehretia*	*rigida*	92	50
64441	Salvadoraceae	*Azima*	*tetracantha*	92	50
496617	Tiliaceae	*Grewia*	*occidentalis*	91	52
496619	Tiliaceae	*Grewia*	*robusta*	91	53
65592	Apocynaceae	*Carissa*	*bispinosa*	91	56
459442	Anacardiacea	*Rhus*	*lucida*	91	57
63431	Plumbaginaceae	*Plumbago*	*auriculata*	91	57
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	57
89362	Asteraceae	*Brachylaena*	*elliptica*	90	62
24191	Portulacaceae	*Portulacaria*	*afra*	90	62
73794	Solanaceae	*Lycium*	*cinereum*	90	67

Table 2.10. Comparison of *B. elliptica* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
89362	**Asteraceae**	***Brachylaena***	***elliptica***		
24191	Portulacaceae	*Portulacaria*	*afra*	98	7
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	49
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	52
459442	Anacardiacea	*Rhus*	*lucida*	91	52
70432	Boraginaceae	*Ehretia*	*rigida*	91	53
640416	Ebenaceae	*Euclea*	*undulata*	91	54
73794	Solanaceae	*Lycium*	*cinereum*	91	54
35061	Fabaceae	*Schotia*	*afra*	91	55
344923	Fabaceae	*Acacia*	*karroo*	91	56
65592	Apocynaceae	*Carissa*	*bispinosa*	91	56
44331	Euphorbiaceae	*Jatropha*	*capensis*	91	58
496617	Tiliaceae	*Grewia*	*occidentalis*	91	57
496619	Tiliaceae	*Grewia*	*robusta*	91	58
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	90	59
46281	Celastraceae	*Putterlickia*	*pyracantha*	90	62
44926	Euphorbiaceae	*Euphorbia*	*bothae*	90	64
64441	Salvadoraceae	*Azima*	*tetracantha*	89	66

Table 2.11. Comparison of *P. afra* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
24191	**Portulacaceae**	***Portulacaria***	***afra***		
89362	Asteraceae	*Brachylaena*	*elliptica*	98	7
65592	Apocynaceae	*Carissa*	*bispinosa*	93	39
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	49
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	53
70432	Boraginaceae	*Ehretia*	*rigida*	91	53
459442	Anacardiacea	*Rhus*	*lucida*	91	53
73794	Solanaceae	*Lycium*	*cinereum*	91	54
35061	Fabaceae	*Schotia*	*afra*	91	55
640416	Ebenaceae	*Euclea*	*undulata*	91	56
344923	Fabaceae	*Acacia*	*karroo*	91	57
44331	Euphorbiaceae	*Jatropha*	*capensis*	91	58
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	90	59
496617	Tiliaceae	*Grewia*	*occidentalis*	90	59
496619	Tiliaceae	*Grewia*	*robusta*	90	60
46281	Celastraceae	*Putterlickia*	*pyracantha*	90	62
44926	Euphorbiaceae	*Euphorbia*	*bothae*	90	64
64441	Salvadoraceae	*Azima*	*tetracantha*	89	67

Table 2.12. Comparison of *E. undulata* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
640416	**Ebenaceae**	***Euclea***	***undulata***		
344923	Fabaceae	*Acacia*	*karroo*	94	37
70432	Boraginaceae	*Ehretia*	*rigida*	94	37
65592	Apocynaceae	*Carissa*	*bispinosa*	93	39
35061	Fabaceae	*Schotia*	*afra*	93	42
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	93	42
46281	Celastraceae	*Putterlickia*	*pyracantha*	93	42
44331	Euphorbiaceae	*Jatropha*	*capensis*	93	43
77222	Bignoniaceae	*Rhigozum*	*obovatum*	93	44
44926	Euphorbiaceae	*Euphorbia*	*bothae*	93	44
459442	Anacardiacea	*Rhus*	*lucida*	93	44
64441	Salvadoraceae	*Azima*	*tetracantha*	93	46
73794	Solanaceae	*Lycium*	*cinereum*	93	46
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	46
496617	Tiliaceae	*Grewia*	*occidentalis*	92	48
496619	Tiliaceae	*Grewia*	*robusta*	92	49
89362	Asteraceae	*Brachylaena*	*elliptica*	91	54
24191	Portulacaceae	*Portulacaria*	*afra*	91	56

Table 2.13. Comparison of *P. auriculata* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
63431	**Plumbaginaceae**	***Plumbago***	***auriculata***		
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	93	42
640416	Ebenaceae	*Euclea*	*undulata*	92	46
77222	Bignoniaceae	*Rhigozum*	*obovatum*	92	46
344923	Fabaceae	*Acacia*	*karroo*	92	47
65592	Apocynaceae	*Carissa*	*bispinosa*	92	48
89362	Asteraceae	*Brachylaena*	*elliptica*	92	49
24191	Portulacaceae	*Portulacaria*	*afra*	92	49
44331	Euphorbiaceae	*Jatropha*	*capensis*	92	50
35061	Fabaceae	*Schotia*	*afra*	92	51
70432	Boraginaceae	*Ehretia*	*rigida*	92	51
64441	Salvadoraceae	*Azima*	*tetracantha*	91	52
496617	Tiliaceae	*Grewia*	*occidentalis*	91	52
496618	Tiliaceae	*Grewia*	*robusta*	91	53
73794	Solanaceae	*Lycium*	*cinereum*	91	56
44926	Euphorbiaceae	*Euphorbia*	*bothae*	91	56
459442	Anacardiacea	*Rhus*	*lucida*	91	56
46281	Celastraceae	*Putterlickia*	*pyracantha*	91	57

Table 2.14. Comparison of *L. cinereum* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
73794	**Solanaceae**	***Lycium***	***cinereum***		
77222	Bignoniaceae	*Rhigozum*	*obovatum*	99	5
459442	Anacardiacea	*Rhus*	*lucida*	99	6
70432	Boraginaceae	*Ehretia*	*rigida*	94	35
65592	Apocynaceae	*Carissa*	*bispinosa*	93	39
496617	Tiliaceae	*Grewia*	*occidentalis*	93	43
496619	Tiliaceae	*Grewia*	*robusta*	93	44
640416	Ebenaceae	*Euclea*	*undulata*	93	45
89362	Asteraceae	*Brachylaena*	*elliptica*	91	54
24191	Portulacaceae	*Portulacaria*	*afra*	91	54
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	91	55
64441	Salvadoraceae	*Azima*	*tetracantha*	91	55
44331	Euphorbiaceae	*Jatropha*	*capensis*	91	55
35061	Fabaceae	*Schotia*	*afra*	91	56
63431	Plumbaginaceae	*Plumbago*	*auriculata*	91	56
344923	Fabaceae	*Acacia*	*karroo*	91	57
44926	Euphorbiaceae	*Euphorbia*	*bothae*	90	60
4728	Celastraceae	*Putterlickia*	*pyracantha*	90	67

Table 2.15. Comparison of *A. tetracantha* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
64441	**Salvadoraceae**	***Azima***	***tetracantha***		
496617	Tiliaceae	*Grewia*	*occidentalis*	93	42
496619	Tiliaceae	*Grewia*	*robusta*	93	43
640416	Ebenaceae	*Euclea*	*undulata*	93	43
44331	Euphorbiaceae	*Jatropha*	*capensis*	93	45
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	93	45
344923	Fabaceae	*Acacia*	*karroo*	92	46
35061	Fabaceae	*Schotia*	*afra*	92	46
44926	Euphorbiaceae	*Euphorbia*	*bothae*	92	49
46281	Celastraceae	*Putterlickia*	*pyracantha*	91	52
63431	Plumbaginaceae	*Plumbago*	*auriculata*	91	52
70432	Boraginaceae	*Ehretia*	*rigida*	91	52
65592	Apocynaceae	*Carissa*	*bispinosa*	91	54
73794	Solanaceae	*Lycium*	*cinereum*	91	55
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	55
459442	Anacardiacea	*Rhus*	*lucida*	91	55
89362	Asteraceae	*Brachylaena*	*elliptica*	89	67
24191	Portulacaceae	*Portulacaria*	*afra*	89	67

Table 2.16. Comparison to *E. fimbriata* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
44987	**Euphorbiaceae**	***Euphorbia***	***fimbriata***		
44926	Euphorbiaceae	*Euphorbia*	*bothae*	97	15
44331	Euphorbiaceae	*Jatropha*	*capensis*	97	19
344923	Fabaceae	*Acacia*	*karroo*	94	33
46281	Celastraceae	*Putterlickia*	*pyracantha*	94	36
35061	Fabaceae	*Schotia*	*afra*	93	43
496617	Tiliaceae	*Grewia*	*occidentalis*	93	43
496619	Tiliaceae	*Grewia*	*robusta*	93	44
640416	Ebenaceae	*Euclea*	*undulata*	93	42
64441	Salvadoraceae	*Azima*	*tetracantha*	93	45
63431	Plumbaginaceae	*Plumbago*	*auriculata*	93	42
70432	Boraginaceae	*Ehretia*	*rigida*	91	53
65592	Apocynaceae	*Carissa*	*bispinosa*	91	53
77222	Bignoniaceae	*Rhigozum*	*obovatum*	91	54
459442	Anacardiaceae	*Rhus*	*lucida*	91	54
73794	Solanaceae	*Lycium*	*cinereum*	91	55
89362	Asteraceae	*Brachylaena*	*elliptica*	90	59
24191	Portulacaceae	*Portulacaria*	*afra*	90	59

Table 2.17. Comparison of *R. obovatum* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
77222	**Bignoniacea**	***Rhigozum***	***obovatum***		
73794	Solanaceae	*Lycium*	*cinereum*	99	5
7722	Anacardiaceae	*Rhus*	*lucida*	99	5
70432	Boraginaceae	*Ehretia*	*rigida*	94	36
65592	Apocynaceae	*Carissa*	*bispinosa*	93	39
640416	Ebenaceae	*Euclea*	*undulata*	93	44
496617	Tiliaceae	*Grewia*	*occidentalis*	93	44
496619	Tiliaceae	*Grewia*	*robusta*	93	45
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	46
89362	Asteraceae	*Brachylaena*	*elliptica*	91	52
24191	Portulacaceae	*Portulacaria*	*afra*	91	53
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	91	53
44331	Euphorbiaceae	*Jatropha*	*capensis*	91	54
35061	Fabaceae	*Schotia*	*afra*	91	55
64441	Salvadoraceae	*Azima*	*tetracantha*	91	55
344923	Fabaceae	*Acacia*	*karroo*	91	56
46281	Celastraceae	*Putterlickia*	*pyracantha*	91	57
44926	Euphorbiaceae	*Euphorbia*	*bothae*	90	60

Table 2.18. Comparison of *E. rigida* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
70432	**Boraginaceae**	***Ehretia***	***rigida***		
65592	Apocynaceae	*Carissa*	*bispinosa*	95	27
73794	Solanaceae	*Lycium*	*cinereum*	94	35
77222	Bignoniaceae	*Rhigozum*	*obovatum*	94	36
459442	Anacardiaceae	*Rhus*	*lucida*	94	36
640416	Ebenaceae	*Euclea*	*undulata*	94	37
344923	Fabaceae	*Acacia*	*karroo*	93	44
35061	Fabaceae	*Schotia*	*afra*	92	47
496617	Tiliaceae	*Grewia*	*occidentalis*	92	46
496619	Tiliaceae	*Grewia*	*robusta*	92	47
44331	Euphorbiaceae	*Jatropha*	*capensis*	92	49
46281	Celastraceae	*Putterlickia*	*pyracantha*	92	50
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	51
64441	Salvadoraceae	*Azima*	*tetracantha*	91	52
89362	Asteraceae	*Brachylaena*	*elliptica*	91	53
24191	Portulacaceae	*Portulacaria*	*afra*	91	53
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	91	53
44926	Euphorbiaceae	*Euphorbia*	*bothae*	91	56

Table 2.19. Comparison of *R. lucida* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
459442	**Anacardiaceae**	***Rhus***	***lucida***		
77222	Bignoniaceae	*Rhigozum*	*obovatum*	99	5
73794	Solanaceae	*Lycium*	*cinereum*	99	6
70432	Boraginaceae	*Ehretia*	*rigida*	94	36
65592	Apocynaceae	*Carissa*	*bispinosa*	93	39
496619	Tiliaceae	*Grewia*	*robusta*	93	47
496617	Tiliaceae	*Grewia*	*occidentalis*	93	46
640416	Ebenaceae	*Euclea*	*undulata*	93	44
44331	Euphorbiaceae	*Jatropha*	*capensis*	91	54
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	91	54
35061	Fabaceae	*Schotia*	*afra*	91	55
64441	Salvadoraceae	*Azima*	*tetracantha*	91	55
344923	Fabaceae	*Acacia*	*karroo*	91	56
46281	Celastraceae	*Putterlickia*	*pyracantha*	91	57
63431	Plumbaginaceae	*Plumbago*	*auriculata*	91	56
89362	Asteraceae	*Brachylaena*	*elliptica*	91	52
24191	Portulacaceae	*Portulacaria*	*afra*	91	53
44926	Euphorbiaceae	*Euphorbia*	*bothae*	90	60

Table 2.20. Comparison of *C. bispinosa* to the other 17 sequenced plant species investigated.

Genus #	Family	Genus	Species	% Identity	No. of variable nucleotides
65592	**Apocynaceae**	***Carissa***	***bispinosa***		
70432	Boraginaceae	*Ehretia*	*rigida*	95	27
73794	Solanaceae	*Lycium*	*cinereum*	93	39
77222	Bignoniaceae	*Rhigozum*	*obovatum*	93	39
640416	Ebenaceae	*Euclea*	*undulata*	93	39
24191	Portulacaceae	*Portulacaria*	*afra*	93	39
459442	Anacardiaceae	*Rhus*	*lucida*	93	39
496617	Tiliaceae	*Grewia*	*occidentalis*	93	42
496619	Tiliaceae	*Grewia*	*robusta*	93	43
344923	Fabaceae	*Acacia*	*karroo*	93	43
63431	Plumbaginaceae	*Plumbago*	*auriculata*	92	48
35061	Fabaceae	*Schotia*	*afra*	92	50
44331	Euphorbiaceae	*Jatropha*	*capensis*	92	50
44987	Euphorbiaceae	*Euphorbia*	*fimbriata*	91	53
44926	Euphorbiaceae	*Euphorbia*	*bothae*	91	54
64441	Salvadoraceae	*Azima*	*tetracantha*	91	54
89362	Asteraceae	*Brachylaena*	*elliptica*	91	56
46281	Celastraceae	*Putterlickia*	*pyracantha*	91	56

Sequences of the genes were aligned using ClustalW program version 1.8.3, the combined matrix contained 10872 characters for *rbc*L (11628 bp minus the length of the *rbc*L Rev 646 and 1 For primers). There were no insertions or deletions except for *R. lucida* sequence, which has a deletion at position 601. Figure 2.7 shows the phylogenetic tree of all the plants species constructed using ClustalW. The phylogenetic tree shows the similarity of the investigated plants based on the partial *rbc*L gene sequenced and reflects on the nucleotide variation of the plant genes as shown in Table 2.3 to 2.20. The plant species with similar sequences are grouped as clusters.

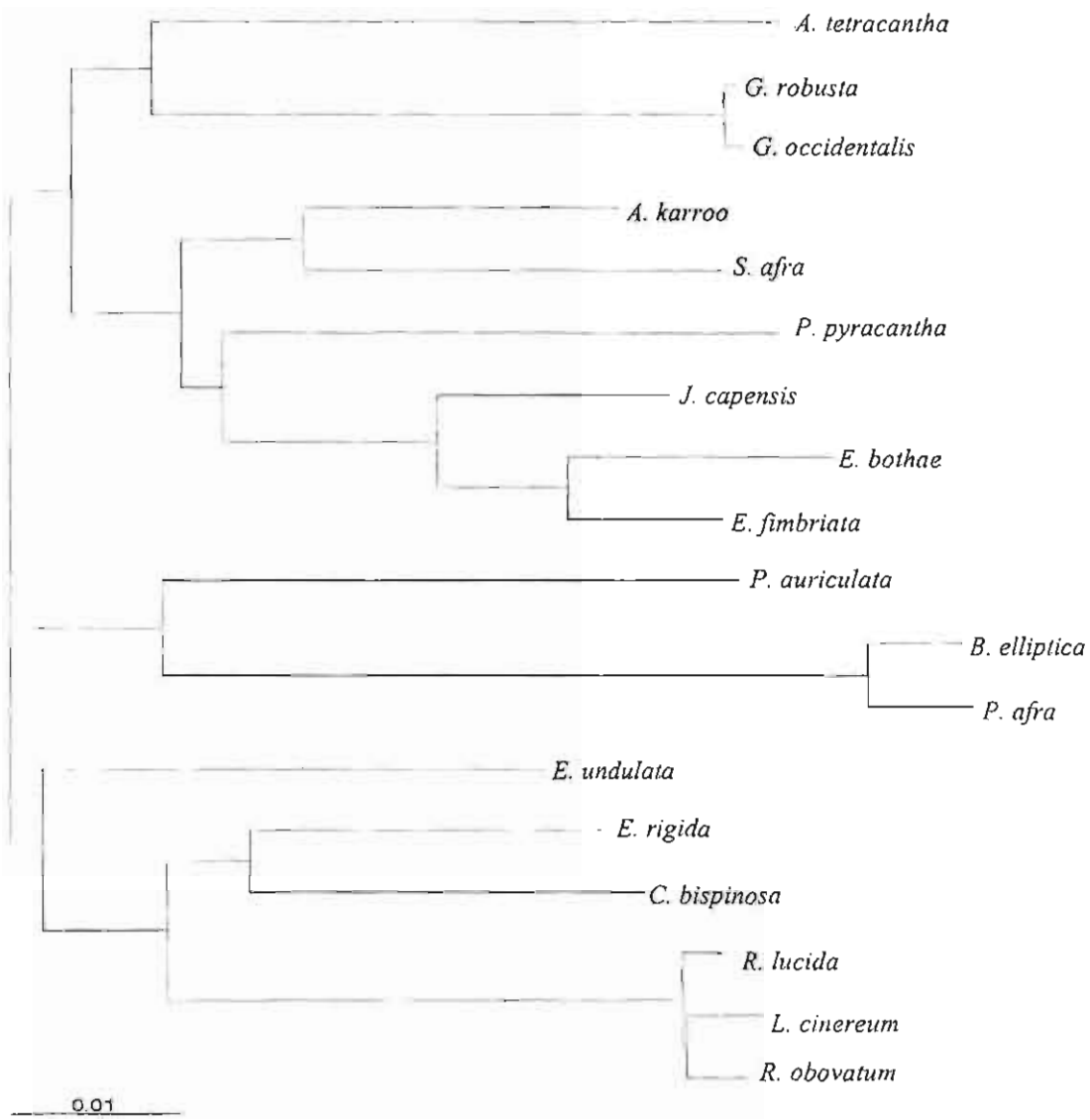


Fig. 2.7. Pylogenetic tree of the investigated plant species from the GFRR based on the *rbc*L gene variation.

2.9 Discussion

The presence of inhibitory compounds such as tannins, phenolics and polysaccharides in leaves are reported to be a major problem in extraction of DNA that can be used for amplification and sequencing genes (Kim *et al.*, 1997; Coyle *et al.*, 2003). In this study, high molecular weight DNA was extracted from the plant tissue samples using the Qiagen DNeasy plant DNA extraction kit as this kit is reported to be effective for extracting high quality DNA from small quantities of fresh leaf material (Coyle *et al.*, 2003). The designed primers could be used to successfully amplify 646 bp of the *rbc*L gene from all plants, except for *R. lucida* which has one deletion in position 601, showing the high degree of conservation and robustness of this gene. The success of amplifying the gene was due to the designed primer (*rbc*L Rev 646) as previous studies by Maweni (2004) using the reverse primer (5'-ATTTTTGGCTTGATAGTACA-3') did not yield any amplified products. The forward primer has been reported for successful amplification of the *rbc*L gene from many plant species (Wanntorp *et al.*, 2001; Sulaiman *et al.*, 2003).

The strategy used to design the reverse primer and the wide taxonomic distribution of the 14 families implies that the two primers employed in this study can be used to amplify the *rbc*L gene from many different families. Amplification of the *rbc*L gene from *B. elliptica* and *E. rigida* was achieved by addition of BSA in the PCR reaction mix. BSA has a widespread use for relieving interference in PCR, and its addition to the PCR mix is now a standard method when the DNA to be amplified is from plants (Meyer *et al.*, 2004). Interference in amplifying the *rbc*L gene from *B. elliptica* is attributed to the presence of phenolic compounds that are present in this plant (section 4.8.4).

For all the plant species studied, generated partial *rbc*L gene sequences were compared with those in the GenBank database (Table 2.2). The GenBank contains a high number of *rbc*L gene sequences, as the gene has been sequenced from a large number of plant species. Sequence data derived from this gene have been used to address phylogenetic relationships of plants at different taxonomic levels (Soltis *et al.*, 1992). Construction of phylogenies using the *rbc*L gene has been preferred due to low substitution rates (Salvolainen *et al.*, 2000).

