Phylogenetic relationships and the effects of edaphic heterogeneity on the distribution of *Wiborgia* (Fabaceae) in the Greater Cape Floristic Region.

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Ntwai Moiloa

ABSTRACT

The Greater Cape Floristic Region (GCFR) is divided into two subregions, the Core Cape Subregion (CCR) and Extra Cape Subregion (ECR), which are mainly characterized by Fynbos and Succulent Karoo biomes, and are recognized among global biodiversity hotspots. The soils in the ECR are mostly shale derived and richer in nutrients compared to the CCR which is characterized by nutrient-poor sandstone soils mainly from the Cape System. The Fabaceae (Leguminosae) is the second largest family in the CCR with a total of about 764 species (belonging to 43 genera, of which 83% of the species are endemic to the CCR), and sixth largest in the ECR with about 140 species currently recognised with 39.3% of these species endemic to the ECR. *Wiborgia* Thunb. is a legume genus made up of 9 perennial shrub species of height 0.5-3.0 metres, with distinct ascending to erect habit, which share morphological similarities with some *Lebeckia*, especially sect. *Viborgoides* currently referred to as *Wiborgiella*. The aim of this dissertation was to understand the evolution and biogeography of the genus *Wiborgia* in the GCFR. This involved (i) inferring phylogenetic relationships within the genus using multiple molecular markers and testing the monophyly and the support of Dahlgren's (1975) morphological subgeneric classification; (ii) determining nutritional characteristics of soils occupied by different *Wiborgia* species and compare them with sites where *Wiborgia* species have not been recorded to occur and testing whether *Wiborgia* species occupy habitats with similar nutrient concentrations; (iii) evaluating the potential of *Wiborgia* species to grow and nodulate in soils from within and outside distribution range and characterizing of rhizobia nodulating *Wiborgia* species in field and glasshouse conditions.

Phylogenetic relationships in *Wiborgia* were inferred using multiple molecular markers (*ITS*, rpl32-trnL, *rps16*, trnS-trnG, and trnT-trnL) and the data were analysed using model based approaches (Maximum Likelihood, Bayesian inference). *Wiborgia* was well supported as monophyletic and sister to both *Wiborgiella* and *Aspalathus*, with *Wiborgiella humilis* well supported as being part of the *Wiborgiella* clade. Within the *Wiborgia* clade, two strongly supported subclades were observed. In subclade 1, *W. tetraptera* was strongly supported as sister to *W. fusca*, whilst *W. monoptera* was strongly supported as sister to *W. incurvata*. In subclade 2, a novel well-supported sister relationship between *W. mucronata* and *W. tenuifolia* was observed. *Wiborgia obcordata,* the only species in Dahlgren's subgenus *Wiborgia,* was found to be embedded within subgenus *Pterocarpia* and thus the subgenera classification of Dahlgren was not supported. It was also identified that sister species pairs (*W. incurvata* and *W. monoptera; W. fusca* and *W. tetraptera; W. tenuifolia* and *W. mucronata)* all showed the tendency to co-occur or have overlapping distribution ranges, and showed subtle differences in floral morphology and habitats.

A number of soil parameters including total P, available P, calcium, potassium, nitrogen, ammonium, carbon, magnesium, sodium, pH, sand, clay, and silt were analysed and similarities among locations and species tested using univariate and multivariate approaches. Soils habitat to *Wiborgia* seems to be generally indifferent and that the species occupy soils of similar nutritional characteristics across their entire distribution range. However, univariate analyses of individual concentrations of the different *Wiborgia* sites showed that nutrient levels of the different *Wiborgia* sites were quite variable. The multivariate analyses results showed that some non-*Wiborgia* sites were comparable (Cape Point) with soils from *Wiborgia* sites, whilst other non-*Wiborgia* sites (Rhodes Memorial and Bainskloof Pass) were distinct and incomparable to soils from *Wiborgia* sites. It was therefore concluded that *Wiborgia* species occupy soil with similar nutritional characteristics but which have a wide range in the individual levels of nutrients, and thus soil type and nutrient availability may be less important in the distribution of species within the *Wiborgia* habitat areas. However, some of the non-*Wiborgia* sites in the CCR were shown to be incomparable in terms of nutritional characteristics, and perhaps soil nutrients in those sites may partially be associated with the absence of *Wiborgia* species.

Using field collected data and common garden experiments; the ability of *Wiborgia* species to grow and nodulate within and outside their current range was tested. Rhizobia nodulating *Wiborgia* species were characterized using standard phylogenetic analyses based on DNA data from *16S* rRNA, *recA*, *nodA*, *nodC*, and *nifH*. Four *Wiborgia* species were grown in different soils which were either habitat (Darling, Grootvlei-Soebatsfontein, Brandvlei, Vanrhynsdorp, and Leliefontein) or non-habitat soils (Cape Point, Bainskloof Pass, and Rhodes Memorial). Some of the four species were able to accumulate biomass levels in the habitat soils that were comparable to or higher than non-habitat soils, whereby *W. incurvata* accumulated higher biomass in the non-habitat (Darling) soils compared to habitat (Leliefontein) soils, and *W. mucronata* accumulated biomass levels in the non-*Wiborgia* site (Rhodes Memorial) which were comparable to those accumulated in the habitat sites (Darling and Brandvlei dam). Furthermore, biomass accumulation seemed to be directly related with the nutrient concentrations in the tissues, being highest in plants which accumulated more biomass and lowest in plants that accumulated the least biomass. Diverse rhizobia were observed in both glasshouse and field conditions belonging to the Alphaproteobacteria (*Mesorhizobium* and *Rhizobium*) and the Betaproteobacteria (*Burkholderia*). *Wiborgia* appears to be promiscuous and showed the ability to be nodulated by rhizobia genera that are widespread and common symbionts of other Fynbos biome legume taxa such as *Aspalathus*. Given that the four species have the ability to establish and grow in non-*Wiborgia* soils, the availability of nutrients and compatible rhizobia does not limit the distribution of *Wiborgia* species in GCFR. Thus the conspicuous absence of *Wiborgia* in the fynbos vegetation may be associated with its inability to compete in a fire-prone environment.

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CHAPTER 1

1.0 General Introduction

1.1 Problem statement

Wiborgia is a Greater Cape Floristic Region (GCFR) endemic genus comprising 9 species, mainly distributed from the lowlands north of the Cape Peninsula extending all the way to the northern parts of Namaqualand. The genus generally occurs on a variety of habitats characterised by different vegetation types as well as with soils derived from the Malmesbury and Cape System (Dahlgren, 1975). The most recent taxonomic revision of the genus was by Dahlgren (1975), and 41 years later no revision of the genus has been done. The other genera in the Crotalarieae have been recently studied using phylogenetic and taxonomic approaches; these include *Lebeckia* (Boatwright *et al*., 2009), *Wiborgiella* (Boatright *et al*., 2010), species identifications via DNA barcoding of *Aspalathus* (Edwards *et al*., 2008); and the most recent phylogenetic study on the Crotalarieae resulted in the monophyly of all 'Cape clade' genera being resolved. Species relationships within *Wiborgia* are unknown as the most sampled phylogeny which includes seven *Wiborgia* species (Boatwright *et al*., 2008) provided an unresolved tree. Therefore a study which includes all species within the genus, including multiple samples from different populations, is needed in order to first identify the phylogenetic relationships that exist within the genus and also to determine if all species are genetically uniform across their entire distribution range, in view of Dahlgren (1975) assertion that the species are morphologically variable.

According to Dahlgren (1975), *Wiborgia* does not belong to the typical fynbos flora and that the distribution of the genus closely overlaps with arid types of the fynbos. He also pointed out that the genus is ecologically different to most sclerophyllous fynbos groups because of the characteristic mesomorphous deciduous leaves (Dahlgren, 1975), which perhaps explains the absence of *Wiborgia* in true fynbos in the Core Cape subregion. Interestingly though, the sister genus *Aspalathus*, has managed to diversify into the true fynbos in Core Cape Sub-Region where it is the second most speciose angiosperm genus. The pattern of patchy distribution gives rise to questions like: which factors are responsible for this restricted distribution of *Wiborgia* (edaphic and/or climatic)? Most species occupy clayey, sandy or are able to occur on both soil types; for example according to Dahlgren (1975), W. leptoptera and W. tenuifolia occur chiefly on clayey soils, whilst W. tetraptera, W. mucronata, W. fusca and W. sericea occur both on clayey as well as sandy soils or a mixture of both, on the other hand W. obcordata chiefly occurs on sandy to loamy soils. The distribution of the different species on different soil types further points to the importance of including edaphic factors when studying the evolution and diversification of *Wiborgia* in the GCFR. Currently however, little is

known about the extent to which edaphic factors such as soil nutrients could be linked with the distribution of *Wiborgia* species.