Soltis *et al.* (1990) used the *rbc*L gene to clarify relationships among members of the taxonomically complex groups of the Saxifragaceae *sensu lato*. In this study the *rbc*L gene sequences from *A. tetracantha*, *P. afra, P. auriculata, G. occidentalis,* and *C. bispinosa* allowed identification to species level. Sequence identification of *P. auriculata* was classified as ambiguous as it gave 100% similarity to two plants from different families. Sequence data analysis between *R. x cultorum* and *P. auriculata* showed a high degree of similarity for the sequenced fragment. Comparison of the partial *rbc*L sequence of *P. auriculata* (accession number Y16906) and the complete sequence of *R. x cultorum* (accession number M77702) from the GeneBank showed a high degree of conservation between these plant sequences (100% identity). Although the sequence gave a 100% similarity to *R. x cultorum,* because its taxonomic affiliation is known, it was identified as *P. auriculata.* For some plants this is to be expected as the gene is highly conserved and considering that about half the gene was sequenced making it difficult to discriminate between the two sequences. Furthermore, the major differences in the *rbc*L gene are towards the 3' end of the gene (Clegg, 1993).

Grewia spp. (*G. occidentalis* and *G. robusta*) *rbc*L gene sequences that were identified down to genus level, showed very low sequence variation as the two plant sequences had one nucleotide difference. Plant sequences that showed a high degree of conservation of the gene at genus level were from *A. karroo, J. capensis, E. bothae,* and *P. pyracantha.* It was noted for 13 plant sequences that there are no identical sequences found in GenBank (Table 2.2). Plant sequences of *E. undulata, L. cinereum, B. elliptica, R. lucida,* and *R. obovatum* were not identified at family level. This number is expected to decrease as the number of *rbc*L gene sequences deposited in the GenBank increases.

Because most of our plant sequences were not available in the GenBank, comparisons between these plant sequences were done to determine *rbc*L sequence variation (Tables 2.3 to 2.20). This is important as analysis of the *rbc*L gene in this study was performed to show *rbc*L sequence variations between the selected plant species from the GFRR so as to perform molecular analysis of the black rhinoceros dung (Chapter 3). The *rbc*L gene is too conserved to show sufficient sequence variation between *G. occidentalis* and *G. robusta*. However, sequence data are able to

discriminate between certain sequences at family, genus and species level. Sequence alignments of the gene fragment for plants in the families, Fabaceae, and Euphorbiaceae have been shown to have different nucleotide composition (Fig. 2.5 and 2.6). Most variations between these sequences are observed towards the 3' end of the gene. Studies by Calie and Manhart (1994) have shown the 3' end of the *rbc*L gene to be highly divergent in several non-flowering land plants.

Sequence variation in *Euphorbia spp.* has been explained by Howis (2004) to be a result of limited seed dispersal causing low rate of gene flow between taxa. Although there is a large sequence variation between the plants in the family Fabaceae (*S. afra* and *A. karroo*) and Euphorbiaceae (*E. bothae, E. fimbriata* and *J. capenis*), with the exception of Tiliaceae (*G. occidentalis* and *G. robusta*) that have low sequence variation, the genus number and the constructed cladogram shows that these plants in each family are closely related. The high level of genetic discrimination between plants of the same family is important for determination of the diet of the black rhinoceros down to species level, as some plants browsed by this megaherbivore may be closely related.

Alignments of all the plants sequences have shown the *rbc*L gene to be less conserved between some families (Appendix G). Sequence alignments between each plant and the other 17 plant species are less conserved between *R. obovatum, R. lucida,* and *L. cinereum.* This is surprising as the genus number of these plants differs significantly. Variation in the *rbc*L gene in some plants is based on the size of the *rbc*L coding region. For example, the *rbc*L gene in the Asteraceae ranges between 1428 to 1458 bp long and variations occur at the 3' end of the gene due to small insertions/ deletions (Kim *et al.*, 1992). The constructed phylogenetic tree shows that these plants are more closely related than any of the other sequenced plants (Fig. 2.7). It is important to point out that the tree was not constructed to define evolutionary pathways, as the plants are from 16 different genera which are in 14 different families and come from 12 different orders (Appendix H), but rather specify only sequence variation among the taxa.

The alignments of the sequences obtained for all the 18 species studied are presented in Appendix G. When looking at the columns of nucleotides it is clear that A-G changes (purine-purine) and C-T changes (pyrimidine-pyrimidine) are the most common. However, occasionally G-T (e.g. *B. elliptica* and *P. afra*, position 45) and A-C (e.g. *A. karroo* and *S. afra*, position 65) changes do occur. It is beyond the scope of this study to pursue this topic in any real depth as it would be best done knowing the complete *rbc*L gene sequence of the plant species investigated and the amino acid sequences of a number of RUBISCO enzymes from other species.

CHAPTER THREE
MOLECULAR ANALYSIS OF BLACK RHINOCEROS DUNG

3.1 Introduction

Interest in the botanical composition of animal diets has led researchers to investigate the diet composition of herbivores using fecal analyses. Most of the data in the literature on dung analysis has involved the use of microhistology. Studies using deposits of coprolites (ancient dung) found in caves that animals used for shelter or visited regularly have revealed the diet composition of certain extinct animals (Poinar *et al.*, 1998; Hofreiter *et al.*, 2000). DNA sequences retrieved from these dung samples have been used, not only to identify the animal species from which the dung originates, but also to identify plants that formed part of the diet of the herbivores (Poinar *et al.*, 1998; Hofreiter *et al.*, 2000). In addition, DNA sequences also have been used to identify microorganisms that inhabited the dung (Höss *et al.*, 1996). Most of the work on ancient dung analysis has studied the 16S ribosomal DNA and the plant chloroplast *rbc*L gene (Höss *et al.*, 1996; Poinar *et al.*, 2001; Hofreiter *et al.*, 2003).

Hofreiter *et al.* (2000) studied the diet composition of the ground sloth coprolite using molecular methods by amplifying a 157 bp fragment of the *rbc*L gene. To trace the animal species from which the dung originates, Hofreiter *et al.* (2003) amplified and sequenced a 537 bp mitochondrial 12S rDNA, and analyzed ancient chloroplast DNA to supplement pollen and cuticle identifications in revealing the animal's diet. Analysis of ancient dung samples is not limited only to animals as it has been used to study DNA sequences from ancient human remains to reveal aspects of their diets (Poinar *et al.*, 2001). Since some plant organs are difficult or impossible to identify from their morphological traits after mastication and digestion, molecular analysis may become a method of choice (Poinar *et al.*, 1998; Hofreiter *et al.*, 2000). In a study conducted by Poinar *et al.* (1998), some of the plants identified by DNA sequence analysis were not detected by morphological analysis.

Amplification and sequencing of scatological samples is limited to short fragments, as amplification of longer fragments has been unsuccessful (Pääbo *et al.,* 1989; Hofreiter *et al.,* 2003). In plant samples, the *rbc*L gene sequence that is routinely amplified from coprolites is limited to less than 200 bp, which compromises the precision for identification of sequences found in the coprolites (Hofreiter *et al.,* 2000). Furthermore, ancient DNA is heavily modified, with reduction in base number, oxidized pyrimidines and cross-links. These modifications are so extensive that less than 3% of the DNA extracted from ancient DNA can be expected to be undamaged. These modifications have made it difficult to clone ancient DNA samples (Pääbo *et al.,* 1989). In addition to DNA degradation of ancient remains that may leave no intact DNA molecules, contamination with modern DNA poses a big concern as it can easily lead to false positive results, which makes it important to use precautionary measures to prevent or minimize contamination (Yang and Watt, 2004).

Application of molecular analysis of dung composition will open a new window for determination of the diet of large herbivores. The specific aim of this part of this investigation was to determine the botanical composition of the black rhinoceros dung using molecular methods.

3.2 Materials and methods

In addition to materials utilized in section 2.3, cetyltrimethyl ammonium bromide (CTAB), tris-hydroxymethyl-aminomethane, sodium dodecyl sulfate (SDS), sodium chloride (NaCl) and polyvinyl pyrrolidone (PVP) were all purchased from Sigma Aldrich. Chloroform, 2-mercaptoethanol, isoamyl alcohol, isopropanol and ethanol were all purchased from Merck, South Africa.

3.2.1 Sample collection

Black rhinoceros dung samples were collected during four different seasons from May 2005 to March 2006 at the GFRR. DNA analysis was carried out immediately upon arrival at the laboratory and remaining dung samples were stored at -70 °C. Collected dung samples were not fresh but had no insects, beetles or fungi, which are characteristics of old dung (Fig. 3.1). The condition of the collected dung was suitable for DNA extraction and amplification using PCR.

Fig. 3.1. Dung of the black rhinoceros from the GFRR (Picture by J. Brand).

3.3 Genomic DNA extraction from dung samples

Air-dried dung was ground using a coffee grinder and the CTAB method (Doyle and Doyle, 1987) was used for extracting genomic DNA from the dung with some modifications as described by Clark (1997). A sample of ground dung (100 mg) was ground further to a fine powder in liquid nitrogen, using a pestle and mortar. The powder was transferred to a 1.5 ml microcentrifuge tube and suspended in 610 µl extraction buffer (100 mM Tris pH 8, 2% CTAB, 20 mM EDTA, 2% PVP, 2% BSA, 1.4 M NaCl and 2 µl of 2-mercaptoethanol (added just before use)), 20 µl NaCl (5 M) and 70 µl SDS (20%) to a final volume 700 µl. The reaction mixture was incubated at 65°C for 1 h, with occasional gentle mixing. This was followed by adding 6 µl of 10 mg/ml RNAse A to the reaction mixture and incubating at 37°C for 10 min, with mixing after 5 min. For purification, 700 µl chloroform - isoamyl alcohol (24:1) was added and the tubes centrifuged for 5 min. The aqueous phase was transferred into a 1.5 ml microcentrifuge tube and the DNA precipitated by

adding 0.7 volumes of isopropanol and kept at -20°C overnight. The overnight samples were centrifuged at 16000 x g for 20 min, the supernatant discarded and the pellet washed twice with 70% ethanol and air-dried. The pellet was resuspended in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA) and 5 μl was subjected to electrophoresis through a 1% agarose gel containing 0.15 μg/ml ethidium bromide, for 45 min at 120 V, and visualized using the chemiluminescence and fluorescence documentation system (UviproChem, UK).

3.4 Amplification of the *rbc*L gene from dung

The same parameters used for amplification of the plant *rbc*L gene (section 2.5.2) were used for amplification of the plant *rbc*L gene in the dung, except that all amplification reactions contained BSA (Meyer *et al.*, 2004). The dung *rbc*L gene PCR products were quantified by gel electrophoresis using a 10 kb Mass Ruler DNA ladder, and cleaned up using a PCR clean up kit (Wizard) following the manufacturer's instructions.

3.5 Cloning and sequencing

PCR products were cloned and sequenced as described in section 2.6 and 2.7.

3.6 Results

3.6.1 DNA recovery

The 646 bp *rbc*L gene was successfully amplified from the dung samples in the presence of BSA in the PCR reaction mix (Fig. 3.2).

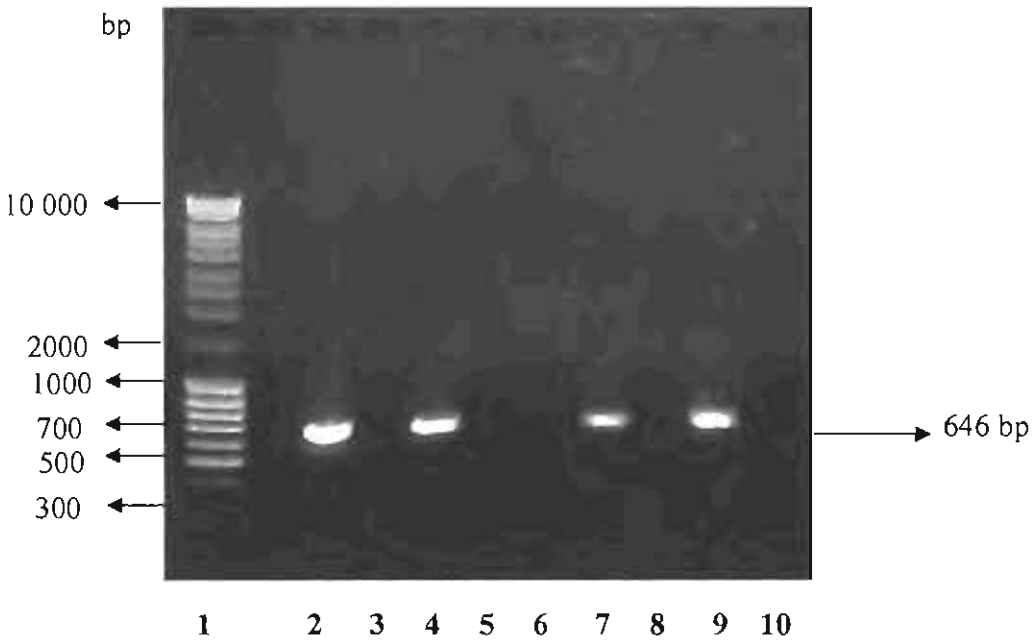


Fig. 3.2. Agarose gel (1%) electrophoresis of 646 bp *rbc*L gene from dung. Lane 1: 10 kb Mass Ruler ladder; lanes 2, 4, 7 and 9: dung *rbc*L gene PCR products from a reaction mix containing BSA; Lane 3, 5, 6 and 8: PCR products from dung samples containing no BSA; and lane 10 negative control (no template added).

3.6.2 Identification of plant species in the black rhinoceros dung

Dung sequences of the *rbc*L gene were assembled and aligned by using Bioedit version 7.04. The sequences were compared to the *rbc*L sequences in GenBank and the sequenced plants obtained in this study (GFRR database) (Chapter 2), using the BLAST program. The number of mismatches to the most similar sequence in the GenBank and the GFRR database were noted. Based on *rbc*L data, identification was performed at family, genus and species level. Tables 3.1-3.5 show BLAST results from both the GenBank and GFRR sequenced plant species.

3.6.3 Preliminary studies (proof of concept)

Before the seasonal study was conducted, a proof of concept study was performed and the results were not included in any season. For proof of concept, 17 clones were sequenced and the sequence data compared to those in the GenBank and the GFRR database (Table 3.1). The sequenced clones were organized into clusters of consensus sequences. The sequences in each cluster were aligned using a BLAST2 program and gave 100% identity to each other. Using GenBank results, the families identified from the sequenced clones from dung were found to be Caesalpinaceae (1), Mesembryanthemaceae (3), Pinaceae (4), Tiliaceae (3), Euphorbiaceae (3), Zamiaceae (2), and Vitaceae (1). Comparison to the created database identified Fabaceae (1), Asteraceae (4), Tiliaceae (3), Euphorbiaceae (3), and Ebenaceae (2), and all sequences that did not match those in the GFRR database were recorded as unidentified.

Most of the *rbc*L gene sequences found in this dung sample were from the family Asteraceae and were identified as *B. elliptica* with BLAST2 alignments. These sequences were positively identified at species level. Other major cluster sequences that were found in the dung were from the families Euphorbiaceae, Mesembryanthemaceae (with no matches found from the GFRR database), and Tiliaceae. Two of the sequences, in the Tiliaceae family, were identified as *G. occidentalis*, which gave 100% and 99% (with one mismatch) similarity, and 99% similarity to *G. robusta* with one and two mismatches respectively. The third sequence which gave a close match (98%) to *G. occidentalis* is, however, not *G. occidentalis* but is another plant species in the family Tiliaceae.

BLAST search showed that three sequences belong to the family Euphorbiaceae, with one sequence giving 99% similarity to *E. bothae.* Although this sequence gave 99% similarity, sequence alignments indicate that the sequence is not *E. bothae* as it has four mismatches. The same applies to its match from the GenBank *E. abyssinica,* as this species is not a South African plant and is not found in the GFRR. In addition to the major taxa, the families Fabaceae (*S. afra*) and Ebenaceae (*E. undulata*) were identified from one and two sequenced clones respectively. One sequence that was found to belong to the family Vitaceae (identified as *R. digitata*) did not give any match to the GFRR database.

Table 3.1. Comparison of the dung sequences with those from the GenBank and GFRR database.

Number of sequences	Match from GenBank	Family of matched plant species	% Identity	GFRR database	Family of matched plant species	% Identity
One	*Brownea spp.*	**Caesalpiniaceae**	98	*S. afra*	**Fabaceae**	99
Three	*Lithops spp.*	**Mesembryanthemaceae**	99	None		
Four	*A. procera*	**Pinaceae**	98	*B. elliptica*	**Asteraceae**	99
One	*G. occidentalis*	**Tiliaceae**	100	*G. occidentalis*	**Tiliaceae**	100
One	*G. occidentalis*	**Tiliaceae**	99	*G. occidentalis / G. robusta*	**Tiliaceae**	99
One	*G. occidentalis*	**Tiliaceae**	98	*G. occidentalis / G. robusta*	**Tiliaceae**	98
One	*E. abyssinica*	**Euphorbiaceae**	99	*E. bothae*	**Euphorbiaceae**	99
One	*E. abyssinica*	**Euphorbiaceae**	98	*E. bothae*	**Euphorbiaceae**	97
One	*E. abyssinica*	**Euphorbiaceae**	97	*E. bothae*	**Euphorbiaceae**	96
Two	*E. natalensis*	**Zamiaceae**	99	*E. undulata*	**Ebenaceae**	99
One	*R. digitata*	**Vitaceae**	99	None		

Randomly sequenced clones were used to determine plant species composition of the dung, and the percentage composition calculated per dung sample. In terms of percentage composition using the generated *rbc*L gene sequences, the diet of the black rhinoceros is expressed in Fig. 3.3.

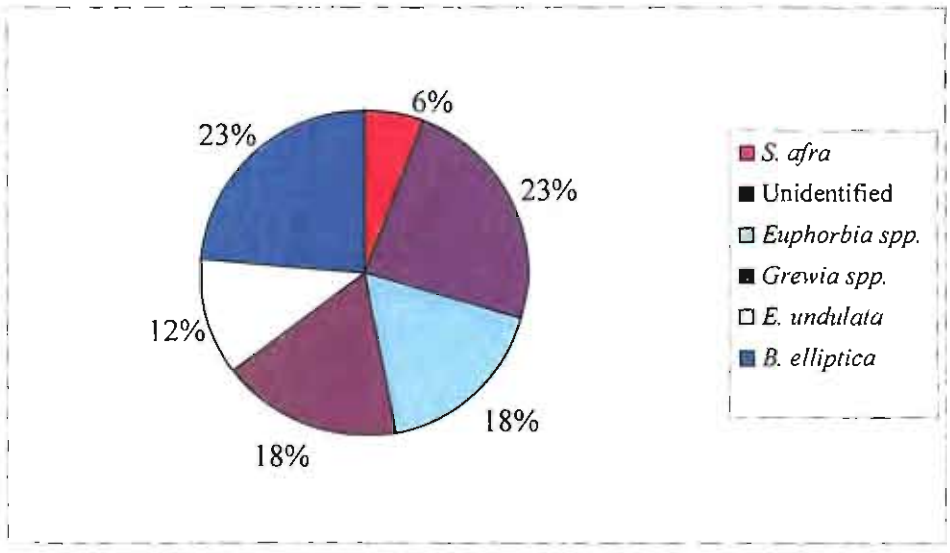


Fig. 3.3. Plant species identified in the black rhinoceros dung using molecular methods expressed as a percentage of plant composition of the dung.

3.6.4 Seasonal dung analysis

After establishing that the botanical composition of the black rhinoceros dung could be determined by using molecular methods, sequence data were generated from dung samples obtained from different seasons. Ten clones were sequenced per seasonal dung sample and the sequenced data compared to the GenBank and the GFRR database. The Tables below show the botanical composition of the black rhinoceros dung from the different seasons. The sequenced clones were organized into clusters of consensus sequences.

3.6.4.1 Winter dung composition

Using GenBank data, families of the plants identified from the winter dung sequences were found to be Tiliaceae (5), Euphorbiaceae (2), Pinaceae (2), and Salvadoraceae (1). BLAST2 search results using the GFRR database identified the plants to be from the families Tiliaceae (5), Euphorbiceae (2), Asteraceae (2), and Salvadoraceae (1). Table 3.2 shows the plants that were identified in the winter dung sample. Based on the alignment results from the GFRR dataset, most of the sequenced clones were identified as *G. occidentalis*, with one sequence giving 100% and two giving 99% (with one mismatch). Other plants that were identified at species level were *B. elliptica* and *A. tetracantha*. Although a 99% similarity to *E. bothae* was observed, the alignment had 4 mismatches and cannot be identified as *E. bothae*.

Table 3.2. Comparison of the sequences from winter dung with those from the GenBank and GFRR database.

Number of sequences	Match from GenBank	Family of matched plant species	% Identity	GFRR database	Family of matched plant species	% Identity
One	*G. occidentalis*	**Tiliceae**	100	*G. occidentalis*	**Tiliaceae**	100
Three	*G. occidentalis*	**Tiliceae**	99	*G. occidentalis / G. robusta*	**Tiliaceae**	99
One	*G. occidentalis*	**Tiliceae**	98	*G. occidentalis*	**Tiliaceae**	98
One	*E. abyssinica*	**Euphorbiaceae**	100	*E. bothae*	**Euphorbiaceae**	99
One	*E. abyssinica*	**Euphorbiaceae**	98	None	**Euphorbiaceae**	96
Two	*A. procera*	**Pinaceae**	98	*B. elliptica*	**Asteraceae**	99
One	*A. tetracantha*	**Salvadoraceae**	99	*A. tetracantha*	**Salvadoraceae**	99

The obtained sequence data were used to estimate the percentage composition of the winter black rhinoceros dung using the results obtained from randomly sequenced clones (Fig. 3.4).