Another factor which has perhaps been historically overlooked, but is now receiving much attention in studying the drivers of distribution in legumes, is the availability of compatible rhizobia or rhizobia specificity. In the GCFR, recent studies on Papilionid tribes include Crotalarieae (*Aspalathus* and *Lebeckia*), Hypocalypteae (*Hypocalyptus*), Indigofereae (*Indigofera*), Phaseoleae (*Bolusafra*, *Dipogon*, *Rhynchosia*), Podalyrieae (*Cyclopia*, *Podalyria*, and *Virgilia*), Psoraleeae (*Psoralea* and *Otholobium*) (Kock, 2004; Elliot *et al*., 2007; Garau *et al*., 2009; Gyaneshwar *et al*., 2011; Gerding *et al*., 2012; Hassen *et al*., 2012; Kanu & Dakora, 2012; Beukes *et al*., 2013; De Meyer *et al*., 2013a & b; Howieson *et al*., 2013; De Meyer *et al*., 2014; Lemaire *et al*., 2015a), but no studies on *Wiborgia* have been carried out to date. Although some genera in the Crotalarieae have been fairly explored, there is still little known about the rhizobia nodulating *Wiborgia* species and their influence on the distribution of species. A further question which is an expansion of the main question highlighted above is whether sister species in the genus utilize the same rhizobia species where they co-occur or have they partitioned and diversified to being compatible with different rhizobia species.

1.2 The Greater Cape Floristic Region (GCFR)

1.2.1 Evolutionary history of the GCFR

The GCFR is divided into two subregions, the Core Cape Subregion (CCR) and Extra Cape Subregion (ECR), and these subregions are mainly characterized by Fynbos and Succulent Karoo biomes, respectively (Manning & Golblatt, 2012; Snijman, 2013). Both the Fynbos and the Succulent Karoo are regarded as some of the most endangered terrestrial ecoregions because of their biological richness (Mittermeier *et al*., 1999), and are recognized among global biodiversity hotspots (Myers *et al*., 2000). Snijman (2013) defined the ECR as an area made up of the southern Namib, western Richtersveld, Namaqualand, western Mountain Karoo and the Ceres-southern Great Karoo areas; whilst Manning and Goldblatt (2012) define the CCR as the entire area which maximizes the inclusion of the overall contiguous fynbos heathlands, a definition consistent with those of Bond & Goldblatt (1984), Goldblatt & Manning (2000). According to Snijman (2013), the CCR and ECR are separated by marked differences in moisture availability, geology, the effect of fire and altitude, where there are sharp dissimilarities of these factors between the two subregions. For example, Snijman (2013) identified dissimilarities in along the west-facing slopes forming part of the Bokkeveld Mountains and dropping down into the low-lying plains of the Knestervlakte (belonging to the CCR and ECR respectively). Gradual transition between vegetation separating the two subregions occur elsewhere; for example in the lowland areas around Klawer in the CCR the vegetation changes

from sandy fynbos to a sandy shrubland when extending into the ECR (see Snijman, 2013), a boundary/transitional zone which is somewhat difficult to be sharply identified (Mucina & Rutherford, 2006; Snijman, 2013).

Rainfall between both regions is predominantly in winter, however the frequency and seasonality of rainfall differs; for example in the ECR there is a difference in rainfall seasonality extending across the Hottentots Bay, Southern Namib and through the area lying to the western of the Great escarpment forming part of both the Northern Cape and Western Cape provinces (Figure 1.1). In addition to this, the rainfall boundaries in the South eastern regions follow differing rainfall seasonality mainly across the mountains of the CCR, Hantam-Roggeveld and the Nuweveld regions (Figure 1.1) (Snijman 2013). According to Linder (2003), the CCR is characterized mainly by winter-rainfall and the Cape Floristic element, which is a system marked by strong seasonality in rainfall in the western parts whilst in the southern coast rainfall patterns sharply change to less seasonal and greatly being experienced throughout the year (Snijman, 2013). Therefore the demarcation of boundaries of these two subregions using modality of rainfall alone is quite difficult and would be very limited because of regional and even lower scale variation in rainfall patterns influenced by various factors which include topography, wind and temperatures to mention a few (Cowling *et al*., 1997; Snijman, 2013). In terms of relating climate and growing seasons between the two subregions, the broadly accepted pattern follows that the ECR is characterized by a short and cool growing season whilst the CCR is characterized by a long and cool growing season; these growing seasons are mainly influenced by the differences in rainfall patterns between the two subregions (Mucina & Rutherford, 2006; Snijman, 2013).

1.2.1 (a) The GCFR: Description of the environmental characteristics of the CCR and ECR

The two subregions making up the GCFR; the CCR and ECR each encompass a land area of 90 760 km^2 and 98 869 km^2 respectively (Snijman, 2013). The CCR is estimated to harbour an estimated 9389 species of vascular plants in total and 68% of those plants are endemic (Manning & Goldblatt, 2012). The ECR has a native flora of 3715 vascular plant species and 40.4% of are endemic (Snijman, 2013). According to Snijman (2013) the somewhat lower levels of endemism in the ECR compared to the CCR could be attributed to the number of shared species by both subregions, where 27% of the native species in the ECR also extend into the CCR thus increasing the endemism levels of the GCFR in total but decreasing that of the ECR when considered as a smaller separate unit (Snijman, 2013).

The CCR

The southern parts of the CCR are characterised by a Mediterranean climate where most of the rain falls in winter (Manning & Goldblatt, 2012). However the eastern half of the CCR receives aseasonal

rainfall with some of the rain substantially in the summer months (Manning & Goldblatt, 2012). This varying pattern of rainfall results in hot and dry summers being characteristic in the west coast whilst in the eastern parts summers are less dry (Bond & Goldblatt 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). For example in the Little Karoo, high levels of precipitation are experienced in late summer but in overall the region still effectively receives its rainfall substantially in winter (Manning & Goldblatt, 2012). Rainfall varies locally across the landscape in the CCR; for example, mountainous areas receive a pronounced increase in the amounts of rainfall mainly due to a phenomenon called orography (Bond & Goldblatt 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Orographic form of precipitation is a phenomenon which occurs when prevailing winds carrying warm air move typically from the sea and inland towards mountainous area where they are caused to move upwards in order to clear the mountain, this results in the air being cooled and then formation of orographic clouds that result in rainfall occurring on the mountain side facing the initial direction of the prevailing winds (Bond & Goldblatt 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Therefore orographic precipitation plays a major role in the amount of rainfall received by coast facing mountains compared to those facing the interior of the CCR, thus leeward to the coastal prevailing winds (Manning & Goldblatt, 2012). There is thus an extreme variation in rainfall patterns across the CCR, where high mountains facing the coast receive up to 2000 mm of rainfall per annum whilst the interior slope ranges experience about 200 mm of rainfall per annum in general (Manning & Goldblatt, 2012). The extreme variation in rainfall patterns is thus largely driven and influenced by slope aspect and elevation (i.e. topography).The differences in seasonality and amount of rainfall received per annum coupled with the mosaic of soils available in the CCR play an important role in the structuring of vegetation across the landscape (Linder, 2003; Manning & Goldblatt, 2012). Moreover the effects caused by this correlation of these factors are mainly expressed by resulting formation of different niches available to a variety of plant species (Bond & Goldblatt, 1984). However, precipitation has been suggested to be more limiting when compared with the effects of soil, for example Manning and Goldblatt (2012) suggest that during periods where rainfall amounts are high, the effects of soil diversity on vegetation composition become reduced, thus suggesting that amounts of received rainfall plays an even greater role in vegetation structuring in the CCR compared to soil diversity. Although climatic gradients within the CCR are not as steep when compared to other Mediterranean type climate regions, the interaction of soil types and climatic variations across the landscape result in a relatively diverse system which is unique to the CCR and sets its apart when compared the other Mediterranean climate regions (Bond & Goldblatt 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012).

Most of the soils covering the CCR are suggested to be mostly derived from a variety of rocks dating back to more than 400 mya in the Pre-Carboniferous age (Manning & Goldblatt, 2012). These rocks form part of sedimentary strata of the Devonian-Ordovician series known as the Cape System, which

is made up mainly of quartzite sandstones and also fine-grained shales mainly derived from the Table Mountain-Witteberg and Bokkeveld groups respectively (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). The components of the Cape System are differentially weathered to result in fundamentally two general soil types which vary in their levels of available plant nutrients. The general soil types are the sandstone derived coarse-grained sandy soils characterized by poor levels of nutrients essential for plants, and the second soil type is shale derived fine-grained clay richer soils characterised by substantially higher levels of nutrients in comparison to the sandy soils (Bond & Goldblatt, 1984; Manning & Goldblatt, 2012), with landscapes broadly divided into four categories based on altitude and geographical position (these include: east montane, east lowland, west montane and west lowland) (Cowling *et al*., 2009). Moreover the coastlands are mainly covered by sandy soils derived from limestones of the Tertiary age, whilst there are also those areas characterized by Aeolian sandy soils mainly derived from the Cape sandstones (Bond & Goldblatt, 1984; Cowling *et al*., 1997; Goldblatt & Manning, 2000; Cowling *et al*., 2009). These results in a mosaic of soils ranging from coastal limestones mixed with deep sands whilst in other cases, nutrient poor sands of mountain ranges alternating with clay rich soils of the lower valleys (Cowling *et al*., 1997; Goldblatt & Manning, 2000). However, although the soils are different they do support a sclerophyllous fire-adapted shrubby vegetation to a certain extent (Bond & Goldblatt, 1984; Manning & Goldblatt, 2012). The differences between these soils becomes apparent when coupled with low precipitation levels, whereby soil type becomes limiting and results in sharp differences in vegetation supported by various different soil types (Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). In addition to soil type and nutrient content, the soils of the CCR also significantly differ in other properties such as their structure and ability to retain water; these differences in turn affect patterns of erosion thus resulting in a general pattern being observed in the CCR where mountains are dominated mainly by presently exposed sandstone rocks whilst the valleys are mainly dominated by clay rich shale soils (Cowling *et al*., 1997; Goldblatt & Manning, 2000; Mucina & Rutherford, 2006; Manning & Goldblatt, 2012). This pattern could be a result of fine-grained soils being eroded from elevated areas and deposited in valleys and low lying areas and thus leaving exposed rock in elevated areas (Bond & Goldblatt, 1984; Manning & Goldblatt, 2012). The mountains also are characterised by winter-freezing which commonly affect the montane vegetation (Goldblatt & Manning, 2000; Manning & Goldblatt, 2012), despite the general elevation of about 1000-2000 m in most areas. It is generally suggested and accepted that the mountains of the CCR offer a wider diversity of habitats compared to the lowlands mainly because variation in nutrients and soil types and rugged varying topography together amplify the already great effects of the variable precipitation patterns characterising the region (Manning & Goldblatt, 2012).