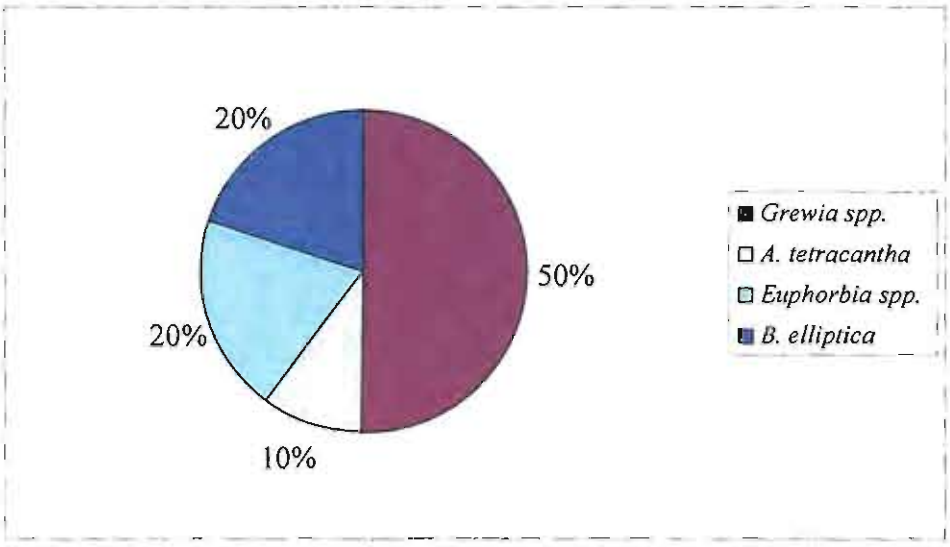


Fig. 3.4. Plant species identified in the winter dung sample of the black rhinoceros using molecular methods expressed as a percentage of plant composition of the dung.

3.6.4.2 Spring dung composition

Using GenBank data, the families of the plants identified from the spring dung sequences were found to be Tiliaceae (4), Zamiaceae (3), Plambaginaceae (1), Euphorbiaceae (1), Tiliaceae (1). BLAST2 search using the created database gave indentities to Tiliaceae (3), Ebenaceae (2), and Plumbaginaceae, with 4 sequences not matching to any of the GFRR dabase sequences. Table 3.3 shows the plants that were identified in the spring dung sample.

Table 3.3. Comparison of the sequences from spring dung with those from the GenBank and GFRR database.

Number of sequences	Match from GenBank	Family of matched plant species	% Identity	GFRR database	Family of matched plant species	% Identity
Three	*G. occidentalis*	**Tiliceae**	99	*G. occidentalis / G. robusta*	**Tiliaceae**	99
Two	*E. natalensis*	**Zamiaceae**	99	*E. undulata*	**Ebenaceae**	99
One	*E. natalensis*	**Zamiaceae**	96	None		
One	*P. capensis*	**Plumbagoniceae**	99	*P. auriculata*	**Plumbagoniceae**	99
One	*E. abyssinica*	**Euphorbiaceae**	96	None	**Euphorbiaceae**	95
One	*H. latifonia*	**Liliaceae**	97	None		
One	*S. ricinocarpa*	**Tiliaceae**	95	None		

The obtained sequence data were used to estimate the percentage botanical composition of the spring black rhinoceros dung using the randomly sequenced clones.

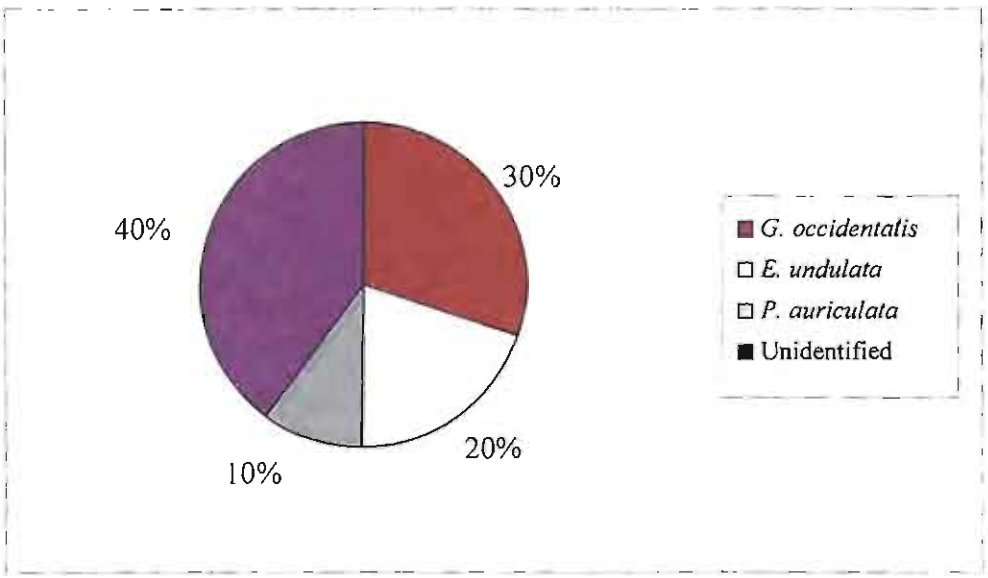


Fig. 3.5. Plant species identified in the spring dung sample of the black rhinoceros using molecular methods expressed as a percentage of plant composition of the dung.

3.6.4.3 Summer dung composition

Using GenBank results, the families identified from summer dung sequences were found in the ratio 2:1:1:2:1:1:1:1 to be Tiliaceae (2), Zamiaceae (1), Cactaceae (1), Euphorbiaceae (2), Pinaceae (1), Salvodoraceae (1), Vitaceae (1) and Mesembryanthemaceae (1). BLAST2 search results using the GFRR database identified the families Tiliaceae (2), Euphorbiaceae (2), Ebenaceae (1) and Salvadoraceae (1), with 4 sequences not matching those in the GFRR databse. Table 3.4 shows the plants that were identified in the summer dung sample.

Table 3.4. Comparison of the sequences from summer dung with those from the GenBank and the GFRR database.

Number of sequences	Match from GenBank	Family of matched plant species	% Identity	GFRR database	Family of matched plant species	% Identity
One	*G. occidentalis*	**Tiliaceae**	99	*G. occidentalis / G. robusta*	**Tiliaceae**	99
One	*E. natalensis*	**Zamiaceae**	99	*E. undulata*	**Ebenaceae**	99
One	*M. guentheri*	**Cactaceae**	95	None		
One	*E. polychroma*	**Euphorbiaceae**	98	None	**Euphorbiaceae**	
One	*E. polychroma*	**Euphorbiaceae**	96	None	**Euphorbiaceae**	
One	*A. procera*	**Pinaceae**	96	None		
One	*S. ricinocarpa*	**Tiliaceae**	98	*G. occidentalis / G. robusta*	**Tiliaceae**	98
One	*A. tetracantha*	**Salvadoraceae**	99	*A. tetracantha*	**Salvadoraceae**	99
One	*R. digitata*	**Vitaceae**	96	None		
One	*Lithops*	**Mesembryanthemaceae**	98	None		

The obtained sequence data were used to estimate the percentage botanical composition of the summer black rhinoceros dung using the randomly sequenced clones (Fig. 3.6).

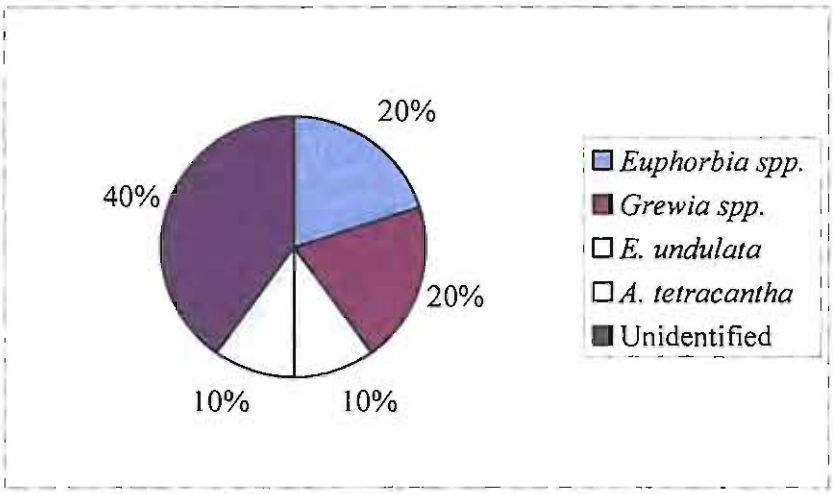


Fig. 3.6. Plant species identified in the summer dung sample of the black rhinoceros using molecular methods expressed as a percentage of plant composition of the dung.

3.6.4.4 Autumn dung composition

Using GenBank results, the autumn dung sequences were identified to the families Tiliaceae (3), Mesembryanthemaceae (3), Calesalpinaceae (2), Zamiaceae (1), and Salvadoraceae (1). BLAST2 search results using the created database identified the families Tiliaceae (3), Fabaceae (2), Ebenaceae (1) and Salvadoraceae (1), with 3 sequences not identified from the GFRR database. Table 3.5 shows the plants that were identified in the autumn dung sample.

Table 3.5. Comparison of the sequences from autumn dung with those from the GenBank and the GFRR database.

Number of sequences	Match from GenBank	Family of matched plant species	% Identity	GFRR database	Family of matched plant species	% Identity
One	*G. occidentalis*	**Tiliaceae**	99	*G. occidentalis / G. robusta*	**Tiliaceae**	99
Two	*G. occidentalis*	**Tiliaceae**	100	*G. occidentalis*	**Tiliaceae**	100
One	*E. natalensis*	**Zamiaceae**	99	*E. undulata*	**Ebenaceae**	99
Two	*Brownea sp.*	**Caesalpiniaceae**	98	*S. afra*	**Fabaceae**	99
One	*A. tetracantha*	**Salvadoraceae**	99	*A. tetracantha*	**Salvadoraceae**	99
Two	*Lithops sp.*	**Mesembryanthemaceae**	99	None		
One	*Lithops sp.*	**Mesembryanthemaceae**	97	None		

The obtained sequence data were used to estimate the percentage botanical composition of the autumn black rhinoceros dung using the randomly sequenced clones (Fig. 3.7).

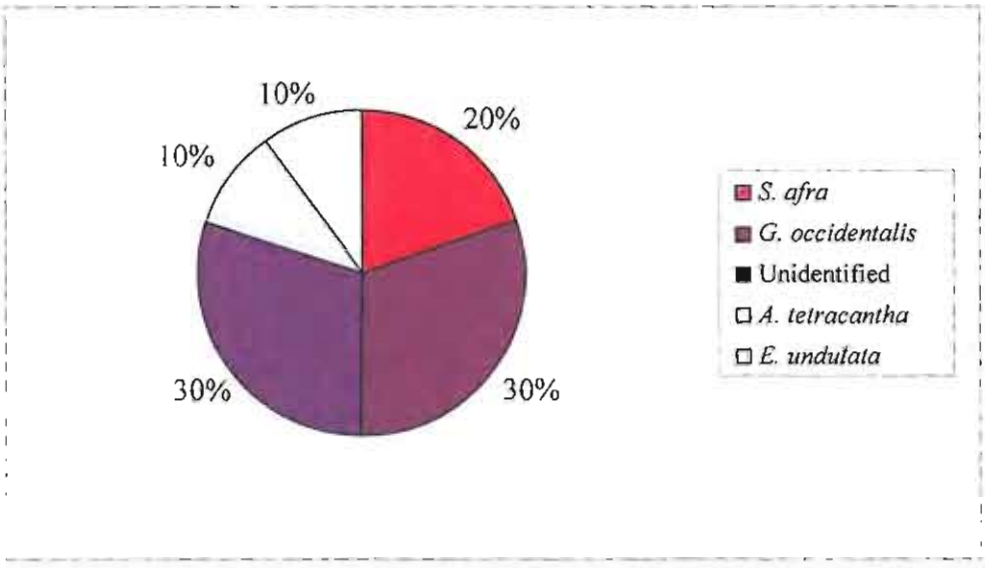


Fig. 3.7. Plant species identified in the autumn dung sample of the black rhinoceros using molecular methods expressed as a percentage of plant composition of the dung.

3.7 Discussion

Studies on the diet composition of the black rhinoceros using backtracking methods at the GFRR have been conducted by Ausland and Sviepe (2000), Brown *et al.* (2003) and more recently by van Lieverloo and Schuiling (2004) who also included microhistology in their work. All these studies have shown that the black rhinoceros utilize a wide variety of plant species at the reserve. Brown *et al.* (2003) produced the top ten plants most frequently browsed by the black rhinoceros from two different vegetation types, some of which are preferred under certain conditions such as season or after rainfall (Tables 1.1 and 1.2). Studies on the diet composition of the black rhinoceros at the GFRR and in published data have reported the use of traditional methods such as direct observation and microhistology. In this study the diet composition of the black rhinoceros was determined from dung by using a molecular method that aimed to sequence the *rbc*L gene.

Molecular analyses have been used to evaluate the diet composition of now extinct animals using coprolites as a source of DNA (Hofreiter *et al.*, 2000; Hofreiter *et al.*, 2003). Although this method has been used in scatology, it has not been extensively employed on living herbivores to reveal their diet, but has been used to trace the animal from which the dung originates (O'Ryan, 1994). Höss *et al.* (1992) amplified the *rbc*L gene to evaluate the diet composition of brown bear dung.

The initial steps in identification of the plants from the black rhinoceros dung involved extraction of genomic DNA and amplification of the *rbc*L gene. The same set of primers (section 2.5.2) used to amplify the *rbc*L gene from plants successfully amplified the *rbc*L gene (646 bp) from dung. Hofreiter *et al.* (2003) used four overlapping primers to amplify 573 bp of the mitochondrial 12S DNA from ancient DNA. This is because ancient DNA is highly fragmented and amplification of longer products (> 250 bp) in most cases is not possible. Difficulty in DNA extraction and amplification of DNA from dung has been associated with cross-links between reducing sugars and primary amines formed by the Maillard reaction (Hofreiter *et al.*, 2000). DNA from the black rhinoceros dung was recovered by addition of BSA during extraction and amplification. Use of BSA has been reported for aiding in DNA recovery during both extraction and amplification (Höss *et al.*, 1992).

To identify the plant species from dung, the study relied more on the GFRR database of plants from the GFRR (Table 2.1) than on the sequences in the GenBank database. This is because most of the investigated plants in this study have been reported as being browsed by the black rhinoceros at the GFRR (Ausland and Sviepe, 2000; Brown *et al.*, 2003; van Lieverloo and Schuiling, 2004), which makes them more likely to be in the black rhinoceros dung. Secondly, although a fairly extensive database of *rbc*L gene sequences from many plant species exists, very few of the plant species on the GFRR have the *rbc*L gene sequences available in the GenBank database. The number of sequenced plant species in this study from the GFRR is however not sufficient for reference analysis considering the wide variety of plants browsed by the black rhinoceros. This low number of plant sequences in the GFRR database limits identification for some sequences from the dung to genus level.

The size of the *rbc*L gene from coprolites amplified and sequenced generally is limited to less that 200 bp, which limits the accuracy of identification of the sequences (Hofreiter *et al.*, 2000; Poinar *et al.*, 2001). Höss *et al.* (1992) sequenced 356 bp fragments of the *rbc*L gene from the brown bear dung to reveal its diet. The partial sequences of the *rbc*L gene (646 bp) from dung were of sufficient size to identify the plant species in the black rhinoceros dung. Preliminary studies indicated that, the major plant species identified from the sequenced clones was *B. elliptica* forming 23% of the sequenced clones. *B. elliptica* has not been reported on the diet of the black rhinoceros at the GFFR before, but rather a plant from the same genus, *B. elicifolia,* has been reported in the top ten plants browsed by the black rhinoceros. Although *B. elicifolia* has not been reported to be browsed, its identification in the preliminary study and winter dung sample (20%) shows that it forms an important part in the diet of the black rhinoceros.

A plant that was found in the black rhinoceros dung frequently was *G. occidentalis*. This plant species was identified in all dung samples analyzed. *Grewia spp.* have been identified by van Lieverloo and Schuiling (2004) in the diet of the black rhinoceros, using both fecal analysis (microhistology) and backtracking methods. However, their methods were unable to identify the plants to species level. In this study *Grewia spp.* were identified mostly as *G. occidentalis,* with 40% of the winter, 30% of spring and autumn, 10% of summer and 20% of the preliminary

sequenced clones from dung identified as originating this plant species. Although the plant sequences from dung were identified to be *G. occidentalis,* there are concerns because the *rbc*L gene of G. *occidentalis* and *G. robusta* is highly conserved, making the sequenced fragment of the gene unable to differentiate between the two species. Sequencing of the complete gene for these two species is important to identify the plants to species level from the dung as *G. robusta* is mostly reported to be favored by the black rhinoceros (Table 1.1 and 1.2) and is common in the GFRR.

Studies by Ausland and Sviepe (2000) at the GFRR reported that *E. bothae* was browsed more than any other plant found in the reserve. This study reports no specific identification of *E. bothae* but rather the identification of plant species from the same genus. *Euphorbia spp.* were identified in all dung samples except that from autumn. The flora of the GFRR is known to be rich with *Euphorbia spp.* and the generated sequences could be one of the many *Euphorbia spp.* found at the reserve such as *E. tetragona* and *E. triangularis* that are found to be favored by the black rhinoceros (Heilmann *et al.*, 2006). Although one sequence from the results gave a 100% identity to *E. abyssinica,* this and all other identities to *E. abyssinica* cannot be correct as this species is not found in South Africa, particularly at the GFRR (Dold, 2006).

Brown *et al.* (2003) found that succulent forbs such as *Lithops spp.* from the family *Mesembryanthemaceae* are highly favored by the black rhinoceros in the GFRR and this species was identified from dung using molecular methods. Other plants species identified from dung were *A. tetracantha, E. undulata* and *P. auriculata* and these species have been reported to be important in the diet of the black rhinoceros (Ausland and Sviepe, 2000; Brown *et al.*, 2003). Identification of *S. afra* from the dung proved the importance of using molecular analysis, as the plant has small leaves that are easily digested and therefore less represented in the dung. Microscopic feacal analyses of giraffe (*Giraffa camelopardalis*) dung by Parker (2004) could not identify *S. afra,* which is known to be favored by the animal. Large numbers of plant fragments remain unidentified when fecal samples are subjected to microscopic analysis as a result of unrecognized epidermis and cuticle fragments and the fact that plant parts may be digested to such an extent that

no stomata or other characteristic structures are found in the dung (Parker, 2004; van Lieverloo and Schuiling, 2004). The advantage of using molecular methods is that some plant species that are not identified to species level in the GFRR database can at least be identified to family or genus level. Due to the large number of *rbc*L gene sequences deposited in the GenBank database, some sequences can be identified to species, genus or family provided that the plant is found in the GFRR. The amplification and sequencing of the *rbc*L gene from dung which produces sequences that are not identified to species level underscores the need to expand the limited GenBank database by sequencing this gene from more plants in the GFRR.

A study by van Lieverloo and Schuiling (2004) found that fecal samples could not be assigned to the vegetation types in where they were found due to the movement of the black rhinoceros and the retention time in the gut of the animal digesting the plant material. The results obtained in this molecular study aimed to reveal its diet and show a more in-depth picture of the black rhinoceros diet than traditional methods used to determine botanical composition of the dung. Molecular methods are able to detect plants that may be rare in the dung, or so modified by masticatory and digestive processes, that they are not easily identified morphologically (Hofreiter *et al.*, 2000). Genetic analysis of the chloroplast *rbc*L gene represents plant material that most likely has been eaten by the animal (Hofreiter *et al.*, 2003).

Molecular analyses of the seasonal black rhinoceros dung samples give an idea of its diet during different seasons at the reserve. All the dung samples show a wide range of plants, with some plants browsed in almost all seasons. Based on the sequenced clones, there was no major difference in plant composition of the four seasonal dung samples analysed. This could be a result of the low number of clones sequenced. *Grewia spp.* were found to dominate the composition of the dung in most seasons, for the clones sequenced. Although the method has potential to determine the diet of herbivores it has its drawbacks. Firstly, it requires a trained molecular biologist to extract DNA, sequence and analyze the obtained data using the available bioinformatics tools. Secondly, sequencing and chemicals used in this method are very expensive. Contrary to this, the method can generate sequence data from those sources of material that are not identified when using microhistology and also it is not labour intensive.

CHAPTER FOUR
ANTIOXIDANT AND PHENOLIC COMPOUNDS FROM SELECTED PLANT SPECIES

4.1 Introduction

The botanical composition of the diet of the black rhinoceros in the GFRR has been studied by observational techniques (Ausland and Sviepe, 2000; Brown *et al.,* 2003; van Lieverloo and Schuiling, 2004) and the chemical composition (e.g. crude protein, lignin, N, P, Na, K and Mg) of some plants has been determined (van Lieverloo and Schuiling, 2004). In addition, a number of studies have shown that the diet given to black rhinoceros in captivity is inappropriate for their sustained good health as the content of vitamin E in their feed has been considered to be inadequate (Dierenfeld and Traber, 1992). Tyrosine and uric acid in red blood cells of black rhinoceros have been reported to have oxidants and /or oxygen free radical scavenging activity (Harley *et al.,* 2004). An earlier study by Ndondo *et al.* (2004), which led to this study, focused on the presence of vitamin E and the fatty acid profiles of selected species of plants browsed by the black rhinoceros in the GFRR. As antioxidants obtained from plants in the diet of many herbivores and omnivores play an important role in their nutrition, selected plant species were investigated for total phenolics and antioxidant capacity.