In terms of topography, the CCR is characterised by a trend of mountain ranges running east to west and north to south parallel to the southern and western coasts respectively (Goldblatt & Manning,

2000; Manning & Goldblatt, 2012). These trending mountain ranges are a result of the folding and warping of the landscape during separation and rifting events where Antarctica separated from the southern African coast and South America rifted from the west coast (Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). The different soil types of the CCR are generally associated with specific vegetation types, but in addition to soil type the association of vegetation type with a particular soil type is also affected and depends on protection from fire and importantly the levels of precipitation associated with the specific area (Manning & Goldblatt, 2012). For example, forest vegetation dominates in fire protected areas characterised by deep soils and high non-seasonal precipitation, but is replaced by shrubby or herbaceous vegetation when there is a change in seasonality, amount of precipitation and a decrease in soil nutrient status (Goldblatt & Manning, 2000; Mucina & Rutherford, 2006; Manning & Goldblatt, 2012). Moreover areas characterised by nutrient-poor sandy soils experiencing high amounts of precipitation give rise to the replacement of forest by fynbos vegetation, whilst the renosterveld vegetation replaces fynbos in areas where soils are clay rich and fairly high levels of nutrients (Goldblatt & Manning, 2000; Manning & Goldblatt 2012). In areas where annual precipitation is less than 300 mm, fynbos is replaced by succulent shrublands, whilst in the case of the renosterveld, areas with precipitation levels below 100 mm per annum results in the replacement of renosterveld being dominated by succulent perennials (Manning & Golblatt, 2012).

The ECR

Snijman (2013) identified eight eco-geographic units making up the ECR; these include the Gariep, Kamiesberg Mountains, Knestervlakte, Namaqualand Hardeveld, Namaqualand Sandveld, southern Namib, Tanqua-southern Succulent Karoo, and Western Mountain Karoo (Figure 1.1). All details regarding the descriptions of the ecogeographic units of the ECR are adapted from Snijman (2013). For a detailed discussion of the ecogeographic units see Snijman (2013).

The Namaqualand Sandveld is mainly composed of coastal plains with sandy soils rich in calcium and nutrients when compared to the inland sands which are characterised by a low pH. The Namaqualand Hardeveld is characterised by granitic gneisses forming large, solid domes with broken koppies, and also including shales and seams of quartzite. The southern Namib is made up of a variety of landscapes ranging from coastal salt pans, high inselbergs, rocky mountain range, and sand plains. The Knestervlakte is a low-lying plain characterised by an undulating landscape of varying altitude from north to south with quartz-veined shales and shale beds, bands of limestone, a mosaic of quartz fields, dolomite outcrops and red sand plumes mainly resulting from the weathering of the paleo-Karoo river delta and the Nama-system. The Gariep is characterised by a landscape with a mountain belt of ancient pre-Gondwanan (>2.5 billion years) rocks with low-lying sandy soils and loamy soils further inland in mountain areas. The Kamiesberg Mountains form part of the highest mountain ranges in Namaqualand, ranging from 1200 to 1700 m elevation, and are characterised by a landscape

made up of an imposing scarp of granite and lower lying hardeveld in the west and eastern slopes respectively. The Western Mountain Karoo is a region abutting the CCR on the south western side where the sediments of the Karoo super-group meet the Cape super-group thus respectively demarcating the boundaries of the ECR and CCR. The Western Mountain Karoo is characterised by scattered hills and low mountains made up of erosion resistant Karoo sandstone and dolerites giving rise to clay-rich soils. The Tanqua-southern Succulent Karoo is characterised by extreme erosion resulting exposed Ecca shales, Dwyka tillites, sandstones and mudstones with sandy soils found mostly within and along drainage lines.

The Namaqualand Sandveld area receives variable amounts of annual rainfall generally decreasing latitudinally from 80 mm to 200 mm per annum from north to south respectively with temperatures ranging respectively from 20–30°C and 5–10°C in summer and winter respectively. Whereas rainfall patterns in the Namaqualand Hardeveld are mainly restricted to winter, however they can be highly variable as it is adjacent to the inland Karoo which receives most of its rainfall in summer. Low-lying areas generally receive 200 mm of rainfall per annum whilst the more elevated areas can receive up to 300mm of rainfall per annum with temperatures ranging from 15–30°C to 5–20°C in summer and winter respectively. The Southern Namib is more arid, with rainfall amounts ranging from 17 mm to 85 mm per annum with additional moisture received in the form of fog mainly on elevated areas. On the other hand, the Knestervlakte experiences precipitation both in the form of rainfall and fog, where the amount of rainfall received reaches 150 mm in most areas, and temperatures ranging from 30–35°C and 5–10°C in summer and winter respectively. The Gariep region experiences precipitation mainly in the form of fog whilst rainfall is extremely low and erratic and mainly falls in winter with amounts ranging from 45 mm to 70 mm and temperatures ranging between 17–40°C in summer and below zero and in mountainous areas. The Kamiesberg region receives the highest precipitation ranging between 355 mm and 400 mm, with temperatures ranging from 10–25°C in summer and 2–15°C in winter accompanied frosts and snowfalls. The Western Mountain Karoo is characterised by rainfall ranging from 150 mm to 250 mm per annum with temperatures of 29.3°C to more than 30°C in summer, with snow and frost common during winter. Abutting the CCR, the Tanqua-southern Succulent Karoo experiences rainfall both in summer and winter ranging from 72–112 mm where 25% of the rain falls in summer, with temperatures ranging between 0°C–35.9°C with a higher prevalence of frost being a characteristic feature of the region.

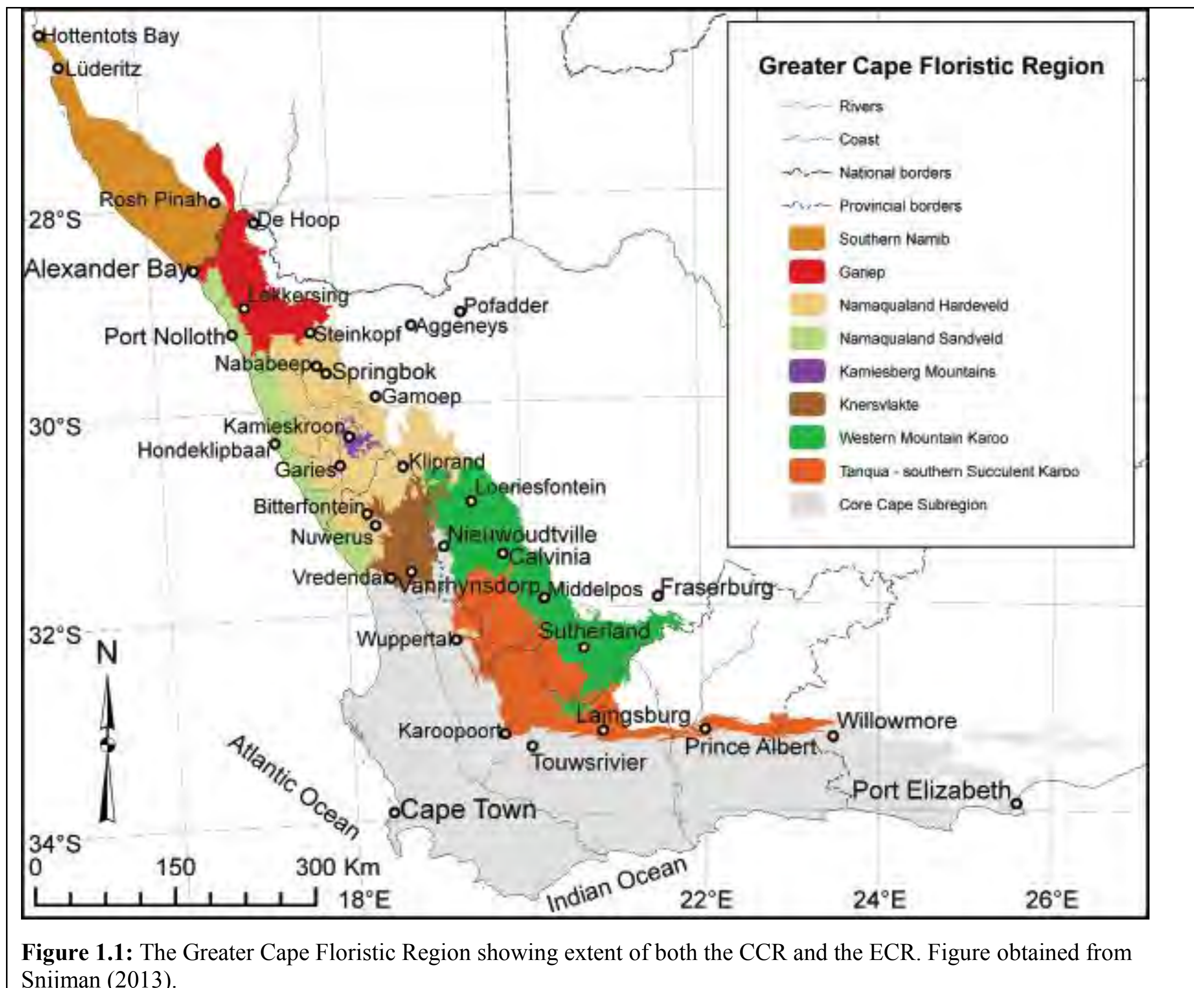


Figure 1.1: The Greater Cape Floristic Region showing extent of both the CCR and the ECR. Figure obtained from Snijman (2013).