4.1.1 Antioxidants

Antioxidants are compounds that decrease or prevent oxidation of substrates from free radical species present in food or in the body. They have been found to aid the body in protecting itself against different types of oxidative damage caused by reactive oxygen species (ROS), which have been linked to a variety of diseases such as cancer, diabetes, shock, arthritis, and acceleration of the aging process (Saha *et al.,* 2004). Low levels of antioxidants such as vitamin E in blood plasma of black rhinoceros have been found to cause haemolytic anaemia (Dierenfeld *et al.,* 1988) Antioxidants are sometimes produced under oxidative stress, a condition that is defined as "the imbalance between oxidants and antioxidants in favour of the oxidants potentially leading to damage". This is suggested to be the underlying cause of various diseases (Katalinic *et al.,* 2006).

The ROS such as the superoxide radical, hydroxyl radicals, and peroxyl radicals, are often generated as byproducts of normal metabolic process (Wong *et al.*, 2006). *In vivo*, some of the ROS can display a positive role in processes such as energy production, phagocytosis, regulation of cell growth and intercellular signaling, or synthesis of biologically important compounds (Chang *et al.*, 2001). Within biological systems there are four general sources of antioxidants: (1) enzymes such as superoxide dismutase, glutathione peroxidase and catalase; (2) large molecules such as certain proteins; (3) small molecules such as vitamin C, α-tocopherol, carotenoids, glutathione, uric acid, polyphenols and (4) hormones such as estrogen, angiotensin and melatonin (Prior *et al.*, 2005). These antioxidants are further classified into two major groups: the antioxidant enzymes and the low molecular weight antioxidants (LMWA). LMWA are major contributors to the total antioxidant capacity as they act as direct chemical scavengers of ROS (Chevion *et al.*, 1997).

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are very effective, but they may possess mutagenic activity. Because of the potential health benefits from plant antioxidants there has been intensive research on this source of natural antioxidants. Data from various studies indicate that plants contain a wide variety of natural antioxidants, such as phenolic acids, flavonoids and tannins, many of which have potent antioxidant activity (Wong *et al.*, 2006). Antioxidants such as vitamin C and E are essential for the protection against ROS, however, most of the antioxidant activity may be from compounds such as phenolic acids and flavonoids rather than from vitamin C or E (Tsao and Deng, 2004).

4.1.2 Phenolic compounds

Plants are unique in that they are able to produce a broad variety of phenolic compounds. Phenolic compounds or polyphenols are known to have many activities in plants including lignin production for structural strength, phytoalexins for protection against photosynthetic stress, and protection from reactive oxygen species formed in wounds and by herbivores (Yang *et al.*, 2001; Baker *et al.*, 2005). Phenolic compounds form one of the most abundant groups of plant secondary metabolites, with more than 8000 phenolic structures currently known. Natural phenolic compounds range from simple molecules (phenolic acids, phenyl propanoids, and flavonoids) to highly polymerized

compounds (lignins and tannins), with flavonoids representing the most common and widely distributed sub-group (Soobrattee *et al.*, 2005).

Phenolic compounds are very important as antioxidants because of their high negative redox potentials which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelating potential. The antioxidant activity of these compounds is predominantly determined by their structures, in particular, electron delocalization over the aromatic nucleus in those based on a phenolic structure. When the compounds react with free radicals, it is the delocalization of the gained electron over the phenolic antioxidant, and the resonance stabilization effect of the aromatic nucleus, that prevents the continuation of the free radical chain reaction. This is often called radical scavenging, but phenolic compounds inhibit oxidation through a variety of mechanisms (Tsao and Deng, 2004).

Phenolic compounds vary in structure, but have at least one aromatic ring bearing one or more hydroxyl groups. Phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) have a single-ring structure whereas flavonoids can be further classified into anthocyanins, flavan-3-ols, flavones, flavanones and flavonols. Many of these compounds are glycosylated by sugars such as glucose, rhamnose, galactose and arabinose. All plant phenolic compounds arise from the common intermediate, phenylalanine (Fig. 4.1), or its close precursor, shikimic acid. They can be divided into at least ten different classes based on their general chemical structures (Yang *et al.*, 2001; Sakihama *et al.*, 2002; Tsao and Deng, 2004).

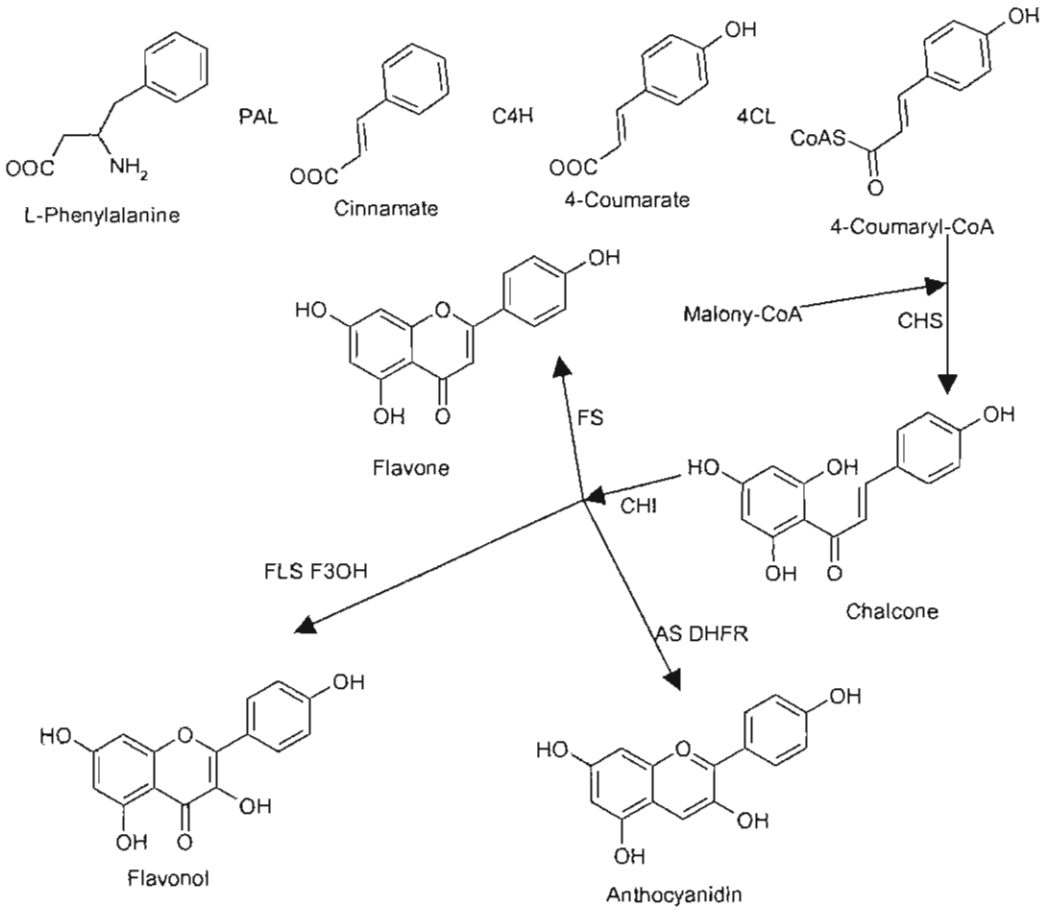


Fig. 4.1. Diagram showing the first common steps in the biosynthesis of certain phenolic compounds. The enzymes involved in each reaction are: phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3OH), isoflavone synthase (ISF), flavone synthase (FS), flavonol synthase (FLS), dihydroxyflavonol reductase (DHFR), and anthocyanin synthase (AS) (Adapted from Geissman and Crout, 1969; Sakihama *et al.*, 2002).

4.1.2.1 Flavonoids

Flavonoids are the largest class of phenolic compounds in plants and may be found accumulating in epidermal cells of flowers, leaves, stems, roots, and fruits in glycosidic and non-glycosidic forms (Sakihama *et al.*, 2002). They are a large group of structurally related compounds with a chromane-type skeleton, with a phenyl substituent in the C_2 or C_3 position (de Rijke *et al.*, 2006).

Flavonoids occur in a variety of structural forms (Fig. 4.2). Flavonoid compounds are C_{15} compounds (exclusive of O-alkyl groups and secondary substitution) composed of two phenolic nuclei connected by a three-carbon unit. They are important in plants as antioxidants, enzyme inhibitors or inducers, and precursors of toxic substances (Kandil *et al.*, 2002).

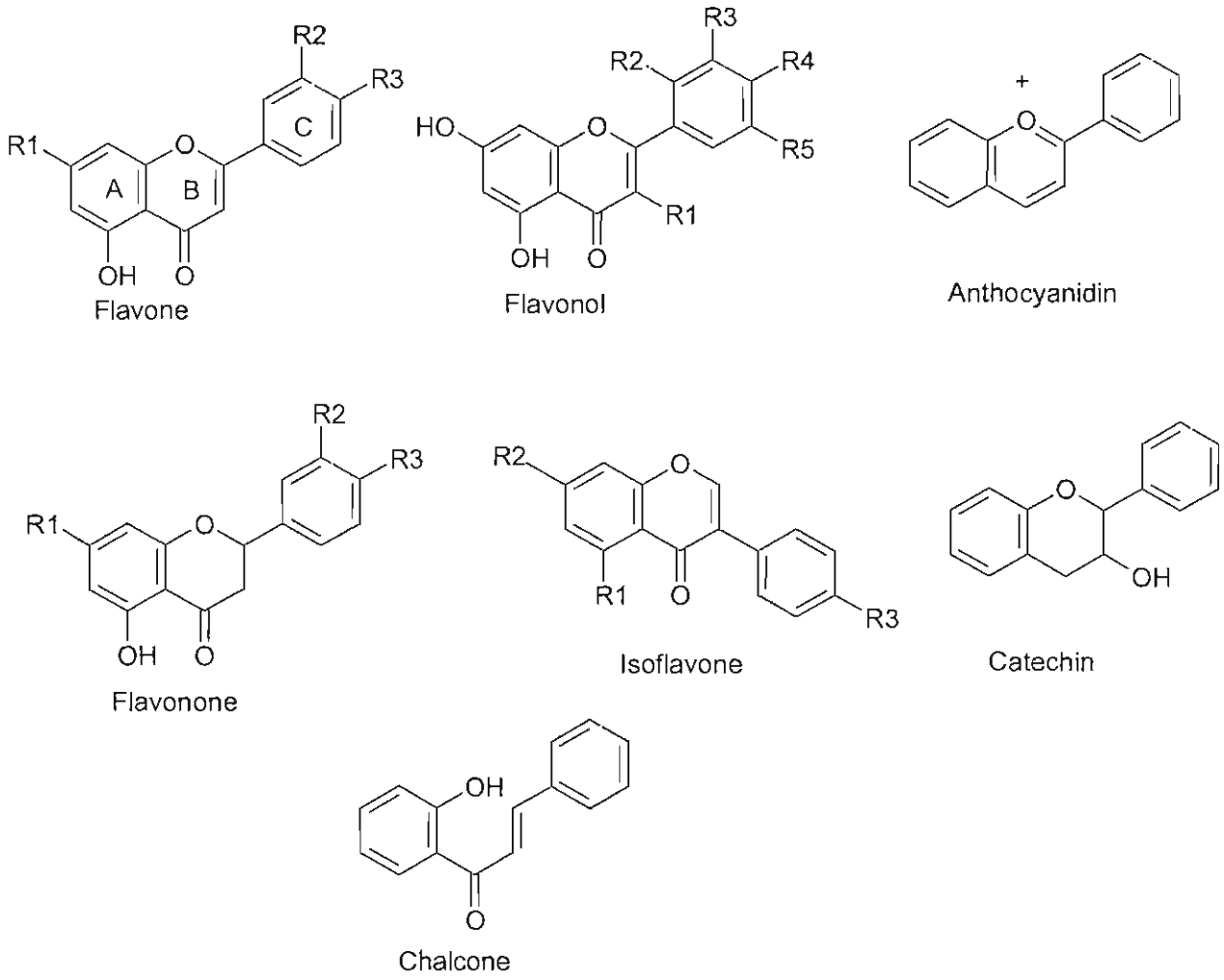


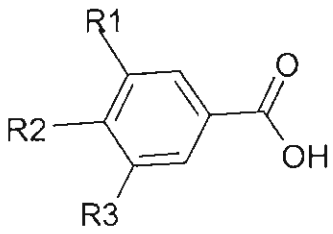
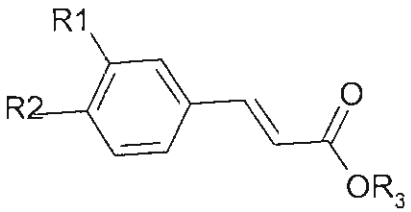
Fig. 4.2. Structures of the main flavonoid subclasses (Adapted from de Rijke *et al.*, 2006).

4.1.2.2 Phenolic acids

Phenolic acids are often found as esters, which are either soluble and accumulate in vacuoles or occur as bound cell-wall components. Hydroxybenzoic acids and hydroxycinnamic acids form about one third of the phenolic compounds in plants with the most abundant being the hydroxycinnamic acids, caffeic acid and ferulic acid (Yang *et al.*, 2001). The hydroxybenzoic acid content of plants is usually low, whereas hydroxycinnamic acids are more common and are found as glycosylated derivatives or esters, rather than in free form (Cimpoiu, 2006).

Phenolic acids are classified according to their structures into different groups. Structures of the common phenolic acids are presented in Table 4.1.

Table 4.1. The structures of common phenolic acids (adapted from Cimpoiu, 2006).

Group	Compound	R1	R2	R3
Hydroxybenzoic acids 	Protocatechuic acid	OH	OH	H
	Gallic acid	OH	OH	OH
Hydroxycinnamic acid 	Coumaric acid	H	OH	H
	Caffeic acid	OH	OH	H
	Ferulic acid	CH_3O	OH	H
	Chlorogenic acid	OH	OH	Quinic acid

4.2 Materials and methods

4.2.1 Chemicals

All chemicals were of analytical reagent grade. Methanol, ethyl acetate, chloroform, formic acid, acetic acid, thin layer chromatography (TLC) plates, polyethylene glycol-4000 were all purchased from Merck, South Africa. Ascorbic acid, Folin-Ciocalteau's phenol reagent, iron (III) chloride, sodium carbonate, gallic acid, 2,4,6-tris-2-pyridyl-*s*-triazine (TPTZ), 2,2-dipheny-l-1-picrylhydrazyl (DPPH), 2-aminoethyl diphenylborinate, Tris-HCl and chlorogenic acid were all purchased from Sigma Aldrich. The 96-well microplates (NUNC, Roskilde, Denmark) were purchased from Ibhayi laboratory, South Africa.

4.2.2 Sample collection

P. auriculata leaves were collected in Grahamstown from Rhodes University grounds and *B. elliptica, G. robusta* and *A. tetracantha* were collected from the GFRR. Collected material was placed in plastic bags containing silica gel and transported to the laboratory for analysis. As these four plant species were identified from the dung in most seasons, they were selected for antioxidant capacity and phenolic content studies.

4.3 Preparation of extracts from the plant leaves

The method for extraction of total antioxidants was performed with modification according to Saha *et al.* (2004) and Chen *et al.* (2006). Plant leaves were cut into small pieces and air-dried in a dark cabinet for 72 h, after which a constant weight was reached. A sample of air-dried leaves (10 g) of each plant species was extracted separately with 120 ml of solvent in a dark cabinet for 4 days. The solvents used for extraction were methanol, ethyl acetate and chloroform. The total extract was filtered through 0.45 μm membrane filters (Whatman, England) using a Buchner funnel with a vacuum pump (Rocker 300, Instruvac) and the filtrate from each of the three solvents collected and concentrated using a rotary evaporator (BÜCHI Rotavapor-R-14, Switzerland). The residue was reconstituted in 2 ml of the solvent used for extraction and the solvent removed under nitrogen. All

samples were stored in a vacuum desiccator containing silica gel for 48 h to remove any remaining moisture before they were subjected to antioxidant assays.

4.3.1 Inter-day variability study

4.3.1.1 Optimum extraction period

Preliminary studies were performed using methanol for extraction from *P. auriculata* on three different days as described in section 4.3. Extractions were done over 4, 7 and 11 days. This was done to establish the optimum period for extraction.

4.3.1.2 Stability studies

Stability studies were investigated on all the plant extracts. The extracts were analyzed for antioxidant capacity after 48 h under vacuum. The samples were then stored at 4°C and the assay repeated after a further 72 h.

4.4 DPPH free radical-scavenging activity assay

The DPPH assay, as described by Chen *et al.* (2006), was followed with modifications. Stock solutions (1 mg/ml) of ascorbic acid and each plant extract were prepared in methanol. Working solutions at different concentrations ranging from 0.5 mg/ml to 0.025 mg/ml were prepared in methanol. An aliquot of 50 μl of each dilution, including the stock solution, was transferred into a 96-well microplate (NUNC, Roskilde, Denmark). A working solution of DPPH (250 μM) in methanol was freshly prepared and 150 μl added to each well. The plates were incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against a methanol blank on a PowerWave X reader (Bio-Tek Instruments INC). Each dilution was analysed in triplicate and the absorbance readings averaged. The ascorbic acid methanol solution was used as a positive control.

The percent of free radical scavenging of the extracts was calculated as a ratio of the absorption of the extracts relative to the control DPPH solution without the extract using the following equation (Saha *et al.*, 2004; Karawita *et al.*, 2005):

$$\% \text{ Inhibition} = \frac{\text{Absorbance}_{517\,nm}\,(\text{DPPH}) - \text{Absorbance}_{517\,nm}\,(\text{Sample} + \text{DPPH})}{\text{Absorbance}_{517\,nm}\,(\text{DPPH})} \times 100$$

Free radical scavenging activity of the plant extracts and ascorbic acid were expressed as IC_{50}, which is the concentration of a sample required to decrease the absorbance at 517 nm by 50% compared to the control response (Chen *et al.*, 2006).

4.5 TLC analysis

4.5.1 DPPH spray

The 4 day methanol plant extracts (20 μl) and 10 μl of ascorbic acid (1 mg/ml) were applied as spots to an activated silica gel F_{254} plate. The developing solvent consisted of chloroform – acetic acid – methanol – water (60:32:12:8) and was allowed to migrate through the applied spots to focus the material into a fine line. The plate was removed immediately and dried with a hair dryer and the plate developed in the same solvent in a pre-saturated chromatographic chamber. The developed chromatoplates were dried with a hair dryer and the spots located by spraying with 250 μM DPPH in methanol.

4.5.2 Ferric Reducing Antioxidant Power (FRAP) spray

The antioxidant activity of the methanol extracts was visualized using FRAP as a TLC spray reagent. The 4 day methanol plant extracts (40 μl) and 2.5 μl of chlorogenic acid (1 mM) were applied as spots to an activated silica gel F_{254} plate. Chlorogenic acid served as a positive control. The applied samples were focused in a mixture of ethyl acetate – formic acid – acetic acid – water (100:11:11:26), dried with a hair dryer and the plate developed with the same developing solvent in a pre-saturated chromatographic chamber. The developed chromatoplates were dried with a hair

dryer and antioxidants located by spraying with a solution of 10 mM TPTZ and 20 mM ferric chloride made in 0.25 M acetate buffer, pH 3.6.

4.5.3 Phenolic compounds

Natural product-polyethylene glycol reagent (NP/PEG) was prepared according to Wagner and Bladt (1996) and consisted of 1% methanolic diphenylboric acid-β-ethylamino ester (diphenylboryloxyethylamine, NP) and 5% ethanolic polyethylene glycol-4000 (PEG) prepared separately. The 4 day methanol plant extracts (10 µl) were applied as spots to an activated silica gel F_{254} plate. The applied samples were focused in ethyl acetate and the plate was dried with a hair dryer. The samples were re-focused in a mixture of chloroform - acetic acid - methanol - water (60: 12: 24: 8), dried and the plate developed with the same solvent in a pre-saturated chromatographic chamber. The developed chromatoplates were dried with a hair dryer and sprayed with the NP/PEG spray reagent (with NP first followed by PEG) to increase the fluorescence of various compounds and the chromatoplates visualized under UV (366 nm) light.

4.6 Cyclic voltammetry analyses

Analyses of antioxidant reducing ability were based on the method of Brimecombe and Limson (2007). Cyclic voltammograms were recorded on the Autolab PGSTAT 30. Cyclic voltammetry tracings were recorded at a scan rate of 100 mV/sec. A 3 mm diameter glassy carbon electrode (GCE) was employed as the working electrode, and a silver/silver chloride (Ag/AgCl) (saturated in 3 mM KCl) and a platinum wire were employed as reference and auxiliary electrodes, respectively. Care was taken to ensure that the spacing between the electrodes was equidistant. The working electrode was thoroughly cleaned before use and between scans by polishing with a paste of alumina oxide powder (Sigma Aldrich) on a Buëhler felt pad, followed by a rinse with triple distilled water, 5% nitric acid to remove any interferences from the working electrode and a final rinse with triple distilled water. For all experiments 100 µl aliquots of the 4 day plant solvent extracts (1 mg/ml) (section 4.3) were introduced into the electrochemical cell to a final concentration of 107.14 µg/ml. Ascorbic acid (1 mM) was used as a positive control. The electrolyte used was 0.2 M Tris-HCl buffer, pH 7.

4.7 Total phenols

4.7.1 Preparation of working solutions

The total phenol content of *B. elliptica, G. robusta, P. auriculata* and *A. tetracantha* solvent extracts were determined with modification according to the method of Wintersteen *et al.* (2005) and Wong *et al.* (2006) for micro-volume analysis. Gallic acid stock solution was prepared by dissolving 0.5 g of gallic acid in 10 ml of ethanol and made up to 100 ml with triple distilled water. Gallic acid samples for the standard curve were prepared by adding 0, 1, 2, 3, 5, and 10 ml of the stock solution to a total volume of 100 ml made up with triple distilled water. The concentration range of gallic acid was 0, 50, 100, 150, 250, and 500 mg/L gallic acid (Appendix I). A 10% solution of Folin Ciocalteau reagent and 20% of sodium carbonate were prepared. All solutions were stored at 4°C for the duration of the study. The stock solutions (1 mg/ml), extracted as described in section 4.3, of *B. elliptica, P. auriculata, G. robusta, and A. tetracantha* were assayed for total phenol content.