1.2.1 (b) Vegetation diversity and floristic composition within the CCR and ECR

The Fabaceae is the second largest family in the CCR, comprising 43 genera, with a total of 764 species of which 83% of the species are endemic to the CCR (Manning & Goldblatt, 2012), and sixth largest in the ECR with 140 species currently recognised with 39.3% of these species endemic to the ECR (Snijman, 2013). The Proteaceae, Ericaceae, Fabaceae, Restionaceae, Rutaceae and Cyperaceae have a diversity strongly linked with nutrient-poor sandstone soils mainly in the Cape mountain ranges (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Conversely, these families have poorly diversified in regions dominated by other soil types (Manning & Goldblatt, 2012). For example, the Fabaceae have a less diverse representation in the ECR where the soils are mostly shale derived and richer in nutrients compared to the CCR (Manning & Goldblatt, 2012; Snijman, 2013). Also this family is known to be generally well represented and more diverse in semi-arid areas, thus the poor representation in the ECR is a peculiar case (Manning & Goldblatt, 2012). Likewise, the Iridaceae are also poorly represented in more nutrient rich soils as compared to the sandstone and limestone soils where a third of the family mainly in the larger genera in are restricted to these limestone and sandstone derived soils (Manning & Goldblatt, 2012). A summary of the 10 largest families and their genera is summarized in Table 1.1 below.

Table 1.1 Summary of the largest families and genera in the GCFR. Adapted from Manning & Goldblatt (2012) and Snijman (2013).

Subregion	Largest families (# of species)	Largest genera (# of species)	Comments
CCR	1. Asteraceae (1077) 2. Fabaceae (764) 3. Iridaceae (758) 4. Ericaceae (680) 5. Aizoaceae (624) 6. Scrophulariaceae (419) 7. Restionaceae (342) 8. Proteaceae (333) 9. Rutaceae (295) 10. Orchidaceae (234)	1. *Erica* (680) 2. *Aspalathus* (273) 3. *Restio* (163) 4. *Agathosma* (163) 5. *Pelargonium* (150) 6. *Phylica* (132) 7. *Cliffortia* (125) 8. *Oxalis* (122) 9. *Moraea* (122) 10. *Senecio* (144)	Extremely high contribution made by the families *Aizoaceae*, *Iridaceae*, *Ericaceae* to the flora (Manning & Goldblatt, 2012); as well contribution by the *Ericaceae*, *Restionaceae* and *Proteaceae* which are the key elements to identifying the fynbos heathland vegetation (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). The *Poaceae* is more species rich in the CCR but less dominant across the landscape compared to Savanna and Grasslands where they dominate the flora. Feature is shared by both the CCR and southwestern Australian flora, where *Poaceae* habitats are dominated by other families such as Cyperaceae and Restionaceae (Cowling *et al*., 1997; Manning & Goldblatt, 2012).
ECR	1. Aizoaceae (658) 2. Asteraceae (495) 3. Iridaceae (286) 4. Scrophulariaceae (230) 5. Hyacynthaceae (177) 6. Fabaceae (140) 7. Crassulaceae (134) 8. Poaceae (117) 9. Asphodelaceae (111) 10. Apocynaceae (110)	1. *Oxalis* (87) 2. *Conophytum* (81) 3. *Crassula* (81) 4. *Mesembryanthemum* (77) 5. *Pelargonium* (77) 6. *Ruschia* (69) 7. *Moraea* (68) 8. *Antimima* (62) 9. *Lanchenalia* (59) 10. *Euphorbia* (58)	Remarkable representation of succulent components mainly from families like the Aizoaceae, however no families are endemic to the region (Snijman, 2013). Thirty-eight genera which are mostly monotypic are endemic to the ECR. Generic representation is unequal with only a few genera dominating, characterised by geophytic or succulents (Snijman, 2013).

The CCR

The Fynbos biome is dominant across most parts of the CCR, occupying 82.5% of the total area of the CCR (Manning & Goldblatt, 2012). Apart from Fynbos, the CCR also encompasses biomes such as the Succulent Karoo, Albany thicket and Afrotemperate forest, each occupying 12%, 3.2% and 0.1% of land area in the CCR respectively (Manning & Goldblatt, 2012). The Fynbos biome is characterised by three major vegetation types which differ in their physical characteristics as well as the dominant species composition, growth and life-forms (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). These vegetation types include the fynbos heathland, renosterveld and strandveld thicket each occupying 55%, 24.2% and 3.3% of land area within the CCR respectively (Manning & Goldblatt, 2012). The fynbos heathland is the most dominant vegetation type in the CCR, occupying about half of the total land area occupied by the Fynbos biome (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). This vegetation type is characterised by oligotrophic sandstone derived soils dominated by shrubby species which have narrowed short needle-like ericoid leaves; in addition to these shrubs there is also shrubs with broad sclerophyllous leaves mainly from the Proteaceae also dominating the floristic composition of the fynbos heathland (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012).

Soils with fine-grained particles, richer clay and thus in essential nutrients in the CCR support the renosterveld vegetation, another distinctive vegetation type in the after the fynbos heathland (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). The renosterveld is mainly dominated by species from the Asteraceae characterised mainly by microphyllous shrubs which are highly prone to fire (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Moreover, apart from the microphyllous shrubs the renosterveld also supports a grassy element and also a herbaceous component which is normally supressed in the understory of old mature shrubs (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Areas of the CCR along the west and south coast support the strandveld thicket vegetation, which like the fynbos heathland, is dominated by broad-leaved sclerophyllous shrubs and in some areas where amounts of precipitation are generally lower, succulent shrubs become more dominant in the vegetation (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Generally, the strandveld thicket vegetation at the south coast is characterised by more sclerophyllous shrubs whilst that of the west coast is mainly dominated by succulent shrubs (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012).

The Succulent Karoo biome in the CCR is less dominant compared to its extent and importance in the ECR, characterised by succulent shrubland vegetation occurring mainly in semi-arid areas (Manning & Goldblatt, 2012). The arid intermontane basin found in the Little Karoo supports most of the succulent shrubland vegetation within the CCR (Manning & Goldblatt, 2012; Snijman, 2013). On the

extreme eastern parts of the CCR the Forest as well as the Albany thicket biomes are more dominant and supported along the landscape (Manning & Goldblatt, 2012). In areas where the Albany thicket biome is dominant, the area is characterised by a thicket mainly composed of succulent plants, thus forming succulent thicket vegetation (Manning & Goldblatt, 2012). The southern coastal and southern Afrotemperate forests are the two main types of forests found in the CCR (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012).

The ECR

The ECR is mainly dominated by three biomes which include the Succulent Karoo, fynbos, Desert and also some elements which are not characteristic of any of these three biomes, these are regarded as Azonal elements (Snijman, 2013). The Succulent Karoo is the most dominant biome followed by the fynbos, each respectively occupying 91.9% and 4.5% of total land area of the ECR (Snijman, 2013). The Desert biome is less dominant and covers areas mostly in the southern Namib and the Gariep ecogeographical regions, covering only up to 1.7% of the total land area of the ECR (Snijman, 2013). Lastly the remaining parts of the ECR not occupied by either of the three biomes is thus covered by Azonal elements which are less dominant only occupying about 1.9% of the total area (Snijman, 2013). Succulent shrublands locally known as 'vygievelds' are widespread across the entire region and they form in overall a relatively uniform and uniquely composed vegetation which is unlike any other vegetation types of similar semi-arid to arid regions in the world (Cowling *et al*., 1997; Desmet, 2007; Mucina & Rutherford, 2006; Snijman, 2013). A vygie, literally meaning small fig, is an Afrikaans name commonly used to refer to shrubs belonging to the Aizoaceae (Mesembryanthemaceae) mainly species from the genus *Carpobrotus* (Desmet, 2007; Snijman, 2013). The vegetation extend across most of the ecogeographical regions in the ECR and this includes the uplands of the Richtersveld and both the Namaqualand hardeveld and sandveld (Cowling *et al*., 1997; Snijman, 2013). The uplands of these areas (Richtersveld and both Namaqualand hardveld and sandveld) are composed of a mixture of dominant succulents and also the less dominant non-succulents, whilst the quartz fields of the same areas including the Knestervlakte are composed of almost exclusively dwarf uniform succulent 'vygieveld' (Cowling *et al*., 1997; Desmet, 2007; Snijman, 2013).