4.7.2 Folin-Ciocalteau total phenol assay

Each gallic acid standard (0-500 mg/L) and the 4 day plant solvent extracts (20 µl) were transferred into separate cuvettes, to which 1.58 ml of triple distilled water added. Folin Ciocalteau reagent (100 µl of 10% solution) was then added and the solutions incubated at room temperature for 8 min, followed by adding 250 µl of 20% sodium carbonate solution. The reaction mix was kept at 40°C for 30 min after which 200 µl of each solution was transferred into a 96-well microplate (NUNC, Roskilde, Denmark). The absorbance of each solution was measured at 765 nm using PowerWave X reader (Bio-Tek Instruments INC). All analyses were carried out in triplicate and the absorbance readings averaged.

4.8 Results

4.8.1 DPPH assay

Preliminary studies using *P. auriculata* methanol extracts after 4, 7 and 11 days were performed to determine the optimum extraction period and the stability of the extracts after storing for 72 h using the DPPH assay. Table 4.2 shows percentage DPPH inhibition by *P. auriculata* methanol extracts at time zero (0 h), which is the first assay after extraction. The *P. auriculata* methanol extracts had a potent free radical scavenging activity against the DPPH free radical, but their percentage scavenging activity was lower than that of the control ascorbic acid. DPPH inhibition assay results obtained from day 4 and day 11 were similar and slightly higher than those obtained from day 7. Stability studies carried out after standing for 72 h showed no appreciable decrease in the percentage DPPH inhibition from all the extracts. Table 4.2 shows percentage DPPH inhibition by the extracts after 72 h and all values are presented as the mean of triplicate analyses.

Table 4.2. Percentage DPPH inhibition by *P. auriculata* methanol extracts after extraction for 4, 7 and 11 days and after standing for 0 h and 72 h.

Concentration of extracts (mg/ml)	**Day 4 Extract (%)**		**Day 7 Extract (%)**		**Day 11 Extract (%)**		**Ascorbic Acid (%)**
	0 h	**72 h**	**0 h**	**72 h**	**0 h**	**72 h**	**0 h**
1	86.9	85.0	78.7	75.0	86.1	86.2	97.0
0.5	57.7	56.9	55.3	54.0	60.2	59.5	97.5
0.4	52.4	54.6	49.2	56.9	51.2	52.2	97.1
0.3	43.8	42.6	42.2	39.6	47.1	45.0	97.2
0.2	37.2	36.1	36.1	35.7	39.0	38.7	97.3
0.1	30.6	30.9	30.9	30.4	34.9	32.8	96.7
0.05	25.9	28.4	26.5	28.7	28.7	30.2	94.4
0.025	24.8	24.1	23.0	26.5	26.5	26.5	60.1

Based on the *P. auriculata* results shown in Table 4.2, extraction on *B. elliptica, G. robusta* and *A. tetracantha* was done for 4 days and the extracts stored at 4°C. The percentage of DPPH free radical inhibition by the methanol extracts is shown in Table 4.3. The methanol extracts from *B. elliptica* exhibited strong antioxidant activity against the DPPH free radical scavenger. The methanol extract from *G. robusta* showed moderate activity whereas *A. tetracantha* extract showed a very weak free radical scavenging ability. Percentage DPPH inhibition effects increased in the order of ascorbic acid > *B. elliptica* > *P. auriculata* > *G. robusta* > *A. tetracantha*. Stability studies done on the methanol extracts of *B. elliptica, G. robusta* and *A. tetracantha* showed that the compounds in the extracts were stable for 72 h as no appreciable decrease in the percentage DPPH inhibition was observed (Table 4.3).

Table 4.3. Percentage DPPH inhibition by methanol extracts (4 day) of *B. elliptica, G. robusta* and *A. tetracantha* at 0 h and 72 h.

Concentration of extracts (mg/ml)	***B. elliptica* (%)**		***G. robusta* (%)**		***A. tetracantha* (%)**	
	0 h	**72 h**	**0 h**	**72 h**	**0 h**	**72 h**
1	95.2	95.7	65.6	67.7	37.7	37.3
0.5	85.6	82.2	47.1	53.1	30.4	33.5
0.4	76.6	80.3	47.7	44.4	31.0	24.9
0.3	66.1	64.7	39.3	40.1	26.9	24.8
0.2	49.5	52.4	33.3	39.0	26.2	23.8
0.1	35.7	37.9	27.2	30.9	24.5	24.8
0.05	26.5	27.2	26.8	28.0	22.8	20.0
0.025	25.3	23.8	23.1	21.8	22.5	23.5

Chloroform plant extracts exhibited low antioxidant activity and were found to be unstable (Table 4.4). Although there was a great decrease in antioxidant activity of the extracts, results from *A. tetracantha* indicated an 8.2% increase relative to the methanol extract at 1 mg/ml concentration.

Table 4.4. Percentage DPPH inhibition by *B. elliptica, P. auriculata, G. robusta,* and *A. tetracantha* chloroform extracts (4 day).

Concentration of extracts (mg/ml)	***B. elliptica* (%)**		***P. auriculata* (%)**		***G. robusta* (%)**		***A. tetracantha* (%)**	
	0 h	**72 h**	**0 h**	**72 h**	**0 h**	**72 h**	**0 h**	**72 h**
1	49.6	46.5	42.1	36.5	43.3	47.1	45.9	52.4
0.5	39.2	34.2	32.4	27.5	34.7	31.4	35.7	37.8
0.4	32.7	31.4	30.1	26.1	29.8	32.0	31.4	32.2
0.3	20.3	26.4	29.4	24.8	20.5	29.5	31.0	34.2
0.2	19.2	25.4	28.6	21.4	26.7	26.4	27.1	25.8
0.1	16.6	19.5	24.2	20.3	26.9	20.5	28.4	33.9
0.05	19.4	18.5	24.8	21.8	26.4	20.2	26.1	32.9
0.025	15.3	18.9	24.8	22.3	23.1	17.9	25.6	32.8

The ethyl acetate plant extracts showed moderate antioxidant free radical scavenging activity for *G. robusta* and *B. elliptica*, and very weak activity from *A. tetracantha* and *P. auriculata* (Table 4.5). The order of DPPH free radical scavenging activity increased in the order of *G. robusta* > *B. elliptica* > *A. tetracantha* > *P. auriculata*. *G. robusta* ethyl acetate and methanol extracts gave similar percentage DPPH inhibition. Stability studies carried out on antioxidant capacity of *B. elliptica, G. robusta* and *A. tetracantha* ethyl acetate extracts showed no appreciable decrease on the free radical scavenging activity of the extracts (Table 4.5).

Table 4.5. DPPH free radical scavenging activity assay of *B. elliptica, P. auriculata, G. robusta,* and *A. tetracantha* ethyl acetate extracts (4 days).

Concentration of extracts (mg/ml)	***B. elliptica*** **(%)**		***P. auriculata*** **(%)**		***G. robusta*** **(%)**		***A. tetracantha*** **(%)**	
	0 h	**7 2h**	**0 h**	**72 h**	**0 h**	**72 h**	**0 h**	**72 h**
1	64.2	61.2	46.5	40.8	67.4	67.2	49.3	52.7
0.5	42.8	42.6	43.9	29.3	47.2	43.1	35.9	34.2
0.4	36.9	39.0	33.9	28.4	41.2	39.4	32.4	32.7
0.3	32.7	37.1	32.0	26.6	34.8	36.7	28.6	31.8
0.2	28.1	33.1	29.4	24.3	26.3	30.1	26.3	27.7
0.1	26.9	26.8	26.0	20.8	22.0	24.9	22.6	22.0
0.05	22.9	23.8	34.9	20.7	22.2	22.7	23.4	20.2
0.025	22.2	21.9	25.6	21.2	17.4	24.5	18.2	24.2

The concentration of the antioxidants required to obtain 50% DPPH inhibition was calculated. Table 4.6 shows the IC_{50} values of all the solvent extracts. The lower the IC_{50} value the greater the free radical scavenging activity. The IC_{50} values of the extracts were found to be higher than that of ascorbic acid, but results obtained from *B. elliptica* methanol extracts showed that the plant extract has a very high antioxidant activity comparable to that of ascorbic acid.

Table 4.6. Percentage DPPH inhibition by the different solvent plant extracts and their IC_{50} values.

Solvent	**Plant species**	**IC_{50} (mg/ml ± SD)***	**%DPPH inhibition ****
	Ascorbic acid	0.019 ± 0.008	97.0
1. Methanol	*B. elliptica*	0.224 ± 0.01	95.2
	P. auriculata	0.400 ± 0.018	86.9
	G. robusta	0.592 ± 0.004	65.6
	A. tetracantha	1.750 ± 0.033	37.0
2. Chloroform	*B. elliptica*	0.954 ± 0.040	49.6
	P. auriculata	1.460 ± 0.022	42.1
	G. robusta	1.400 ± 0.027	43.3
	A. tetracantha	1.220 ± 0.019	45.9
3. Ethyl acetate	*B. elliptica*	0.681 ± 0.019	61.1
	P. auriculata	1.110 ± 0.052	46.5
	G. robusta	0.627 ± 0.022	67.0
	A. tetracantha	1.000 ± 0.019	49.3

Data are presented as the mean of triplicate analyses.

* The antioxidant scavenging activity was evaluated as the concentration of the test sample required to decrease the absorbance at 517 nm by 50% in comparison to the control response.

** The % DPPH inhibition values are from 1 mg/ml concentration solvent extracts.

4.8.2 TLC analysis

Methanol extracts of the four plant species were separated by TLC and each plant species gave a chromatographic pattern illustrative of the compounds in the extract. The free radical scavenging activity of all extracts screened with DPPH spray on a TLC plate showed zones of inhibition due to the compounds present in each extract. Fig. 4.3 shows a developed TLC plate spotted with the methanol extracts of four plants and ascorbic acid and sprayed with DPPH solution.

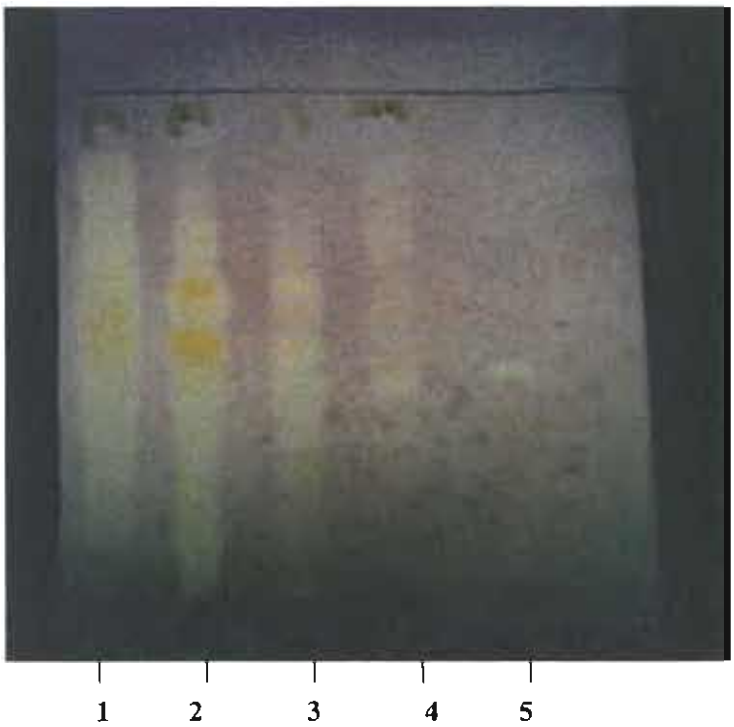


Fig. 4.3. Thin Layer Chromatoplate showing compounds separated from the methanol extracts and sprayed with DPPH (conditions: chloroform – acetic acid – methanol – water ; 60:32:12:8). Lane 1: *B. elliptica,* lane 2: *P. auriculata,* lane 3: *G. robusta,* lane 4: *A. tetracantha,* and lane 5: Ascorbic acid.

To obtain additional information on the reducing ability of the methanol extracts, the same extracts were developed on a TLC plate and sprayed with ferric chloride-TPTZ solution (Fig. 4.4). By using ferric chloride-TPTZ as a TLC spray reagent, three spots of *B. elliptica* extract appeared blue on the TLC plate, which shows their ability to reduce ferric ion. The *P. auriculata* extract showed distinct brown-blue and two brown spots. Similar results were observed with *G. robusta* extracts, where one blue and two brown spots were observed. No blue spots were observed from *A. tetracantha* extracts. Chlorogenic acid, which was used as a positive control, reduced ferric ion and a blue spot was observed.

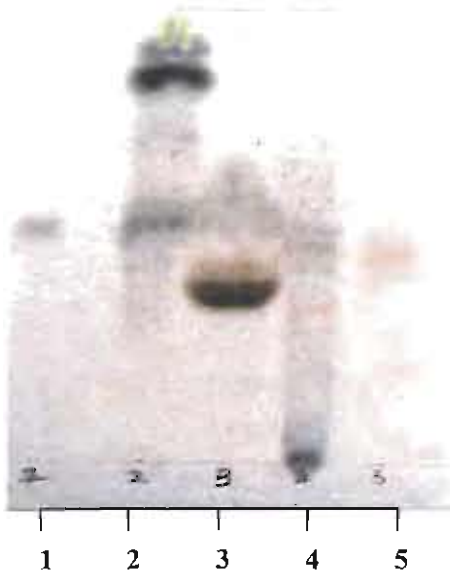


Fig. 4.4. Thin Layer Chromatoplate showing compounds separated from methanol extracts and sprayed with TPTZ solution (conditions: ethyl acetate – formic acid – acetic acid – water ; 100:11:11:26). Lane 1: chlorogenic acid, lane 2: *B. elliptica*, lane 3: *P. auriculata*, lane 4: *G. robusta* and lane 5: *A. tetracantha*.

TLC was used to separate and identify phenolic acids and flavonoids in the methanol extracts of *B. elliptica, P. auriculata, G. robusta* and *A. tetracantha.* The R_f values and the colours, visualized under UV light (366 nm), spraying with the NP/PEG spray reagent were used to identify the class of compounds in each extract by comparing with details given by Wagner and Bladt (1996). Also the colour and R_f value of chlorogenic acid is similar to that reported by Wagner and Bladt (1996). Fig. 4.5 shows phenolic compounds present as blue or white fluorescent spots and flavonoid glycosides as coloured (yellow, orange, green or brown) in the methanol extracts of *B. elliptica, P. auriculata, G. robusta,* and *A. tetracantha.*

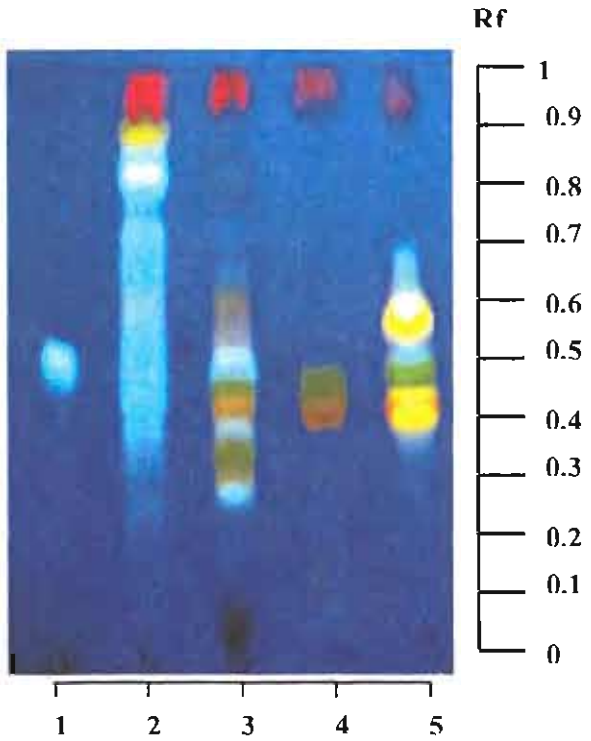


Fig. 4.5. Thin Layer Chromatoplate showing compounds separated from a methanol extract and sprayed with NP/PEG spray and visualized under UV (366 nm). Conditions: chloroform – acetic acid – methanol – water (60: 12: 24: 8). Lane 1: chlorogenic acid, lane 2: *B. elliptica,* lane 3: *G. robusta,* lane 4: *A. tetracantha* and lane 5: *P. auriculata.*

4.8.3 Cyclic voltammetry

The cyclic voltammogram (CV) of ascorbic acid showed a broad anodic wave at 181 mV (Fig. 4.6 A).

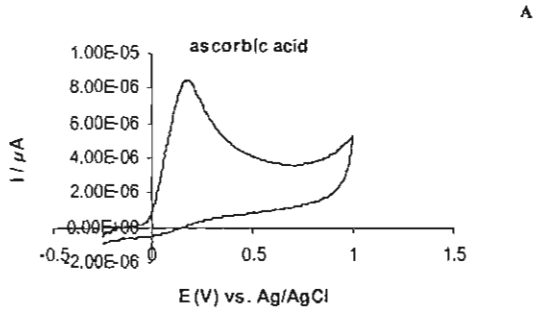


Fig. 4.6. Cyclic voltammogram of ascorbic acid (1 mM) in 0.2 M Tris-HCl buffer (pH 7). Scan rate at 100 mV/sec.

B. elliptica methanol extract exhibited both a reduction and oxidation potential. The CV for *B. elliptica* showed a peak potential at the anodic and cathodic waves at 301 mV and -106 mV vs. Ag/AgCl, respectively as shown in Fig. 4.7 B1. The CV of *B. elliptica* ethyl acetate showed a weak anodic and cathodic peak potential at 319 mV and -182 mV, respectively (Fig. 4.7 B2). The CV of *B. elliptica* chloroform extract showed a weak anodic and cathodic peak potential at 322 mV and -158 mV vs. Ag/AgCl, respectively (Fig. 4.7 B3).

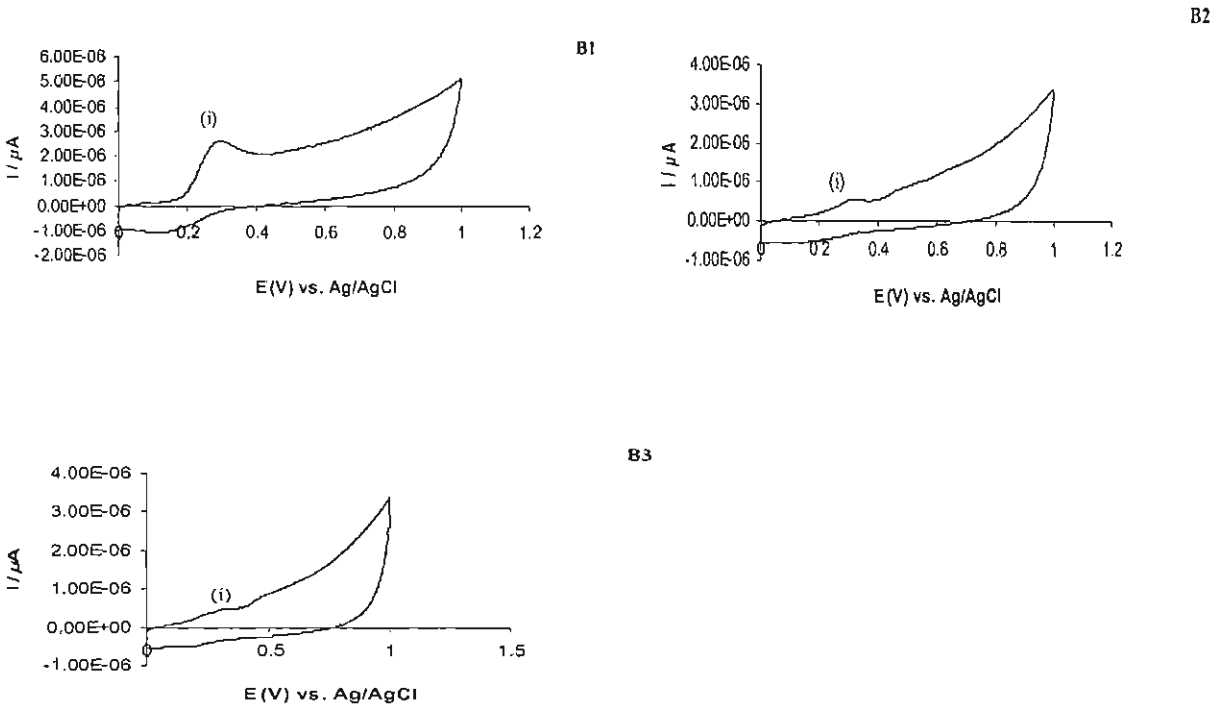


Fig. 4.7. Cyclic voltammograms of *B. elliptica* solvent extracts (107.4 μg/ml) in 0.2 M Tris-HCl buffer (pH 7). Scan rate at 100 mV/sec. B1 (methanol extract), B2 (ethyl acetate extract) and B3 (chloroform extract).

The CV of *P. auriculata* methanol extract showed two anodic waves at 264 mV and 850 mV (Fig. 4.8 C1). No peak potentials vs. Ag/AgCl were observed for both ethyl acetate and chloroform extracts of *P. auriculata*.

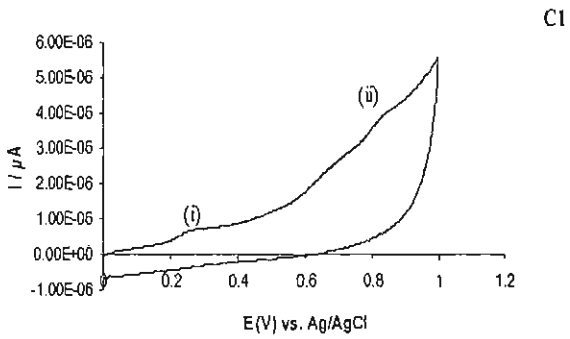


Fig. 4.8. Cyclic voltammogram of *P. auriculata* methanol extract (107.4 μg/ml) in 0.2 M Tris-HCl buffer (pH 7). Scan rate at 100 mV/sec.

The CV of *G. robusta* methanol extract showed a weak anodic wave at 275 mV (Fig. 4.9 D1). No peak potentials vs. Ag/AgCl were observed on both the ethyl acetate and chloroform extracts of *G. robusta.*

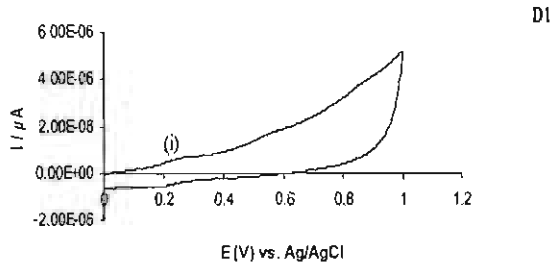


Fig. 4.9. Cyclic voltammogram of *G. robusta* methanol extract (107.4 µg/ml) in 0.2 M Tris-HCl buffer (pH 7). Scan rate at 100 mV/sec.

No peak potentials vs. Ag/AgCl were observed in all *A. tetracantha* extracts in 0.2 M Tris-HCl buffer (pH 7). CV of increasing concentration of the extracts, except for *A. tetracantha*, and ascorbic acid showed that the anodic peaks increased with an increase in the volume of the samples added in the analyte, with slight potential shifts towards the less positive potentials, except for peak (ii) of *P. auriculata* which shifted towards the positive potentials. The cathodic peak of *B. elliptica* broadened with an increase in the volume of the extract added to the analyte.

4.8.4 Phenol content

Gallic acid was used to plot the standard curve for the Folin Ciocalteau assay. Appendix I shows the gallic acid standard curve. The total phenol content of the plant extracts was determined using the Folin Ciocalteau assay and results expressed as mg/L gallic acid equivalence (GAE) as shown in Table 4.7. The phenolic contents of methanol extracts varied from 38.7 to 255.9 mg/L GAE, with *B. elliptica* having the highest phenolic content followed by *P. auriculata* and *G. robusta* having a relatively high phenolic content, with *A. tetracantha* showing the lowest phenolic content.

The phenolic content of ethyl acetate extracts ranged from 20.1 to 51.6 mg/L GAE. The order of phenolic content in the decreasing order was *B. elliptica* > *G. robusta* > *P. auriculata* > *A. tetracantha*. In the chloroform extracts, *P. auriculata* had the highest phenolic content followed by *B. elliptica*, with *G. robusta* giving relatively high phenolic content and *A. tetracantha* showed the lowest phenolic content. The phenolic content for the chloroform extracts varied from 17.3 to 41.6 mg/L GAE.

Table 4.7. Phenolic content in *B. elliptica*, *P. auriculata*, *G. robusta*, and *A. tetracantha* organic solvent extracts.

Solvent	Plant species	GAE/L* ± SD
1. Methanol	*B. elliptica*	255.9 ± 0.008
	P. auriculata	185.9 ± 0.004
	G. robusta	87.30 ± 0.059
	A. tetracantha	38.70 ± 0.003
2. Ethyl acetate	*B. elliptica*	51.60 ± 0.005
	P. auriculata	37.30 ± 0.001
	G. robusta	41.60 ± 0.034
	A. tetracantha	20.10 ± 0.0
3. Chloroform	*B. elliptica*	35.90 ± 0.001
	P. auriculata	41.60 ± 0.0
	G. robusta	25.90 ± 0.002
	A. tetracantha	17.30 ± 0.001

Data are the mean of triplicate analyses.

* Gallic acid equivalence per litre (GAE/L)

The best correlation coefficient (R^2) between the antioxidant activity and the total phenol content was found in methanol extracts (Fig. 4.10), whereas chloroform and ethyl acetate extracts showed very poor correlation between antioxidant activity and phenol content (Fig. 4.11 and Fig. 4.12).

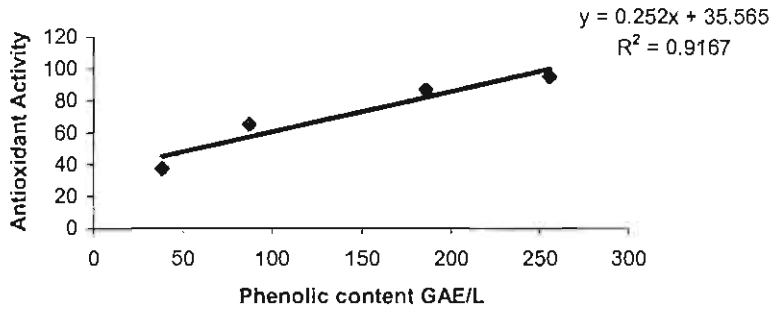


Fig. 4.10. Correlation of the antioxidant activities determined by the DPPH free radical assay and the phenolic content of the methanol extracts.

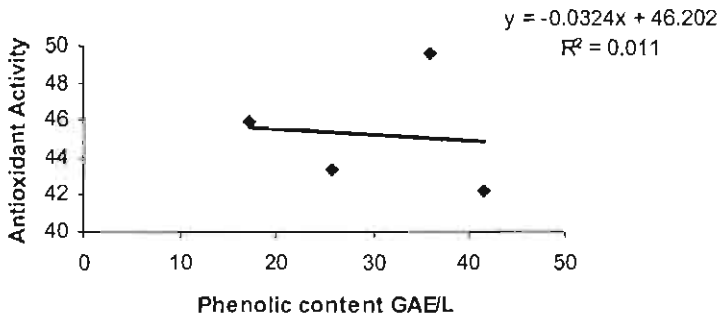


Fig. 4.11. Correlation of the antioxidant activities determined by the DPPH free radical assay and the phenolic content of the chloroform extracts.

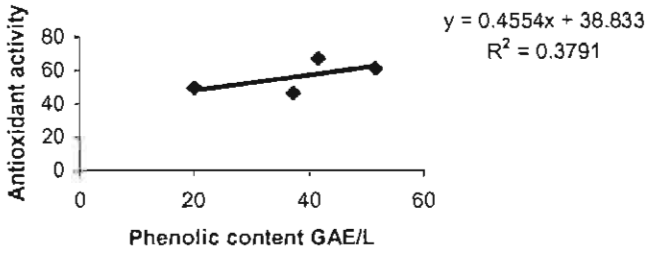


Fig. 4.12. Correlation of the antioxidant activities determined by the DPPH free radical assay and the phenolic content of the ethyl acetate extracts.

4.9 Discussion

4.9.1 DPPH assay

Antioxidant activity of the plant solvent extracts was evaluated using the DPPH free radical scavenging activity assay. DPPH is a stable free radical, which has been widely accepted as a method of estimating free radical scavenging activity of antioxidants (Chang *et al.*, 2001; Chen *et al.*, 2006). Antioxidant activity studies on the three different plant solvent extracts indicated that the extracts have scavenging activity against the DPPH free radical at all concentrations assayed. However, the free radical scavenging activity of each extract differs depending on the particular solvent used for extraction, reflecting the extent that different solvents will extract different groups of compounds to a different degree.

Preliminary studies to determine the optimum extraction period using *P. auriculata* methanol extracts showed slight variations in antioxidant capacity of the extracts and established that total antioxidants can be extracted from leaf material in four days. This is illustrated by no appreciable increase in total antioxidant activity against DPPH being observed in leaf material extracted for longer than 4 days (Table 4.2). In some studies shorter extraction periods have been used. Karawita *et al.* (2005) extracted total antioxidant for 24 h, but the leaf samples were first freeze-dried and ground into fine powder before extraction. Unlike the work we carried out, these studies employ solvent-solvent extraction (Karawita *et al.*, 2005; Chen *et al.*, 2006).

Antioxidant stability studies carried out on the plant extracts by investigating inter-day variability studies, indicated that both methanol and ethyl acetate extracts were stable, whereas chloroform plant extracts were unstable. The good stability of methanol extracts has been reported by Yamakazi *et al.* (2005). Both ethyl acetate and chloroform extracts showed a lower percentage DPPH inhibition compared to methanol plant extracts, with the exception of *G. robusta* and *A. tetracantha*, in which the ethyl acetate extract gave slightly higher values. The order of free radical scavenging activity from the organic solvents increased in the order of methanol > ethyl acetate > chloroform. The IC_{50} values of the chloroform extracts were very high (Table 4.6), suggesting that low amount of compounds with antioxidant capacity was extracted from the leaf material.

The results obtained in this study are not in agreement with those of Chen *et al.* (2006) and Karawita *et al.* (2005) both of whom employed solvent-solvent extraction of antioxidants using different plant species from those of this study. Their results showed that the free radical scavenging activity of the extracts increased in the order ethyl acetate > chloroform > methanol. Studies by Karawita *et al.* (2005) have shown that potential antioxidant activities increase with the hydrophilicity of extracts of the plant species used. The overall antioxidant scavenging activity assay results indicated the superiority of the more polar extracts to the less polar extracts investigated. Methanol was a more suitable organic solvent for antioxidant extraction, although this does not apply to all plant species. The results in this study demonstrate that most of the free-radical scavenging compounds were extracted by methanol, and moderately by ethyl acetate. A high percentage inhibition of DPPH free radical scavenging ability, in a concentration dependent assay, was found using *B. elliptica* methanol extract (Table 4.3). Although the antioxidant free radical scavenging activity of *B. elliptica* was comparable to that of ascorbic acid at 1 mg/ml concentration, its IC_{50} value was higher than that of ascorbic acid (Table 4.6). The DPPH free radical scavenging activity of the methanol extracts increased in the order *B. elliptica* > *P. auriculata* > *G. robusta* > *A. tetracantha.*

4.9.2 TLC analysis

The DPPH free radical scavenging assay performed directly on the TLC plate was more informative than the spectrophotometric DPPH method, as it showed the contribution to antioxidant activity of different compounds separately (Fig. 4.3). Although the TLC plates sprayed with DPPH give an insight into the contribution of the antioxidant ability of the compounds present in the extract, they do not show the types of compounds present in the methanol plant extracts, whereas TLC plates sprayed with NP/PEG indicate that the extracts contain phenolic acids and flavonoid glycosides, which are shown by their characteristic colour (Fig. 4.5).

The calculated R_f value of the control chlorogenic acid and the R_f values from Wagner and Bladt (1996), using the same solvent system as in this study, indicates that *B. elliptica* methanol extracts may contain chlorogenic acid ($R_f = 0.5$) and other phenolic acids such as isochlorogenic acid ($R_f = 0.6$). Chlorogenic acid is an ester of caffeic acid and quinic acid, and is one of the major phenolic compounds found in plants and its synthesis is known to be stimulated by environmental stress (Kono *et al.*, 1998). The antioxidant activity of *B. elliptica* methanol extract is related to its phenolic acids and the flavonoid ($R_f = 0.9$), which maybe present in this plant extract (Fig. 4.5). The flavonoid contributes to the antioxidant capacity of the extract, as it gives a zone of inhibition against the DPPH free radical (Fig. 4.3).

B. elliptica methanol extracts contain the highest phenolic acids content compared to the other methanol plant extracts investigated in this study. In the diet of animals, phenolic acids such as chlorogenic acid are reported to decrease low-density lipid (LDL) oxidation, remove ROS by scavenging alkylperoxyl radicals and prevent degenerative age-related diseases (Niggeweg *et al.*, 2004). Of the plant species identified from the black rhinoceros dung using molecular methods to determine its plant composition, *B. elliptica* contributed 20 % in the preliminary study and 23 % in the winter seasonal study (section 3.6). *B. ilicifolia*, which is from the same family and genus with *B. elliptica*, has been reported by Brown *et al.* (2003) to form an important part in the diet the black rhinoceros. Members of this family are known to have potent antioxidants such as caffeic acid and quercitin (Viturro *et al.*, 1999).

P. auriculata methanol extracts, which exhibited DPPH free radical scavenging activity, has different phenolic profile compared to that of *B. elliptica* methanol extract (Fig. 4.5). The R_f values of the compounds indicated that *P. auriculata* methanol extract may contain flavonoid glycosides such as rutin (quercetin-3-rutinoside) (R_f = 0.43), other flavonoids (R_f = 0.4) and phenolic acids such as chlorogenic acid (R_f = 0.5) (Fig. 4.5). These compounds are responsible for the antioxidant activity of *P. auriculata* methanol extracts as shown by the clear zones of inhibition on the TLC plate sprayed by DPPH (Fig. 4.3). The flavonol rutin is reported to be one of the major antioxidants involved in prevention of LDL oxidation (Katsube *et al.*, 2006). The genus *Plumbago* is marked by the presence of flavonoids and terpenoids (de Paiva *et al.*, 2004). Studies by van Lieverloo and Schuiling (2004) at the GFRR identified *P. auriculata* as one of the preferred plant species of the black rhinoceros.

The methanol extracts of *G. robusta* exhibited moderate antioxidant free radical scavenging activity. The scavenging activity of this extract maybe related to its flavonols such as hyperoside (quercetin-3-O-galactoside) (R_f = 0.56), quercetine-rutinoside (R_f = 0.41) and chlorogenic acid (R_f = 0.5) and traces of other phenolic acids (Fig. 4.5). Although *G. robusta* methanol extract has more flavonoid compounds compared to *B. elliptica* and *P. auriculata*, its DPPH free radical scavenging ability was found to be lower when the spectrophotometric DPPH assay was employed. *G. robusta* dominates the diet of the black rhinoceros (Brown *et al.*, 2003). Another plant that is preferred by the black rhinoceros is *A. tetracantha.* Methanol extracts of this plant exhibited the lowest antioxidant free radical scavenging activity. The extracts of this plant contains few phenolic compounds, one of which maybe identified as quercetin-rutinoside (R_f = 0.4). Studies by Bennet *et al.* (2004) showed that the leaves of this plant contain a lower concentration of phenolic compounds, whereas its roots and seeds may have a complex mixture of 26 flavonoids.

The results indicate that the scavenging activity of various plant extracts is due to the presence of different types of phenolic compounds. The concentration of phenolic compounds in plants can vary during the year and depends on the type of extraction method used, which may influence quantitatively and qualitatively in the extraction composition (de Paiva *et al.* 2004). A study by Ndondo (2003) on vitamin E of these plant species, with the exception of *B. elliptica*,

obtained from the GFRR showed an increased vitamin E content in the order of *A. tetracantha* > *P. auriculata* > *G. robusta*. Non-polar compounds such as vitamin E, which have good antioxidant capacity, will not be efficiently extracted using a polar solvent such as methanol. Therefore, this study cannot suggest that the feeding habits of the black rhinoceros is influenced by high levels of antioxidants in its diet as there are other compounds, such as N, P, Na, Ca, K and Mg, that are important in the diet of animals. Ras (1993) reported no correlation between the phenolic compound profiles and the palatability of *P. afra* by animals.

The FRAP assay was modified and used for the first time in this study as a spray for TLC. The FRAP assay is used spectrophotometrically to measure the change in absorbance at 593 nm as a result of the formation of the blue coloured Fe^{2+}-TPTZ compound from colourless oxidized Fe^{3+}-TPTZ form by the action of electron donating antioxidants (Katalinic *et al.*, 2006). The results obtained from TLC plates sprayed with Fe^{3+}-TPTZ spray were found to be different from those observed when the TLC plate was sprayed with DPPH (Fig. 4.4 and Fig. 4.3, respectively). Some compounds in the extracts on the TLC plate sprayed with Fe^{3+}-TPTZ were able to reduce ferric iron as observed in Fig. 4.4, with most of the compounds not reacting with ferric ion. Because FRAP measures only the reducing capability based upon ferric ion, which is not relevant to antioxidant activity (Prior *et al.*, 2005), it does not measure other antioxidants that were observed to reduce DPPH. Compounds such as quercetin, chlorogenic acid and caffeic acid have been reported to form complexes with ferric iron (de Maria *et al.*, 2000; Kono *et al.*, 1998).

Unlike quinic acid and *p*-coumaric acid, these compounds will reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. The antioxidant effectiveness of these compounds is mainly attributed to the presence of a 3',4'-dihydroxy (ortho-dihydroxy) in the aromatic ring (de Maria *et al.*, 2000). Because phenolic compounds possessing multiple hydroxyl groups as substituents in the benzene ring are generally the most efficient antioxidants and metal chelators (de Maria *et al.*, 2000), they will reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. The antioxidant activity of a compound in the FRAP assay depends mainly on the electron transfer from the compound to Fe^{3+}, which is determined by the redox potential of the involved compound (Firuzi *et al.*, 2005).

The blue coloured spots on the TLC plate sprayed with FRAP indicate the reduction of Fe^{3+} to Fe^{2+} by some of the compounds present in the methanol plant extracts. For some compounds that exhibited clear zones of DPPH inhibition, showed brown spots whereas some did not reduce the ferric ion.

Spectrophotometric assays, which use a single wavelength, will cause inconsistencies between various plant extracts as they contain different compounds some of which will not reduce a ferric ion, but have free radical scavenging ability. The FRAP spray was able to show compound(s) from *G. robusta* that did not give a bright colour when sprayed with NP/PEG at the origin of the TLC plate. These compounds from *G. robusta* also showed a clear zone of inhibition when sprayed with DPPH. Although the FRAP spray exhibit no reduction ability for some of the compounds present in the extracts, it does show reduction capacity (showed by blue colour) for some of the compounds that are not detected using NP/PEG spray, such as the compounds in *G. robusta* (R_f = 0) (Fig. 4.4).

4.9.3 Cyclic voltammetric analysis

The electron potential of all the solvent plant extracts was evaluated using cyclic voltammetry. Cyclic voltammetry has been found to be an efficient method for evaluating the total antioxidant capacity of antioxidants in plant extracts (Chevion *et al.*, 2000). The electron potential of ascorbic acid, which was used as a positive control in this study, has been evaluated by Chevion *et al.*, (1997). Ascorbic acid exhibits strong reduction at low anodic potential vs. Ag/AgCl.

Only extracts of *B. elliptica* showed good antioxidant activity. The compounds present in these extracts exhibited both reduction and oxidation potential vs Ag/AgCl. The oxidation and reduction potential of the extracts increased from methanol > ethyl acetate > chloroform. The extracts exhibited broad peaks for both the anodic and cathodic scans (Fig 4.7). Because the peaks are relatively broad, each is likely to originate from a group of compounds with similar redox potentials rather than from a single antioxidant (Neill *et al.*, 2002).

Although results in this study have shown that *B. elliptica* methanol extract constitute largely phenolic acids (Fig. 4.5), the electrochemical behaviour of the compounds in the extract depends not only on the amount and specific compounds present in the extract but rather on the their structural features such as the number and positions of phenolic hydroxyl or methyl groups (Cosio *et al.,* 2006). *B. elliptica* methanol extract most likely contained a flavonoid glycoside that exhibited antioxidant activity against DPPH free radical and caused ferric ion reduction. This compound could be involved in the oxidation/reduction potential of the extract. The extracts showed a correlation between the electron potential and the antioxidant activity against DPPH free radical. Good correlations have been reported between redox potentials and antioxidant properties of plant extracts (Firuzi *et al.,* 2005; Cosio *et al.,* 2006).

The methanol extract of *P. auriculata*, which exhibited ferric ion reduction and strong scavenging activity against DPPH free radical, showed weak potential anodic wave scan vs. Ag/AgCl. Although the wave scans were weak, the position of the peak (i) in the voltammogram was found at a low potential (Fig. 4.8). The position of the peaks in the voltammograms indicate the antioxidant ability of the compounds present in the extracts, with peaks at the lower potentials signifying the more powerful reducing agents (Neill *et al.,* 2002). The methanol extract of *P. auriculata* contained flavonoids and traces of phenolic acids. The flavonol quercetin, which was identified from this extract using the R_f values of Wagner and Bladt (1996), has been found to exhibit oxidation peak scans at low potential due to its –OH groups present on the B ring (Cosio *et al.,* 2006). The unidentified flavonoids and phenolic acids could be involved in the oxidation potential of the *P. auriculata* methanol extract. The results obtained in the cyclic voltammetric analyses of both ethyl acetate and chloroform extracts of *P. auriculata* correlate with those of DPPH scavenging ability and phenolic content of the extracts, as no peak potentials vs. Ag/AgCl were observed.

G. robusta methanol extract, which showed moderate antioxidant scavenging activity against DPPH free radical and ferric ion reduction for some of the compounds present in the extract, exhibited very weak anodic potential wave scan vs. Ag/AgCl (Fig. 4.9). Methanol extracts showed better reducing power than both ethyl acetate and chloroform extracts of *G. robusta,* as there were

no peak potential scans vs. Ag/AgCl for these solvent extracts. All *A. tetracantha* solvent extracts did not show any potential vs. Ag/AgCl, confirming the weak antioxidant activity of this plant leaf extracts. The results obtained on the cyclic voltammetric analysis of all the solvent extracts of *G. robusta* and *A. tetracantha* correlate with those of DPPH scavenging ability and phenolic content of the extracts.