Apart from the Succulent Karoo there are also non-succulent elements in the ECR, amongst which is the fynbos which covers more land area than the latter two (Desert and Azonal) (Snijman, 2013). Along the coast there are large areas of sand fynbos vegetation, dominated Proteaceae and Restionaceae which are mostly evident along areas with a shallow water table and high water-retention capacity of the acidic sandy soils (Cowling *et al*., 1997; Desmet, 2007; Snijman, 2013). Further inland, the fynbos vegetation is restricted to moist areas of high altitude quartzite patches (e.g. Stinkfontein Mountains and Richtersveld regions and granite gneisses (Kamiesberg Mountains) (Desmet, 2007; Snijman, 2013). The renosterveld vegetation, dominated mostly by evergreen shrubby

species from the Asteraceae and also containing *Wiborgia* (Fabaceae), occurs mostly on dolerites and shale derived (clay rich soils) mainly of the Namaqualand hardeveld and the south -facing slopes of the Kamiesberg Mountains (Cowling *et al*., 1997; Desmet, 2007; Snijman, 2013). The Desert biome and its elements mainly occur along the inland and lower Gariep valley region; these elements are mainly low Aizoaceae shrubs (Desmet, 2007; Snijman, 2013).

1.2.2 Factors affecting plant distribution

Environmental factors refer to all external forces and matter which affect and influence the growth, structure, and reproduction of a plant (Billings, 1952). Distribution patterns of plants are mainly influenced by the interaction of environmental factors, including climatic, topographic and biotic factors, which interact to create unique conditions which in turn results in the variability of plants from region to region (Salisbury, 1926; Billings, 1952; Grace, 1987; William & Palmanis, 1998; Essl *et al*., 2009; Reed *et al*., 2009). Amongst all environmental factors affecting the biogeography of plants, climatic factors have been identified as the most important factor influencing the presence or absence of plants in a particular area and structuring of plant groups into biomes (Polunin, 1960; Eyre & Woodward, 1988; Woodward *et al*., 2004; Silva *et al*., 2012). In plant biogeography context, climatic factors mainly refer to temperature, moisture, light, and wind; which all interact to create unique climatic environment that is associated with a particular vegetation type (Davis & Shaw, 2001; Pearson & Dawson, 2003; Adams, 2010). Secondary to climatic factors, edaphic factors rank as one of the major environmental determinants of plant distribution (Eyre & Woodward, 1988). Edaphic factors mainly refer to all components associated with the substratum (soil) which provides the plants with nutrients, water supply and also growth medium as well as anchorage (Rajakaruna, 2004). Soil is mainly derived from parent-rock material which interacts with climatic and living organisms to form a variety of complexes; for example, soil texture is mainly influenced by water and frost action amongst other forms of weathering, whilst organic matter content (humus) is mainly influenced by the input and activities of the habitat plants and animals (Polunin, 1960). Much research has focussed on exploring the effects of edaphic factors on the distribution of plants (e.g. Polunin, 1960; Good, 1974; Eyre & Woodward, 1988; Rendig & Taylor, 1989; Power *et al*., 2010; Maistry *et al*., 2013). Apart from the climatic and edaphic factors, other environmental factors which are often overlooked and not regarded as having great influence in plant distribution when compared to climatic and edaphic factors are the biotic factors. Biotic factors generally refer to all living organisms, which include animals and plants such as man, herbivores, as well as soil microorganisms; and majority of these factors, seem to largely be external in origin and may affect plant distribution directly or indirectly (Polunin, 1960). For example, herbivore activity may affect plant distribution directly by reducing plant fitness, whilst simultaneously also indirectly affecting the distribution by confounding the effects of competition on plants which have reduced fitness (Maron & Crone, 2006; Huang *et al*., 2012). Humans directly affect plant distribution in a number of ways such as the clearing of land for

habitation, whilst the indirect effects may include industrialization which alters the overall environmental characteristics essential for a particular plant species to be able to proliferate (Polunin, 1960; Kelly & Goulden, 2008).

1.2.2 (a) Climatic factors

Temperature

Amongst all climatic factors, temperature has been suggested to be the most important and influential in plant distribution mainly because it is a direct function of the position and shape of the earth in relation to the sun (Good, 1974). Plant distribution is therefore limited by temperature in a twofold manner expressed as either the maximum or minimum temperatures required by a particular species for its proliferation, thus the two extremes largely determine the occurrence of a plant species in a particular area. In addition, vast number of studies has suggested that the correlation between plant distribution and climate may best be explained in the case of temperature as compared to any other climatic variable (Eyre & Woodward 1988; Richardson & Bond, 1991; Prentice *et al*., 1992; Walther *et al*., 2002). This close correlation between temperature and plant distribution thus inspired the development of broad plant classifications according to their tolerance to different temperature gradients, thus giving rise to names such as tropical, temperate, hardy, or tender (Good, 1974). Several studies have also predicted that as temperatures are currently increasing there will be widespread shifts in plant distributions (Parmesan & Yolie, 2003; Parmesan, 2006; Kelly & Goulden, 2008; Silva *et al*., 2012). And in the GCFR context, temperature and the changes thereof have been identified to be one of the major factors which played a role in the genesis of the current characteristic vegetation distribution of the subcontinent (Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). The establishment of the cold Benguela current during the Miocene was the most important climatic shifts which resulted in temperatures of the West coast of southern Africa being characteristically cool with a drying effect (Meadows & Sugden, 1991). Unlike the characteristic succulent or sclerophyllous shrublands characterizing the GCFR today, the work of Coetzee & Rogers (1982) show that in the late mid-Miocene, the vegetation of the West coast, mainly around the Saldanha Bay region comprised of fairly rich subtropical flora. In addition, the work of Meadows & Sugden (1991) suggests that fluctuations in temperature resulting from climate change may be responsible for the current vegetation distribution. One of the major plant families which have been shown to have radiated and diversified as a result of the vegetation change in response to the arising Mediterranean-type climatic conditions during the late-Miocene/early-Pliocene is the Fabaceae (Edwards & Hawkins, 2007).

Moisture/Precipitation

Amongst the climatic factors which affect plant distribution, secondary to temperature is the moisture levels, which are mainly referred to as precipitation or rainfall, but may also refer to humidity, dew, snow and fog (Polunin, 1960). Rainfall characteristics (availability and amount) of a particular area, in concert with other factors, is suggested to be of high importance because of its role in the regulation of the occurrence and primary productivity of plants (Polunin, 1960); thus the variation in the distribution of plants between different areas is often mainly linked with being as a result of a combination of rainfall differences as well as temperatures characterizing a particular area (Osmond *et al*., 1987; Polis, 1999). For example a correlation between the characteristic feature of grass community complexity and the pronounced east to west rainfall gradient across the Namib Desert, was reported by Jacobson (1997). Similarly, a study by Osmond *et al*. (1987) suggested that the distribution of C_4 plants to be associated with low rainfall areas, characterized by water limited environments; this was mainly suggested to be linked to the high water-use efficiency of most C_4 plants which thus enable those plants to proliferate even under water limited environments (Osmond *et al*., 1987). Generally, the distribution of vegetation types of the world seems to be correlated with rainfall distribution; and authors like Good (1974) further illustrated this point by pointing out that areas of maximum rainfall such as the lowlands of Brazil, parts of West Africa, as well as Malaysia were generally all equatorial, whilst areas such as South Africa fall under 'nearly continuous" ranges of low rainfall (Good, 1974). In addition, rainfall distribution as one of the major plant distribution determinants become more apparent in consideration of the close correspondence of some of the floristic regions of the world in relation to rainfall distribution; however, this correlation could not be shown solely by rainfall totals (Good, 1974). Moreover, a study by Toledo *et al*. (2012) exploring the distribution patterns of tropical woody species, identified rainfall to be the major factor influencing 91% of the species distribution. A Mediterranean-type climate characterizes the greater portion of the GCFR, which experiences orographic winter rainfall (Bond & Goldblatt, 1984); where the differences in rainfall vary according to topography where the lowlands generally receive rainfall ranging between 300-500 mm whilst in the mountainous areas, rainfall levels are beyond 1000 mm due to cloud persistence as well as other precipitation modes such as fog and snow which normally all in winter (Goldblatt, 1978; Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Linder, 2003; Manning & Goldblatt, 2012). In the GCFR, areas receiving high precipitation which is evenly distributed throughout the year, are characterized mainly by forest vegetation, and as the precipitation levels decrease and become more seasonal and erratic then the forest vegetation is replaced by shrubby vegetation, and successively in areas where rainfall levels becomes even lower (ranging between 200-300 mm annually) the landscape becomes mainly dominated by a succulent shrubland (Bond & Goldblatt, 1984; Fraser, 1988; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012).

Light and wind

Light and wind are regarded as being secondary to temperature and rainfall in their effects on plant distribution mainly because their influence on plant distribution is by mainly modifying temperature or rainfall (Good, 1974). A close relation between light and temperature has been suggested mainly because both are directly influenced by the sun, however because light is available and relatively sufficient in almost all areas, light has thus been regarded as the least important climatic variable influencing the distribution of plants (Salisbury, 1926; Polunin, 1960; Good, 1974; Osmond *et al*., 1987). Wind influences both temperature and precipitation by mainly affecting the humidity as well as temperature levels of a particular area and thus indirectly playing a role in the distribution of plants (Good, 1974). In the GCFR context, the one major event which is evidence of the role that wind plays in plant distribution was the establishment of the cold Benguela current during the Miocene which resulted in a change in vegetation type from tropical vegetation to proliferation of plants characterizing the GCFR presently (Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Studies showing more effects of light and wind in the GCFR are still lacking in literature.

1.2.2 (b) Edaphic factors

Edaphic factors mainly refer to all factors associated with the soil/substratum which provides most essential nutrients, water and a medium for the plants to grow on (Rajakaruna, 2004). Ranked second to climatic factors as one of the major environmental factors affecting plant distribution, edaphic factors have mostly been presented as a little world characterized by its unique physical structure, chemical composition, atmosphere as well as biota (Polunin, 1960; Good, 1974; Eyre & Woodward, 1988). The role of edaphic factors becomes more pronounced at regional levels with similar climatic conditions. The variation in vegetation type is then most likely best explained by variation in soil types, therefore highlighting the importance of soil in plant geography (Polunin, 1960). In terms of physical structure, soil is made up of depth, texture and chemistry, which are all related to climatic factors. For example the importance of soil depth is mainly related to it having much influence on determining the available moisture content of the soil; shallow soils are mainly characterized by the ability to retain only a limited amount of moisture and also its ability to provide anchorage to mainly vegetation dominated by small herbaceous shrubs and grasses, whilst deeper soils are conversely characterized by increased ability to retain higher amounts of moisture and also providing anchorage to vegetation mainly dominated by trees (Osmond *et al*., 1987; Clark *et al*., 1998). In addition, soil texture is a physical factor mainly related to the physical constitution and proportionate combinations of the basic components of soil which include sand, clay and humus (Good, 1974). Similar to soil depth, soil texture is also mainly associated with water relations and aeration of the soil. For example, sandy soil is generally characterized by good aeration, whilst clay is conversely characterized by poor aeration, however with a high water holding capacity while humus is characterized by an even more

enhanced water retention capacity (Good, 1974). Therefore a good soil texture would ideally be that containing all three different soil textures evenly divided in their right proportions. A study by Toledo *et al*. (2012) found that soil texture influenced the distribution of 44% of tropical woody species, whilst a study by Prentice *et al*. (1992) also further found that soil texture and depth were important and affected the growth, success, diversity and distribution of plants in areas of highly seasonal climates.

Amongst the three variables of the soil, chemistry of the soil is regarded as the most complex soil factors limiting plant distribution mainly due to the variety of chemical compounds occurring in nature (Good, 1974). Plant distribution in nature has been suggested to be influenced either favourably or unfavourably by the availability of certain minerals in the soil; and furthermore some studies have further reported a detailed account of plant species suggested to have a distribution pattern correlated with the availability of certain minerals (Polunin, 1960). However the four main chemical constituents of rocks which have been identified to generally have a higher level of importance in plant distribution include: quartz which gives rise to sandy soils, aluminium silicate which gives rise to mainly clay soils, calcium carbonates which gives rise to chalk and limestone, and lastly humus which is mainly made up of organic compounds (Good, 1974). Thus the differences in the proportional representations of these four chemical components determine the chemical distinction between soils, giving rise to a variety of soils with different characteristics which then ultimately influence the occurrence of a particular plant in an area. For example, a study by Rajakaruna (2004) exploring the role of edaphic factors in plant evolution found that edaphic islands such as limestone outcrops gave rise to localized patterns of plant distributions. Furthermore, these four chemical components have also been suggested, in concert with other environmental factors, to determine soil pH which has been identified by a number of studies to be correlated with plant distribution patterns (Osmond *et al*., 1987; Eyre & Woodward, 1988). Some plants may be acid tolerant and may thus be excluded in soils of high pH due to their being alkaline sensitive, whilst some species may be alkaline tolerant and due to being acid sensitive they may thus be excluded in soils of low pH.

The GCFR is geologically characterized by a mosaic of sandstone and shale parent substrates which give rise to a variety of soil types. Generally, mountainous areas are made up of coarse grained sandy soils derived from erosion-resistant quartzite rock, whilst the valleys and lower plains are made up of nutrient richer soils derived from shale substrates (Bond & Goldblatt, 1984; Fraser, 1988). The GCFR soils have long been suggested to play a role in influencing the distribution of plants (Richards *et al*., 1997a & b); for example a study by Richards *et al*. (1997a) which investigated the effects of the environment in vegetation composition in the Soetanysberg Hills found that the structuring of five plant communities were closely correlated with distinct soil types. In addition to this, the effects of soil type diversity in the GCFR were more pronounced when in conjunction with the precipitation gradients which exist in the different regions (Fraser, 1988; Linder, 2003). In the GCFR, forest

vegetation is generally predominant in areas of high yearly spread precipitation with deep soils, and as the soil becomes more sandy then forest vegetation becomes replaced by the fynbos vegetation; on the other hand, areas overlaid with clayey soils are mainly dominated by the renosterveld vegetation (Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). Thus the mosaic of soil types have been identified by several authors as one of the major contributing factors responsible for the increased plant diversity in the GCFR, but moreover for its role in role in influencing the type of a vegetation supported by a specific soil type (Bond & Goldblatt, 1984; Fraser, 1988; Meadows & Sugden, 1991; Dean *et al*., 1995; Goldblatt & Manning, 2000; Linder, 2003). In the CCR, Asteraceae is reported as the largest plant family and it has been identified to mostly be associated with clayey soils, whilst the second largest, Fabaceae, has not identified to be closely associated with only one single soil type but rather it has been associated with a variety of soil types, along different habitats (Manning & Goldblatt, 2012).

1.2.2 (c) Biotic factors

Biotic factors in the non-strict sense refer to living organisms such as man, herbivores, plants as well as the often disregarded microorganisms, and their effects in influencing plant distribution range from reduced plant fitness which normally goes hand in hand with competition, as well as clearing of land and industrialization in the case of herbivores, plants and man respectively (Polunin, 1960; Maron & Crone, 2006; Huang *et al*., 2012). Recent studies have identified soil microbes as having an important role in plant distribution; for example, van der Heijden *et al*. (2008) reported that soil microbes are important regulators of plant productivity mainly in nutrient poor ecosystems where the microbes assist the plants by enhancing the acquisition of the limiting nutrients in those ecosystems (van der Heijden, 2008). Since the time of Darwin, earthworms were among the few smaller animals which were identified to play a role in influencing the aeration and physical structure of the soil (Good, 1974), and recently Mishra *et al*. (2012) identified the two major microbes (bacteria and fungi) which are suggested to be vital microbes limiting plant distribution. Plants growing in nutrient poor soils, like those of the CCR, depend primarily on the relationships they form with microbes such as rhizobia and mycorrhiza for enhancing their acquisition of limited nutrients such as N and P (for rhizobia and mycorrhiza respectively). Rhizobia is a term collectively used to refer to a group of genetically diverse and physiologically heterogeneous nitrogen fixing bacteria that have the ability to form a symbiosis with plants mainly from the family Fabaceae (Willems 2000; Sprent 2007). The legume-rhizobia symbiosis occurs through a process where the root tissue of the legumes are infected and formation of structures known as nodules that contain nitrogen fixing bacteria (Willems 2000; Sprent 2007; Masson-Boivin *et al*., 2009; Sprent 2009; Bontemps *et al*., 2010; Lemaire *et al*, 2012). However, some non-leguminous plants have been reported to also form symbiosis, e.g. *Parasponia* in the family Ulmaceae (Sprent, 2007). Initially, rhizobia were known to mainly be restricted to the

family Rhizobiaceae, where two main groups (*Rhizobium* and *Bradyrhizobium*) were identified based on their fast-growing or slow-growing nature (Jordan 1982; Young & Haukka 1996). The concept of rhizobia has been expanded over the years to encompass all bacteria capable of nodulating legumes and fixing nitrogen (Willems 2006; Raychaudhuri *et al*., 2007; Garci, 2010). This expansion saw the Rhizobiaceae being extended from five traditionally known genera (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium*), to the inclusion of other Alpha-rhizobia which were initially not part of the Rhizobiaceae, these included *Methylobacterium*, *Devosia*, *Ochrobactrum* and *Phyllobacterium* (Moulin *et al*., 2001; Willems, 2006). In addition to this, other genera from the Beta subclass were also discovered, these included *Burkholderia*, *Ralstonia* and *Cupriavidus* (Willems, 2006). Presently, rhizobia that have been proven to nodulate legumes are known to belong to four families mainly from the Alpha and Beta subclasses of the Proteobacteria, these include the Rhizobiaceae, Phyllobacteriaceae, Nitrobacteriaceae and Hyphomicrobiaceae (Moulin *et al*., 2001; Willems, 2006; Raychaudhuri *et al*., 2007; Sprent, 2007; Garci, 2010). Benhizia *et al*. (2004), however, reported bacteria isolated from the nodules of *Hedysarum carnosum*, *H. spinosissimum*, and *H. pallidum* with the ability to nodulate legumes to belong to the Gamma subclass.