4.9.4 Phenol content

Because antioxidant activity is attributed to phenolic compounds, the total phenolic content of the extracts was assayed using the Folin-Ciocalteau reagent method. Folin-Ciocalteau is an accepted method for the determination of phenols. This method is based on the reduction of tungstate and/or molybdate in the Folin-Ciocalteau by oxidizable compounds in an alkaline medium resulting in a blue coloured product which is measured spectrophotometrically at 765 nm (Singh *et al.*, 2003).

The variation of the total phenolic content was significant between the plant solvent extracts (Table 4.7). On comparing the efficiency of extraction with methanol, chloroform, and ethyl acetate, a trend similar to the antioxidant activity was found. The methanol extracts showed higher phenolic content than those of ethyl acetate and chloroform. The methanol extracts ($R^2 = 0.9167$) showed a good correlation between the antioxidant activity and the total phenolic content (Fig. 4.10), which is supported by the results of Wong *et al.* (2006). The linear relationship that exists between the antioxidant activity and phenolic content of methanol extracts indicates that phenolic compounds are major contributors to antioxidant activity in plants (Wong *et al.*, 2006). The methanol extracts investigated contain groups of phenolic compounds that need to be identified and characterized. Ethyl acetate ($R^2 = 0.3781$) and chloroform ($R^2 = 0.001$) extracts showed no correlation between antioxidant activity and total phenolic content (Fig. 4.11 and Figure 4.12). This suggests that these two solvents were unable to extract polar phenols that will exhibit good antioxidant activity.

CHAPTER FIVE
Conclusion and future work

This thesis investigated *rbc*L gene sequence variation between selected plant species from the GFRR, and sought to determine the botanical composition of the black rhinoceros dung during different seasons. The *rbc*L gene was readily amplified from all plants, except for *B. elliptica* and *E. rigida,* which required BSA to improve recovery of the gene. Although the *rbc*L gene is conserved, alignments of the investigated plants indicate that the nucleotide composition of the gene between closely related plant species, in particular those of the families Euphorbiaceae and Fabaceae, is variable. The molecular method employed in this study was able to distinguish between different plant species using the available bioinformatics tools. The gene was found to be highly conserved for the plants *R. obovatum, R. lucida* and *L. cinereum*, which are from different families, yet showed enough nucleotide differences to distinguish between each plant species. Other plants sequences that showed low sequence variations are *G. occidentalis* and *G. robusta,* which have only one mismatch. Although they are highly conserved, the single mismatch is enough to show variation if it constantly occurs at the same position in the sequence. These highly conserved *rbc*L gene plant sequences emphasize the need to sequence the complete *rbc*L gene, as most nucleotide variations are observed to occur towards the 3' end of the gene (Clegg, 1993; Calie and Manhart, 1994).

Of the 18 studied plants, only five were found to have been deposited in the GenBank database and they are *A. tetracantha, P. afra, C. bispinosa, G. occidentalis* and *P. auriculata.* The other thirteen sequences obtained have not been reported previously. Based on the results obtained in this study, a conclusion can be drawn that the size of the sequenced *rbc*L gene fragment is sufficient to confer differences between plant species using the designed primers. This is further shown by the wide variety of plant *rbc*L gene sequences generated from dung samples. The molecular method used in this study was able to reveal the botanical composition of the black rhinoceros dung. However, further studies are required to sequence more clones from dung so as to identify more of the plants browsed by this megaherbivore. This will also require expanding the GFRR database by

sequencing more plant species from the GFRR. The ITS (Internal Transcribed Spacer) and the spacer region between the *rbc*L gene and the *AtpB* could also be sequenced and compared between plant species from the GFRR and those generated from dung, as these two genes are reported to be less conserved than the *rbc*L gene, and have been used to distinguish between plants down to species level (Chiang *et al.*, 1998; Soltis *et al.*, 1998). This may allow for distinguishing between *G. occidentalis* and *G. robusta*, as the *rbc*L gene is highly conserved between these two plant species. A future study should perform a phylogenetic analysis, for example, parsimony, using a more advanced programme such as PAUP (Phylogenetic Analysis Using Parsimony) to generate a phylogenetic tree that illustrates the relationships of the plants with much greater resolution. Although, the method used in this study has the potential to identify plants down to species level, it should not be used to substitute traditional methods such as microhistology, but rather complement those methods to obtain better knowledge of the botanical composition of dung.

This study also investigated the antioxidant capacity and phenolic content of some of the plants browsed by the black rhinoceros. Methanol extracts of the investigated plants, particularly *B. elliptica* and *P. auriculata*, were found to exhibit potent antioxidant activity and high phenolic content compared to chloroform and ethyl acetate plant extracts. Methanol extracts showed a good correlation between antioxidant capacity, phenolic content and electron reducing power. Cyclic voltammetric analyses show that *B. elliptica* extracts exhibit both oxidation and reduction potential vs Ag/AgCl. The oxidation and reduction potential of the extracts increased from methanol > ethyl acetate > chloroform. Methanol extracts of *P. auriculata* and *G. robusta* exhibited weak reduction potential vs Ag/AgCl, with other solvent extracts showing no peak potentials similarly to those of *A tetracantha*.

TLC studies showed that the extracts consist of a wide variety of both phenolic acids and flavonoid glycosides. The TLC investigations on the compounds exhibited different results based on the method used. Further studies are required to identify the phenolic acids and flavonoid glycosides in the methanol extracts of *B. elliptica* and *P. auriculata.*

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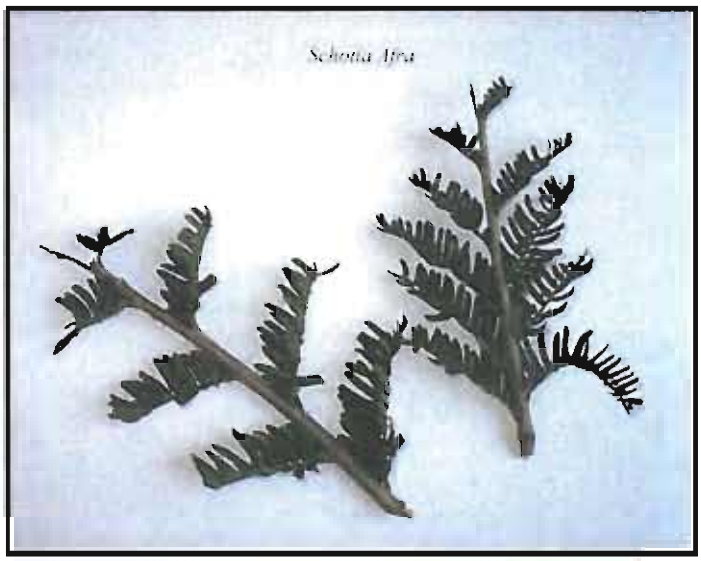
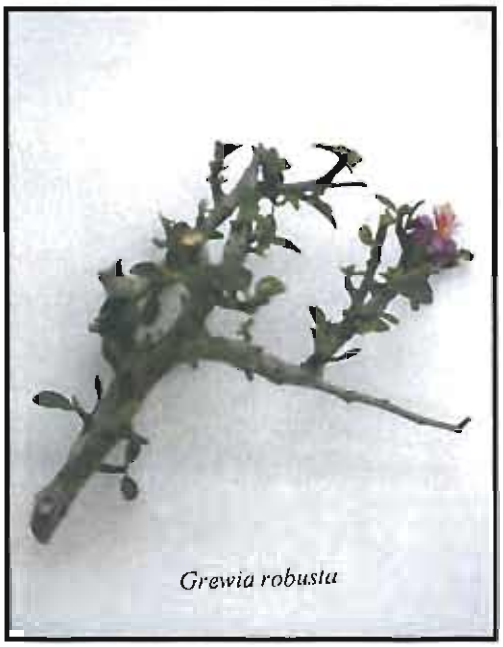
APPENDIXES

Appendix A: Plant sample photographs



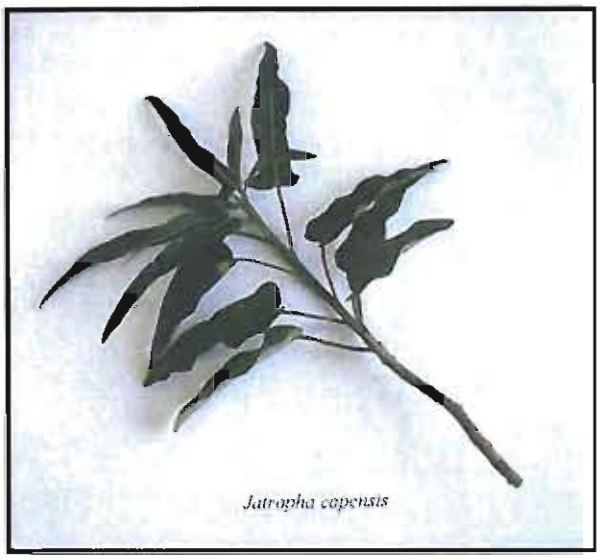



Carissa bispinosa

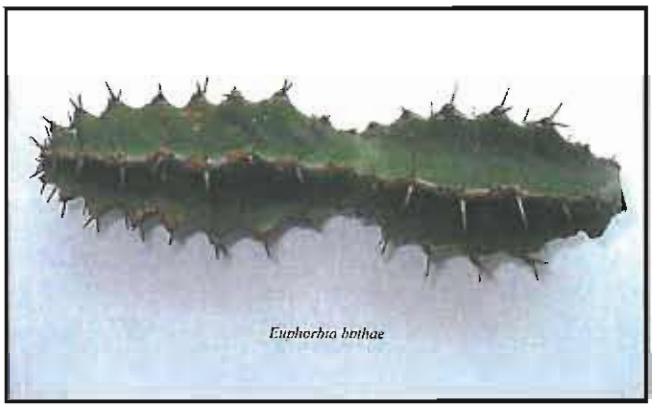

Rhus luciola


Ehretia rigida


Putterlickia pyracantha

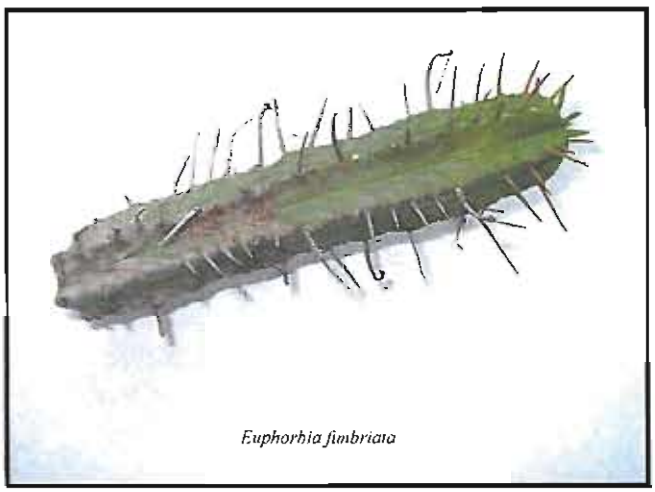


Jatropha capensis



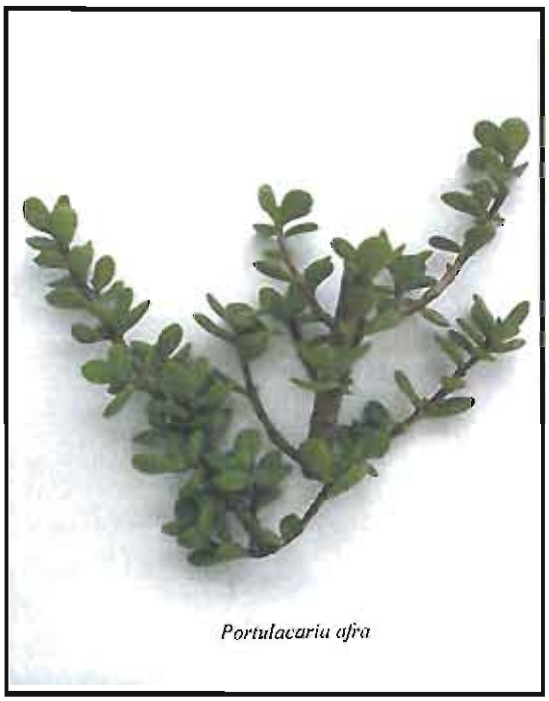
Euphorbia bothae

Azima tetracantha



Euphorbia fimbriata


Carissa bispinosa


Portulacaria afra


Euclea undulata

Appendix B: Preparation of TAE buffer

A stock solution (50 x) was prepared by dissolving 242 g of tris base in 57.1 ml glacial acetic acid and 100 ml EDTA (0.5 M), pH 8. The running buffer (0.5 x) was prepared by diluting 10 ml of the 50 x TAE buffer with triple distilled water to a final volume of 1 l.

Appendix C: PCR reagents and volumes

Reagents	**Volume (μl)**	**Final concentration**
$MgCl_2$	2	1.4 mM
Thermophilic buffer*	3.5	0.7 X
dNTP mix	0.7	200 μM
Forward primer	3.5	0.15 μM
Reverse primer	3.5	0.15 μM
Taq DNA polymerase	0.25	1.25u/35 μl
Template DNA	2	17.14 ng
Nuclease free water	19.55	

*Thermophilic DNA polymerase 10 X reaction buffer, $MgCl_2$-free

Appendix D: Preparation of JM109 *E. coli* competent cells

A test tube containing 5 ml of LB was inoculated with JM109 *E. coli* strain and left overnight to grow at 37°C, with shaking at 200 rpm. Four Erlenmeyer flasks (250 ml), containing 100 ml of LB each, were inoculated with 1.5, 1.0, 0.7 and 0.3 ml of the overnight cultures respectively and incubated at 37°C for approximately 2 h until they reach an OD_{600} absorbance of 0.8. The flasks were then cooled 5 to 10 min on ice and centrifuged in a Beckman centrifuge (JA14 rotor) 5000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 50 ml RF1 (100 mM KCl, 50 mM, $MgCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15% glycerol, pH 5.8) followed by a further 20 min incubation on ice. The cells were again pelleted by centrifugation as above and the supernantant discarded. The pellets were pooled together by resuspending in afinal volume of 4 ml of RF2 (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15% glycerol, pH 6.8). The competent cells were aliquoted into 200 μl volumes and stored at -70°C until required.

Appendix E: Transformation of JM109 *E. coli* competent cells

JM109 cells (50 μl) were thawed on ice and mixed with the 2 μl of ligation reaction mixture in a sterile Eppeadorf tube and incubated on ice, for 20 min. The cells were then subjected to heat shock by inoculating at 42°C for 45-50 sec and immediately placed on ice for 2 min. Room temperature SOC* (950 μl) then added and the cells incubated at 37°C for 1.5 h, after which the cells were spread plated onto the LB plates with ampicillin/ IPTG/ X-Gal.

*SOC medium (2.0 g Bacto-tryptone, 0.5 Bacto-yeast, 1 ml of 1 M NaCl, 0.25 ml of 1 M KCl, 1 ml of 2 M $MgCl_2$ and 1 ml of 2 M glucose, all solutions sterilized) made up in 100 ml and autoclaved.

Appendix F: Preparation of plates and broth

LB plates were prepared by dissolving 10 g Bacto-tryptone, 5 g Bacto-yeast, 15 g agar and 5 g NaCl in 1 litre of Milli Q water, followed by autoclaving. The medium was allowed to cool to 50°C and ampicillin, IPTG, X-Gal added to a final concentration of 100 μg/ml, 0.5 mM and 80 μg/ml, respectively.

Appendix G: Sequence alignments of all plant sequences based on the *rbc*L gene

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A. karroo               CTCTCCAACGCATAAATGGTTGGGAATTCACATTTTCATCATCTTTGGTAAAATCAAGTC 60
S. afra                 CTCTCCAACGCATAAATGGTTGGGAATTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
E. bothae               CTCTCCAACGCATAAATGGTTGAGAGTTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
E. fimbriata            CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTCCCATCATCTTTGGTAAAATCAAGTC 60
J. capensis             CTCTCCAACGCATAAATGGTTGGGAATTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
P. pyracantha           CTCTCCAACGCATAAATGGTTGGGAGTTTACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
G. occidentalis         CTCTCCAACGCATAAATGGTTGGGAGTTCACATTCTCATCATCTTTGGTAAAATCAAGTC 60
G. robusta              CTCTCCAACGCATAAATGGTTGGGAGTTCACATTCTCATCATCTTTGGTAAAATCAAGTC 60
A. tetracantha          CTCTCCAACGCATAAATGGTTGGGAGTTCACATTCTCATCATCTTTGGTAAAATCAAGTC 60
E. undulata             CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
C. bispinosa            CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTTTCATCATCTTTGGTAAAATCAAGTC 60
E. rigida               CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTTTCATCATCTTTGGTAAAATCAAGTC 60
L. cinereum             CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
R. obovatum             CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
R. lucida               CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTCTCATCATCTTTGGTAAAATCAAGTC 60
B. elliptica            CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTTTCATCATCTTGGGTAAAATCAAGTC 60
P. afra                 CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTTTCATCATCTTTGGTATAATCAAGTC 60
P. auriculata           CTCTCCAACGCATAAATGGTTGGGAGTTCACGTTTTCATCATCTTTGGTAAAATCAAGTC 60
                        ************************ ** ** ** **  ********* **** *********

A. karroo               CACCACGAAGACATTCATAAACCGCTCTACCGTAATTCTTAGCGGATAATCCCAATTTTG 120
S. afra                 TACCGCGGAGAACTTCATAAACCGCTCTACCGTAATTCTTAGCGGATAACCCCAATTTAG 120
E. bothae               CCCCGCGAAGACATTCATAAACCGCTCTACCATAATTCTTAGCGGATAGCCCCAATTTTG 120
E. fimbriata            CATCGCGAAGACATTCATAAACCGCTCTACCATAATTCTTAGTGGATAGCCCCAATTTTG 120
J. capensis             CACCGCGAAGACATTCATAAACCGCTCTACCATAATTCTTAGCGGATAGCCCCAATTTAG 120
P. pyracantha           CACCGCGGAGACATTCATAAACTGCTCTACCATAATTCTTAGCGGATAATCCCAATTTAG 120
G. occidentalis         CACCACGTAGACATTCATAAACTGCTCTACCGTAGTTCTTAGCGGATAACCCCAATTTAG 120
G. robusta              CACCACGTAGACATTCATAAACTGCTCTACCGTAGTTCTTAGCGGATAACCCCAATTTAG 120
A. tetracantha          CACCGCGTAGACATTCGTAAACCGCTCTACCGTAGTTCTTCGCGGATAACCCCAATTTAG 120
E. undulata             CACCGCGGAGACATTCATAAACTGCTCTACCGTGGTTTTTAGCAGATAACCCCAATTTCG 120
C. bispinosa            CACCACGAAGACACTCATAAACTGCCCTACCGTAGTTTTTAGCGGATAACCCCAATTTAG 120
E. rigida               CACCACAAAGACATTCATAAACTGCTCTACCGTAGTTTTTAGCAGATAATCCCAATTTAG 120
L. cinereum             CACCGCGAAGACATTCATAACATGCTCTACCGTAGTTTTTAGCAGATAACCCCAATTTAG 120
R. obovatum             CACCGCGAAGACATTCATAACATGCTCTACCGTAGTTTTTAGCAGATAACCCCAATTTAG 120
R. lucida               CACCGCGAAGACATTCATAACATGCTCTACCGTAGTTTTTAGCAGATAACCCCAATTTAG 120
B. elliptica            CACCGCGAAGACATTCATAAACTGCTCGACCATAGTTTTTAGCAGATAACCCCAATTTCG 120
P. afra                 CACCGCGAAGACATTCATAAACTGCTCGACCATAGTTTTTAGCAGATAACCCCAATTTCG 120
P. auriculata           CGCCGCGAAGACATTCATAAACAGCTCGACCGTAGTTCTTAGCGGACAACCCCAATTTAG 120
                          * *  ***   ** ***   ** * *** *  ** ** *  ** *  ******** *

A. karroo               GTTTAATAGTACATCCCAATAGGGGACGGCCGTACTTGTTCAATTTATCTCTCTCAACTT 180
P. afra                 GTTTAATAGTACATCCCAATAGGGGACGGCCATACTTGTTCAATTTATCTCTCTCAACTT 180
E. bothae               GTTTAATAGTACAACCCCATAGAGGGCGACCATATTTGTTCAATTTATCTCTCTCAACTT 180
E. fimbriata            GTTTAATAGTACAACCCAATAGAGGGCGACCATACTTGTTCAATTTATCTCTCTCAACTT 180
J. capensis             GTTTAATAGTACAACCCAATAGGGGGCGACCATACTTGTTCAATTTATCTCTCTCAACTT 180
P. pyracantha           GTTTAATAGTACACCCCAATAGAGGGCGTCCATACTTGTTCAGTTTATCTCTCTCAACTT 180
G. occidentalis         GTTTAATAGTACATCCCAATAGGGGACGACCGTACTTGTTCAATTTATCTCTTTCAACCT 180
G. robusta              GTTTAATAGTACATCCCAATAGGGGACGACCGTACTTGTTCAATTTATCTCTTTCAACCT 180
A. tetracantha          GTTTAATAGTACATCCTAATAGGGGACGACCATACTTGCTCAATTTATCTCTTTCAACTT 180
E. undulata             GTTTAATAGTACATCCCAACAGGGGACGACCATACTTGTTCAATTTATCTCTTTCAACTT 180
C. bispinosa            GTTTAATAGTACATCCCAACAGGGGACGACCATATTTGTTCAATTTATCTCTCTCAACCT 180
E. rigida               GTTTAATAGTACATCCCAACAGAGGACGACCATACTTGTTCAATTTATCTCTCTCAACTT 180
L. cinereum             GTTTAATAGTACATCCCAATAGGGGACGACCATACTTGTTCAATTTATCTCTCTCACTTT 180
R. obovatum             GTTTAATAGTACATCCCAATAGGGGACGACCATACTTGTTCAATTTTCCTCTCTCACTTT 180
R. lucida               GTTTAATAGTACATCCCAATAGGGGACGACCATACTTGTTCAATTCATCTCTCTCACTTT 180
B. elliptica            GTTTAATAGTGCATCCCAATAGAGGACGGCCATACTTGTTCAATTTATCTCTCTCGACTT 180
P. afra                 GTTTAATAGTGCATCCCAATAGAGGACGGCCATACTTGTTCAATTTATCTCTCTCAACTT 180
P. auriculata           GTTTAATAGTACATCCCAATAGGGGACGCCCATATTTGTTCAATTTATCTCTTTCAACTT 180
                        ********** ** **  * ** ** ** ** *** *** **  **** **     *