In an attempt to understand the distribution of bacteria, earlier researchers hypothesized that bacteria were present everywhere, and this was based on the point that bacteria can easily be dispersed through both abiotic and biotic means (Staley, 1999). This view was further supported through the use of molecular phylogenetic methods which showed that based on the 16S sequence analysis, some cyanobacteria species were cosmopolitan (Staley, 1999); this view thus leading to the suggestion that microbes such as rhizobia being adapted to a wide range of climatic and soil conditions may also be cosmopolitan. To partially concur with this point, several authors further added that rhizobia, in the absence of their host plants are free-living in the soil as saprophytes with their reproductive organs having the ability of remaining viable in dormancy during times when environmental conditions are not favourable (Somasegaran & Hoben, 1985; Silva *et al*., 2005; Sprent, 2007). However, other authors only agree with the suggestion that rhizobia are free-living, but were unconvinced about the theory that rhizobia are everywhere (Woomer *et al*., 1988; Staley, 1999; Bala *et al*., 2003a; Makatiani & Odee, 2007). The uncertainty of these authors could perhaps be based on the fact that some studies (e.g. Bala *et al*., 2003b) showed that the diversity of rhizobia is highest in the centre of diversity of their host, this thus suggesting the co-evolution of the hosts and their compatible bacteria. Furthermore, more studies have shown that legume distribution and productivity is strongly linked with the presence of compatible symbionts (Kuper *et al*., 2006; Essl *et al*., 2009; Santos *et al*., 2011; Sprent, 2012). Therefore, studies aimed at understanding the interaction of these different factors (edaphic and biotic), particularly how the soil environment influences the distribution of rhizobia and how this distribution in-turn influences the distribution of legumes in the GCFR are still needed. In the GCFR context, the most dominant legume subfamily is the Papilionoideae, which is the subfamily

where nodulation ability is most common (Elliot *et al*., 2007; Mishra *et al*., 2012). The general distribution of legumes in the GCFR is well documented (Bond & Goldblatt, 1984; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012; Snijman, 2013), where some legume species have a widespread distribution, whilst others have a somewhat narrowed distribution. Despite these distribution records there are very few studies which have investigated the role that availability and distribution of rhizobia may play on the current of these legumes in the GCFR, and perhaps most parts of other Mediterranean regions. This is particularly important because legumes have been reported in literature to be one of the most speciose and widely distributed plant families in the world, and this has most of the time been suggested to be due to their ability to fix nitrogen (Young & Haukka, 1996; Moulin *et al*., 2001; Willems, 2006; Raychaudhuri *et al*., 2007; Sprent, 2012) and hence overcome the limitations of nutrient deficiency in nutrient poor soils such as those of the Cape (Cowling *et al*., 1997; Manning & Goldblatt 2012; Stock & Verboom, 2012).

1.3 The genus *Wiborgia* Thunb.

1.3.1 Classification history and phylogenetic position of *Wiborgia*

The Crotalarieae is the largest tribe within the papilionoid legumes and comprises 51% of the genistoid alliance (Van Wyk 2005; Lewis *et al.,* 2005; Boatwright *et al.,* 2008). The tribe is made up of a total of approximately 1208 species which are distributed within 13 genera, namely: *Aspalathus*, *Bolusia*, *Calobota*, *Crotalaria*, *Lebeckia*¸ *Lotononis*, *Pearsonia*, *Rafnia*¸ *Robynsiophyton, Rothia*, *Spartidium*, *Wiborgia*, *Wiborgiella*; and within these genera is the *Crotalaria* with *ca* 690 species (Lewis *et al.,* 2005; Boatwright *et al.,* 2008). Van Wyk (1991) identified two broad groups within the tribe, these include the “Cape Group” comprising of *Aspalathus*, *Lebeckia*, *Calobota*, *Rafnia*, *Wiborgiella* and *Wiborgia*; all of which are endemic/sub-endemic to the Greater Cape Floristic Region (GCFR), with the exception of species of *Lebeckia* which extend into Namibia; and second the *Lotononis* group which comprises of *Lotononis*, *Pearsonia*, *Robynsiophyton*, and *Rothia*. However, although the two main groups have been identified, the placement of *Spartidium*, *Crotalaria* and *Bolusia* remained unclear until most recently, when a molecular and morphological study by Boatwright *et al.* (2008) have suggested the placement of *Spartidium* within the “Cape Group” whilst *Crotalaria* and *Bolusia* are nested within the *Lotononis* group. The tribe is well known for its commercial and medicinal importance, with commercially important species like *Aspalathus linearis* which is used to produce Rooibos tea (van Wyk *et al*., 1997), *Lotononis bainesii* which has been reported to be an important fodder plant (Boatwright *et al.,* 2008), and also *Crotalaria* and *Lotononis* species have been reported to be important in traditional medicine used to cure a variety of illnesses like broken-heart (Moteetee & Van Wyk 2007; van Wyk 2005); but also some species within

the *Crotalaria* and *Lotononis* have also been reported to be poisonous (van Wyk *et al.,* 2002). The Crotalarieae as a tribe has a very wide and interesting distribution, with most of the species from the Cape Group being confined to the Cape region with some extending to KwaZulu-Natal and Namibia; whilst the *Lotononis* group extends beyond Africa with a distribution range that widely represents the group in almost all continents like Asia, North and South America and Australia (Lewis *et al.,* 2005; LOWO, 2014). A summary of how each genus within the Crotalarieae is distributed and the species numbers are compiled in Table 1.2 below.

Table 1.2: Summary of the genera within the Crotalarieae and their distribution. Adapted from Lewis *et al* (2005) and Legumes of the world online (LOWO, 2014)

Genus	Description	# of species	Distribution
***Aspalathus* L.**	Shrubs/shrublets or rarely small trees	278	Sub-endemic to rocky and sandy shrublands of the Cape region and extend slightly eastwards to the grasslands of the KwaZulu-Natal Province
***Bolusia* Benth.**	Perennial herbs	5	South central and southern Africa in rocky and sandy dry tropical/subtropical and xerophytic bushland, shrubland and grassland
***Calobota* Eckl. & Zeyh.**	Spinescent, woody (but without bark) shrubs/shrublets	16	south western parts of South Africa, extending into Namibia, with *Calobota saharae* endemic to the sand dunes of Libya, Algeria and Morocco
***Crotalaria* L.**	Shrubs, shrublets, perennial and annual herbs and small trees	690	Africa, Madagascar, Asia, India, Indonesia, China, Australia, South, North and Central America; mainly in sandy or rocky outcrops of the dry tropical/subtropical forest, woodland, xerophytic shrubland and grassland
***Lebeckia* Thunb.**	Shrubs/shrublets or herbs	15	Southern Africa with most species in the Cape region but also extending to Namibia, Botswana
***Spartidium* Pomel.**	Shrubs	1	Sahara Regional Transitional Zone mainly in North Africa
***Rafnia* Thunb.**	Shrubs or suffrutice	19	Sub-endemic to the Cape region and extend slightly eastwards to KwaZulu-Natal Province
***Lotononis* (DC.) Eckl. & Zeyh.**	Shrubs/shrublets, perennial or annual herbs and suffrutices	150	Southern and Northern Africa, tropical Africa, Asia, Arabia, Pakistan and Spain
***Pearsonia* Dummer.**	Shrublets and herbs	13	Africa, as well as South of the equator and Madagascar
***Rothia* Pers.**	Annual herbs	2	Africa, Asia and Australia
***Robynsiophyton* R. Wilczek.**	Herbs	1	South Central Africa (Angola, Zambia and Congo)
***Wiborgiella* Boatwr. and B.-E. van Wyk.**	Rigid, resprouting, woody shrubs with woody branches	9	Endemic to the GCFR and is mainly distributed in the Extra Cape Region

The genus *Wiborgia* is composed of 9 species which are all endemic to the GCFR of South Africa. These species include: *W. fusca* Thunb., *W. incurvata* E.Mey., *W. leptoptera* R. Dahlg., *W. monoptera* E. Mey., *W. mucronata* (L.F) Druce., *W. obcordata* (P.J. Berg) Thunb., *W. sericea* Thunb. *W. tenuifolia* E. Mey., and *W. tetrapetra* E.Mey. The species within the genus are generally shrubs or shrublets which grow mainly in rocky areas or sandy flat areas of the fynbos and renosterveld vegetation types. This genus is clearly morphologically distinct (indehiscent fruit with wings) from other genera within the Crotalarieae, and recent molecular studies and circumscriptions have shown that the genus is monophyletic (Boatwright *et al.,* 2008). However, similarity between species of *Wiborgia* and *Wiborgiella* was previously suggested, with species of both genera previously included in *Lebeckia* section *Viborgoides* (Bentham, 1844) but later separated by Dahlgren (1975) into *Wiborgia* and *Lebeckia* section *Viborgoides*. Although the two genera show close morphological similarity, recent molecular work has shown that they are indeed two well defined generic clades within the tribe Crotalariae (Boatwright *et al*., 2008). However, it is clear that a more extensive study into the *Wiborgia-Wiborgiella* relationship needs to be undertaken in order to explore much more about the close relationship between the two genera. The genus *Wiborgia* shares much morphological similarities with *Wiborgiella* that Dahlgren (1975) considered *Wiborgia* to be paraphyletic with *Wiborgia humilis* showing a superficially close similarity with the *Lebeckia* section *Viborgoides*. A similar result was shown also by molecular studies done by Boatwright *et al.* (2008) which presented *Lebeckia* as a polyphyletic genus with *Lebeckia inflata* nested with the *Aspalathus* clade whilst all the other sections of *Lebeckia* formed their own individual clades. In light of the generic challenges presented in the study of Boatwright *et al.* (2008), a working conclusion was presented by Boatwright *et al* (2009) to reinstate the genus *Calobota* and also introduce a new genus *Wiborgiella* which includes all the species of the *Lebeckia* section *Viborgoides* including *Wiborgia humilis*. That working conclusion then resulted in *Wiborgia* and *Lebeckia* being monophyletic. There is emerging consensus on the monophyly of the genera of "Cape Clade" Crotalarieae (Van Wyk 1991; Boatwright *et al.,* 2008; Boatwright *et al.,* 2009), but more work is still needed to address the species relationships within each of the genera of the Crotalarieae.