A. karroo               GGATGCCGTGAGGCGGACCTTGGAAAGTTTTAGAATAAGAAGTAGGGATTCGCAAATCCT 240
P. afra                 GGATACCGTGAGGCGGACCCTGGAAAGTTTTAATATAAGCAGTAGGGATTCGCAAATCCT 240
E. bothae               GGATTCCATGAGGTGGCCCTTGGAAAGTTTCAGTATAAGAAGTAGGGATTCGCAAATCCT 240
E. fimbriata            GGATGCCATGAGGTGGCCCTTGGAAAGTTTTAGTATAAGAAGGAGGGATTCGCAAATCCT 240
J. capensis             GGATACCATGAGGCGGCCCTTGGAAAGTTTTAGTATAAGCAGTAGGGATTCGCAAATCCT 240
P. pyracantha           GGACACCATGCGGCGGGCCTTGGAAAGTTTTAGAATAAGCAGGGGGGATTCGCAAATCCT 240
G. occidentalis         GGATGCCATGAGGCGGGCCTTGGAAAGTTTTAATATAAGCAGGAGGGATTCGCAGATCCT 240

G. robusta              GGATGCCATGAGGCGGGCCTTGGAAAGTTTTAATATAAGCAGGAGGGATTCGCAGATCCT 240
A. tetracantha          GGATACCATGAGGTGGTCCCATGAAAGTTTTACTATAAGCAGGAGGGATTCGCAAATCCT 240
E. undulata             GGATACCATGAGGTGGTCCTTGGAAAGTTTTAGAATACGAAGTAGGGATTCGCAAATCTT 240
C. bispinosa            GGATGCCATGAGGCGGGCCTTGGAAGGTTTTAACATAAGCCGTAGGGATTCGCAAATCTT 240
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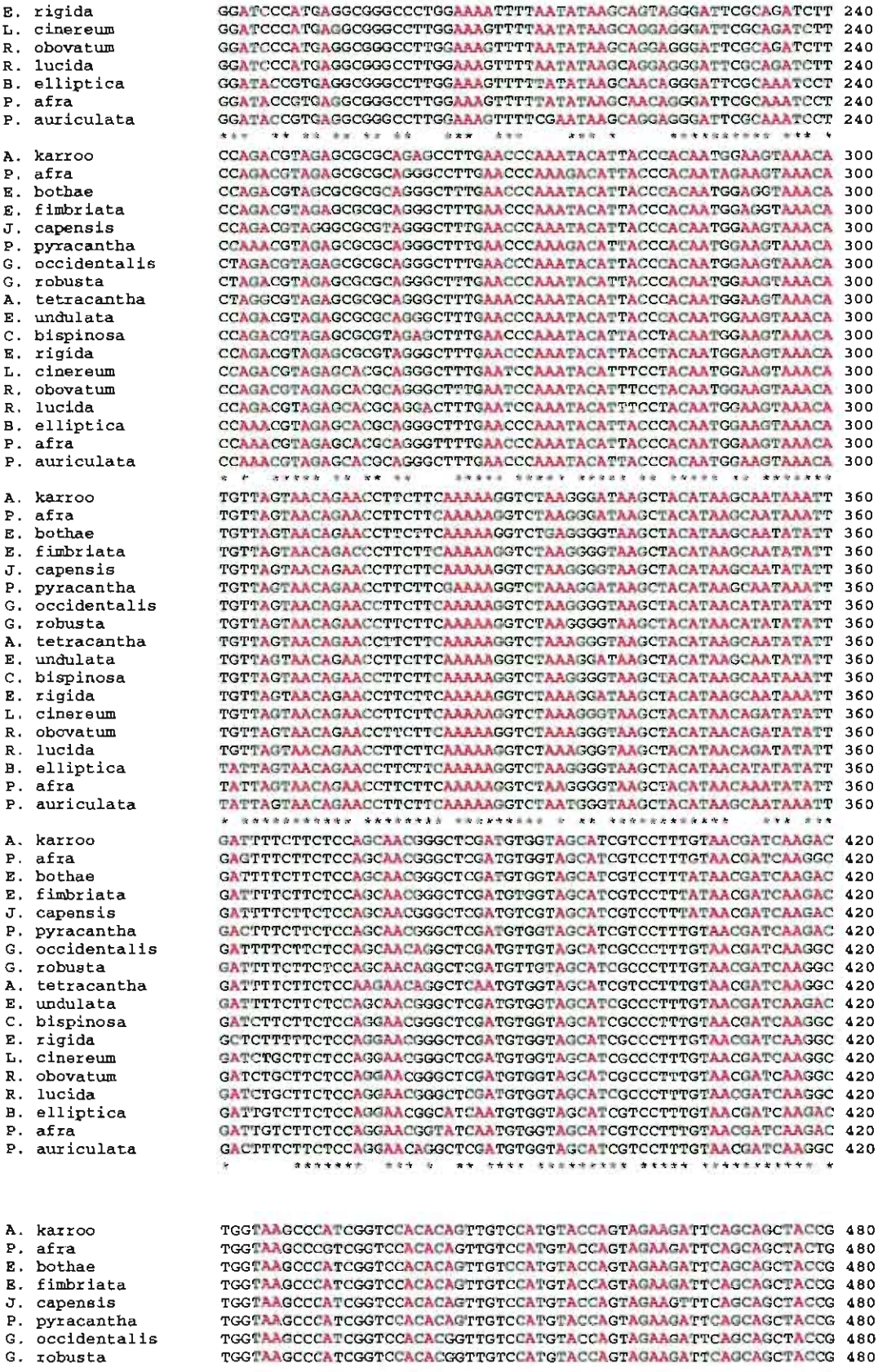
E. rigida GGATCCCATGAGGCGGGCCCTGGAAAATTTTAATATAAGCAGTAGGGATTCGCAGATCTT 240
L. cinereum GGATCCCATGAGGCGGGCCTTGGAAAGTTTTAATATAAGCAGGAGGGATTCGCAGATCTT 240
R. obovatum GGATCCCATGAGGCGGGCCTTGGAAAGTTTTAATATAAGCAGGAGGGATTCGCAGATCTT 240
R. lucida GGATCCCATGAGGCGGGCCTTGGAAAGTTTTAATATAAGCAGGAGGGATTCGCAGATCTT 240
B. elliptica GGATACCGTGAGGCGGGCCTTGGAAAGTTTTTATATAAGCAACAGGGATTCGCAAATCCT 240
P. afra GGATACCGTGAGGCGGGCCTTGGAAAGTTTTTATATAAGCAACAGGGATTCGCAAATCCT 240
P. auriculata GGATACCGTGAGGCGGGCCTTGGAAAGTTTTCGAATAAGCAGGAGGGATTCGCAAATCCT 240
A. karroo CCAGACGTAGAGCGCGCAGAGCCTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
P. afra CCAGACGTAGAGCGCGCAGGGCCTTGAACCCAAAGACATTACCCACAATAGAAGTAAACA 300
E. bothae CCAGACGTAGCGCGCGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAGGTAAACA 300
E. fimbriata CCAGACGTAGAGCGCGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAGGTAAACA 300
J. capensis CCAGACGTAGGGCGCGTAGGGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
P. pyracantha CCAAACGTAGAGCGCGCAGGGCTTTGAACCCAAAGACATTACCCACAATGGAAGTAAACA 300
G. occidentalis CTAGACGTAGAGCGCGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
G. robusta CTAGACGTAGAGCGCGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
A. tetracantha CTAGGCGTAGAGCGCGCAGGGCTTTGAAACCAAATACATTACCCACAATGGAAGTAAACA 300
E. undulata CCAGACGTAGAGCGCGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
C. bispinosa CCAGACGTAGAGCGCGTAGAGCTTTGAACCCAAATACATTACCTACAATGGAAGTAAACA 300
E. rigida CCAGACGTAGAGCGCGTAGGGCTTTGAACCCAAATACATTACCTACAATGGAAGTAAACA 300
L. cinereum CCAGACGTAGAGCACGCAGGGCTTTGAATCCAAATACATTTCCTACAATGGAAGTAAACA 300
R. obovatum CCAGACGTAGAGCACGCAGGGCTTTGAATCCAAATACATTTCCTACAATGGAAGTAAACA 300
R. lucida CCAGACGTAGAGCACGCAGGACTTTGAATCCAAATACATTTCCTACAATGGAAGTAAACA 300
B. elliptica CCAAACGTAGAGCACGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
P. afra CCAAACGTAGAGCACGCAGGGTTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
P. auriculata CCAAACGTAGAGCACGCAGGGCTTTGAACCCAAATACATTACCCACAATGGAAGTAAACA 300
A. karroo TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGATAAGCTACATAAGCAATAAATT 360
P. afra TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGATAAGCTACATAAGCAATAAATT 360
E. bothae TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTGAGGGGTAAGCTACATAAGCAATATATT 360
E. fimbriata TGTTAGTAACAGACCCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAAGCAATATATT 360
J. capensis TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAAGCAATATATT 360
P. pyracantha TGTTAGTAACAGAACCTTCTTCGAAAAGGTCTAAAGGATAAGCTACATAAGCAATAAATT 360
G. occidentalis TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAACATATATATT 360
G. robusta TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAACATATATTT 360
A. tetracantha TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGGTAAGCTACATAAGCAATATATT 360
E. undulata TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGATAAGCTACATAAGCAATATATT 360
C. bispinosa TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAAGCAATAAATT 360
E. rigida TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGATAAGCTACATAAGCAATAAATT 360
L. cinereum TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGGTAAGCTACATAACAGATATATT 360
R. obovatum TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGGTAAGCTACATAACAGATATATT 360
R. lucida TGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGGTAAGCTACATAACAGATATATT 360
B. elliptica TATTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAACATATATATT 360
P. afra TATTAGTAACAGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAACAAATATATT 360
P. auriculata TATTAGTAACAGAACCTTCTTCAAAAAGGTCTAATGGGTAAGCTACATAAGCAATAAATT 360
A. karroo GATTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTGTAACGATCAAGAC 420
P. afra GAGTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTGTAACGATCAAGGC 420
E. bothae GATTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTATAACGATCAAGAC 420
E. fimbriata GATTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTATAACGATCAAGAC 420
J. capensis GATTTTCTTCTCCAGCAACGGGCTCGATGTCGTAGCATCGTCCTTTATAACGATCAAGAC 420
P. pyracantha GACTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGTCCTTTGTAACGATCAAGAC 420
G. occidentalis GATTTTCTTCTCCAGCAACAGGCTCGATGTTGTAGCATCGCCCTTTGTAACGATCAAGGC 420
G. robusta GATTTTCTTCTCCAGCAACAGGCTCGATGTTGTAGCATCGCCCTTTGTAACGATCAAGGC 420
A. tetracantha GATTTTCTTCTCCAAGAACAGGCTCAATGTGGTAGCATCGTCCTTTGTAACGATCAAGGC 420
E. undulata GATTTTCTTCTCCAGCAACGGGCTCGATGTGGTAGCATCGCCCTTTGTAACGATCAAGAC 420
C. bispinosa GATCTTCTTCTCCAGGAACGGGCTCGATGTGGTAGCATCGCCCTTTGTAACGATCAAGGC 420
E. rigida GCTCTTTTTCTCCAGGAACGGGCTCGATGTGGTAGCATCGCCCTTTGTAACGATCAAGGC 420
L. cinereum GATCTGCTTCTCCAGGAACGGGCTCGATGTGGTAGCATCGCCCTTTGTAACGATCAAGGC 420
R. obovatum GATCTGCTTCTCCAGGAACGGGCTCGATGTGGTAGCATCGCCCTTTGTAACGATCAAGGC 420
R. lucida GATCTGCTTCTCCAGGAACGGGCTCGATGTGGTAGCATCGCCCTTTGTAACGATCAAGGC 420
B. elliptica GATTGTCTTCTCCAGGAACGGCATCAATGTGGTAGCATCGTCCTTTGTAACGATCAAGAC 420
P. afra GATTGTCTTCTCCAGGAACGGTATCAATGTGGTAGCATCGTCCTTTGTAACGATCAAGAC 420
P. auriculata GACTTTCTTCTCCAGGAACAGGCTCGATGTGGTAGCATCGTCCTTTGTAACGATCAAGGC 420
A. karroo TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
P. afra TGGTAAGCCCGTCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACTG 480
E. bothae TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
E. fimbriata TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
J. capensis TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGTTTCAGCAGCTACCG 480
P. pyracantha TGGTAAGCCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
G. occidentalis TGGTAAGCCCATCGGTCCACACGGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
G. robusta TGGTAAGCCCATCGGTCCACACGGTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480

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A. tetracantha      TGGTAAGCCCATCGGTCCACACACTTGTCCATGTACCAGTAGAAGATTCAGCAGCTACCG 480
E. undulata         TAGTAAGTCCATCGGTCCACACAGCTGTCCATGTACCAGTAGAAGATTCGGCAGCTACCG 480
C. bispinosa        TGGTAAGTCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCGGCAGCTACCG 480
E. rigida           TGGTAAGTCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCGGCAGCTACTG 480
L. cinereum         TGGTAAGTCCATCGGTCCACACGGTTGTCCATGTACCAGTGGAAGATTCGGCAGCTACCG 480
R. obovatum         TGGTAAGTCCATCGGTCCACACAGTTGTCCATGTACCAGTGGAAGATTCGGCAGCTACCG 480
R. lucida           TGGTAAGTCCATCGGTCCACACAGTTGTCCATGTACCAGTGGAAGATTCGGCAGCTACCG 480
B. elliptica        TGGTAAGTCCGTCGGTCCATACAGTTGTCCATGTACCAGTAGAAGATTCGGCAGCTACTG 480
P. afra             TGGTAAGTCCGTCGGTCCATACAGTTGTCCATGTACCAGTAGAAGATTCGGCAGCTACTG 480
P. auriculata       TGGTAAGTCCATCGGTCCACACAGTTGTCCATGTACCAGTAGAAGATTCGGCAGCTACCG 480
                    * ***** ** ******** **   ********************* **** *** ******** *

A. karroo           CGGCACCTGCTTCTTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
P. afra             CGGCACCTGCTTCTTCGGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
E. bothae           CAGCTCCTGCTTCCTCAGGTGGAACTCCAGGTTGAGGACTTACTCGGAATGCTGCCAAGA 540
E. fimbriata        CAGCTCCTGCTTCCTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
J. capensis         CAGCTCCTGCTTCCTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
P. pyracantha       CCGCCCCTGCTTCTTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
G. occidentalis     CGGCTCCTGCTTCCTCAGGCGGAACTCCGGGTTGAGGAGTTACTCGGAAGGCTGCCAAGA 540
G. robusta          CGGCTCCGGCTTCCTCAGGCGGAACTCCGGGTTGAGGAGTTACTCGGAAGGCTGCCAAGA 540
A. tetracantha      CAGCCCCTGCCTCTTCAGGTGGAACTCCGGGTTGAGGAGTTACTCGGAATGCTGCCAAAA 540
E. undulata         CGGCCCCTGCTTCTTCCGGTGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
C. bispinosa        CGGCCCCTGCTTCTTCGGGTGGAACTCCGGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
E. rigida           CGGCCCCTGCTTCTTCAGGGGGAACTCCGGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
L. cinereum         CTGCCCCTGCTTCTTCAGGGGGAACTCCAGGTCGAGGAGTTACTCGGAATGCTGCCAAGA 540
R. obovatum         CTGCCCCTGCTTCTTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
R. lucida           CTGCCCCTGCTTCTTCAGGCGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
B. elliptica        CGGCCCCTGCTTCTTCTGACGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
P. afra             CGGCCCCTGCTACTTCTGACGGAACTCCAGGTTGAGGAGTTACTCGGAATGCTGCCAAGA 540
P. auriculata       CGGCCCCTGCTTCCTCTGGTGGAACTCCAGGTTGAGCAGTTACTCGAAATGCTGCCAAGA 540
                    * ** ** **  * ** *  ******** *** *** * ******* ** ******** *

A. karroo           TATCACTATCTTTGGTTTCATAGTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
P. afra             TATCAGTATCCTTGGTTTCATAGTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
E. bothae           TATCAGTATCTTTGGTTTCATATTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
E. fimbriata        TATCAGTATCTTTGGTTTCATATTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
J. capensis         TATCAGTATCTTTGGTTTGATACTCAGGAGTATAATAAGTCAATTTATAATCTTGAACAC 600
P. pyracantha       TATCAGTATCTTTGGTTTCATAGTCAGGAGTATAATAAGTCAATTTATAATCTTTAACGC 600
G. occidentalis     TATCAGTATCTTTGACTTGATATTCAGGAGTATAATAAGTCAATTTATACTCTTTAACAC 600
G. robusta          TATCAGTATCTTTGACTTGATATTCAGGAGTATAATAAGTCAATTTATACTCTTTAACAC 600
A. tetracantha      TATCAGTATCTTTGGTTTCATAGTCAGGAGTATAATAAGTCAATTTATAATCTTTAACAC 600
E. undulata         TATCAGTATCTTTGGTTCCATAGTCAGGAGTATAATAAGTCAATTTGTAATCTTTAACAC 600
C. bispinosa        TATCAGTATCTTTAGTTTCGTATTCAGGAGTATAATAAGTCAATTTGTACTCTTTAACAC 600
E. rigida           TATCAGTATCTTTGGTTTGGTATTCAGGAGTATAATAAGTCAATTTGTAATCTTTAACAC 600
L. cinereum         TATCAGTATCCTTGGTTTCGTATTCAGGAGTATAATAAGTCAATTTGTACTCTTTAACAC 600
R. obovatum         TATCAGTATCCTTGGTTTCGTATTCAGGAGTATAATAAGTCAATTTGTACTCTTTAACAC 600
R. lucida           TATCAGTATCCTTGGTTTCGTATTCAGGAGTATAATAAGTCAATTTGTACTCTTTAACAC 600
B. elliptica        TATCAGTATCCTGAGGTTGATATTCAGGAGTATAATAAGTCAATTTGTAATCTTTAACAC 600
P.   afra           TATCAGTATCCTGAGGTTGATATTCAGGAGTATAATAAGTCAATTTGTAATCTTTAACAC 600
P. auriculata       TATCAGTATCTAGGGTTTGATAATCAGGAGTATAATAAGTCAATTTGTAATCTTTAACAC 600
                    ***** ****      *   ** ********************** ** **** *** *

A. karroo           CAGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
P. afra             CAGCTTTGAACCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
E. bothae           CAGCCTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
E. fimbriata        CAGCCTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
J. capensis         CAGCCTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
P. pyracantha       CAGCCTTGAATCCAACACTCGCTTTAGTCTCTGTTTGTGGTGACAT 646
G. occidentalis     CGGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 626
G. robusta          CGGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
A. tetracantha      CAGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
E. undulata         CAGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
C. bispinosa        CGGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
E. rigida           CAGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
L. cinereum         CCGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646

R. obovatum         CCGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
R. lucida           C-GCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 645
B. elliptica        CTGCTTTAAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
P. afra             CTGCTTTAAATCCAACACTTGCTTTAGTCTCTGTTTGTGGTGACAT 646
P. auriculata       CAGCTTTGAATCCAACAAAAGCTTTAGTCTCTGTTTGTGGTGACAT 646
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Appendix H: Family, order and species number of the plant species

SPECIES #	GENUS	SPECIES	FAMILY	ORDER
24191	*Portulacaria*	*afra*	Portulacaceae	Caryophyllales
344923	*Acacia*	*karroo*	Fabaceae	Fabales
35061	*Schotia*	*afra*	Fabaceae	Fabales
44331	*Jatropha*	*capensis*	Euphorbiaceae	Malpighiales
44926	*Euphorbia*	*bothae*	Euphorbiaceae	Malpighiales
44987	*Euphorbia*	*fimbriata*	Euphorbiaceae	Malpighiales
459442	*Rhus*	*lucida*	Anacardiaceae	Sapindales
46281	*Putterlickia*	*pyracantha*	Celastraceae	Rosales
496617	*Grewia*	*occidentalis*	Tiliaceae	Malvales
496619	*Grewia*	*robusta*	Tiliaceae	Malvales
63431	*Plumbago*	*auriculata*	Plumbaginaceae	Caryophyllales
640416	*Euclea*	*undulata*	Ebenaceae	Ericales
64441	*Azima*	*tetracantha*	Salvadoraceae	Celastrales
65592	*Carissa*	*bispinosa*	Apocynaceae	Gentianales
70432	*Ehretia*	*rigida*	Boraginaceae	Lamiales
73794	*Lycium*	*cinereum*	Solanaceae	Solanales
77222	*Rhigozum*	*obovatum*	Bignoniaceae	Lamiales
89362	*Brachylaena*	*elliptica*	Asteraceae	Asterales

Appendix I: Gallic acid standard curve (mg/ml)