1.4 Evolution of nodulation

The evolution of nodulation is currently still a point of interest which is still to be addressed comprehensively. Although the exact point when nodulation evolved has not been identified and supported with robust data, it is generally suggested (Doyle, 2011; Sprent, 2007; Sprent, 2013) that nodulation most probably evolved shortly after 60 Mya (Sprent, 2007; Sprent, 2013) since the evolution of the legumes is believed to be approximately 60 mya (Lavin *et al*., 2005). This suggestion is further coupled with the fact that looking at the entire family (Leguminosae), nodulation has been

identified to have arisen in about 6 to 7 independent evolutionary events (Doyle, 2011). However, nodulation is not only confined to the Leguminosae but rather seems to be a phenomenon occurring across the Rosid orders Cucurbitales, Fagales and Rosales (Sprent, 2007; Sprent, 2013). On the other hand, plants that have the ability to nodulate with unicellular bacteria (collectively known as rhizobia) occur only in the Leguminosae, with an exception of the genus *Parasponia* (Ulmaceae) (Sprent, 2007; Sprent, 2013). Although majority of species within the Leguminosae are known to nodulate, most of the early derived lineages in the family are incapable of nodulation; therefore the evolutionary chronology of nodulation is suggested to have arisen in the Caesalpinioideae followed by the Mimosoideae and then Papilionoideae (Allen & Allen, 1981; Sprent, 2007; Sprent, 2013). This evolutionary sequence of events is however not consistent with recent evidence found by other authors (e.g. Lavin *et al*., 2005; LPWG, 2013). Within the subfamily Caesalpiniodeae, nodulation is currently known to occur only in two of the four tribes within the subfamily (i.e. Cassieae and Caesalpinieae) (Sprent, 2007; Sprent, 2013), whilst within the Mimosoideae nodulation occurs within the tribes Mimoseae, Acacieae and the Ingeae with a few exceptions of forms as well as the older groups which have lost the trait or did not possess the ability to nodulate at all (Sprent, 2007). Within the Papilionoideae, nodulation is considered to be uniformly spread across both the genistoid and dalbergoid clades, with the exception of a few genera which are suggested to not have the ability to nodulate (Sprent, 2007). In terms of the triggers of the evolution of nodulation, it has suggested that a chain of linked environmental changes which include the abrupt increase in temperature across a range of latitudes (a rise of approximately 5–10°C) (Bowen *et al*., 2004; Sprent, 2007), as well as the release of methane and carbon dioxide both from the seafloor sediments (Sprent, 2007). These changes in environmental conditions are suggested to have occurred approximately 55 mya, which is also a period which coincides with the evolution of the two major legume groups (genistoids and dalbergoids) where nodulation is most common, thus leading Sprent (2007) to hypothesize that the evolution of nodulation could most probably have been triggered by these sudden changes in environmental conditions. In addition, Sprent further hypothesized that possibly due to the high CO_2 levels, nitrogen then became the limiting nutrient for plant growth and thus resulting in the favouring of the evolution of nitrogen fixation as a mitigation response (Sprent, 2007). Since the evolution of legumes and nodulation, environmental conditions (especially CO_2 levels) are suggested to have changed considerably leading to the evolution of C_4 photosynthesis which was not identified in any leguminous plants (Sprent, 2007). In addition, during these fluctuations in CO_2 levels, temperatures also started dropping and were followed by the formation of ice at the South and North poles which resulted in legumes colonizing mostly the emergent cooler areas and thus resulting in an increase in the interdependence between legumes and rhizobia (thereby increasing specificity) (Sprent, 2001; Sprent, 2007; Sprent, 2013).

1.5 AIM, OBJECTIVES AND THESIS OUTLINE

1.5.1 Aim and objectives of study

The overall aim of this study was to understand the evolution and biogeography of the genus *Wiborgia* Thunb. in the Greater Cape Floristic Region (GCFR), with the main objectives being:

i. To test the monophyly of *Wiborgia* and infer phylogenetic relationships within the genus using multiple molecular markers.
ii. To test the support of Dahlgren's (1975) morphological subgeneric classification using molecular markers
iii. To determine if there is a difference in nutritional characteristics of soils occupied by different *Wiborgia* species and compare them with sites where *Wiborgia* species have not been recorded to occur previously and presently.
iv. To evaluate potential of *Wiborgia* species to grow and nodulate in soils from within and outside distribution range.
v. To determine the diversity, characterize and infer phylogenetic relationships of rhizobia nodulating *Wiborgia* species.

1.5.2 Thesis outline

CHAPTER 1 provides the literature review and general introduction to the dissertation

CHAPTER 2: MOLECULAR SYSTEMATIC STUDIES ON *WIBORGIA* THUNB.: EXPLORING THE MONOPHYLY AND PHYLOGENETIC RELATIONSHIPS: addresses objectives i. and ii. above using DNA sequences from five markers (*ITS*, rpl32-trnL, *rps16*, trnT-trnL, and trnS-trnG) obtained via standard molecular systematic methods, with the aim of exploring the phylogenetic relationships within the genus, with all the species represented by several accessions, and to test Dahlgren (1975) hypotheses on taxonomy and evolution. It was hypothesized that (1) *Wiborgia* is monophyletic and sister to *Wiborgiella*

CHAPTER 3: DISTRIBUTION PATTERNS IN THE GENUS *WIBORGIA*: EXPLORING THE ROLE OF EDAPHIC HETEROGENEITY: addresses objective iii. using data from soil samples collected from *Wiborgia* and non-*Wiborgia* sites in order to compare nutritional differences between those sites. Parameters such as total P (P), available P (Bray II P), total nitrogen (N), ammonium (NH_4^+), potassium (K), iron (Fe), calcium (Ca), magnesium (Mg), pH, sand, clay, silt and carbon (C). It was hypothesised that (1) *Wiborgia* species occupy soils of similar nutritional levels across their entire distribution range, (2) There are soil nutritional differences between *Wiborgia* and non-*Wiborgia* sites, and that (3) Narrowly distributed species (*W. incurvata* and *W. tenuifolia*) in the genus

are edaphic specialists whilst widely distributed species (e.g. *W. mucronata* and *W. obcordata*) are edaphic generalists.

CHAPTER 4: ARE *WIBORGIA* SPECIES ABLE TO GROW AND NODULATE IN SOILS OUTSIDE THEIR DISTRIBUTION RANGE?: addresses objectives iv., v., and vi. Using data from field collected nodules as well as those glasshouse-grown plants, including plant biomass data; in order to test the ability of *Wiborgia* species to grow outside their current range and evaluate if they can nodulate in diverse soils. Rhizobia phylogenetic relationships were inferred from DNA data (*16S*, *recA*, *nodA*, *nodC*, *and nifH*) obtained using standard molecular systematic methods. It was hypothesized that (1) The distribution of *Wiborgia* species is determined by the presence of compatible rhizobia, (2) *Wiborgia* species occupy habitats with similar nutrient concentrations in the soil and will thus have similar nutrient concentration in the tissues, and that (3) *Wiborgia* species are nodulated by unique and closely related rhizobia species.

CHAPTER 5 provides a synoptic summary of the entire thesis by integrating the results, discussions and conclusions from the three data Chapters summarize collective findings and also provide recommendations and future research prospects.

CHAPTER 2

2.0 MOLECULAR SYSTEMATIC STUDIES ON *WIBORGIA* THUNB.: EXPLORING THE MONOPHYLY AND PHYLOGENETIC RELATIONSHIPS

2.1 Introduction

2.1.1 General morphological features of *Wiborgia*

Wiborgia Thunb. are perennial shrubs of height 0.5-3.0 metres, with distinct ascending/erect habit, except for *W. obcordata* which reaches heights beyond 2.5 metres (Dahlgren, 1975). Morphologically, all species in the genus are characterized by a spreading rounded branching pattern due to sympodial shoot systems where proleptic short-shoots develop from the axis of the longer shoots, with the leaves of the short-shoots radiating from a fixed point of the axil of the leaves of the long-shoots and new branches (long-shoots) developing below the inflorescence in the upper leaf axis (Dahlgren, 1975).The leaves are trifoliate with green semi-cylindrical petioles. They have a more or less flat to concave appearance on the adaxial side, whilst the leaflets generally have an oblanceolate and flat appearance (Dahlgren, 1975).

Species within the genus *Wiborgia* share great similarities with some *Lebeckia*, especially sect. *Viborgoides sensu* Dahlgren (1975), currently referred to as *Wiborgiella sensu* Boatwright *et al*. (2008). For example, the more flattened and broader petioles of *W. fusca* as well as some *Lebeckia* species remain for a longer time on the branches and are shed considerably latter than the leaflets thus resulting in a characteristic appearance as mostly evident in *W. fusca* as well as some *Lebeckia* species (Dahlgren, 1975). During situations where growth is impeded by overgrazing, the short-shoots dominate in growth, thus leading to a very different morphological appearance of the plants, whilst during normal growth, extension of short-shoot becomes limited and the fast growing long-shoots dominate instead (Dahlgren 1975). This variation of shoot and leaf growth during different stages of the plant's life has proven to be puzzling especially when trying to make identifications of the species (Dahlgren, 1975). All species of *Wiborgia* are characterized by deciduous mesomorphous leaves, a character suggested by Dahlgren (1975) to be an adaptation to endure the hot summer seasons and drought, and differs from typical fynbos taxa which are characterized by sclerophyllous evergreen leaves (Dahlgren, 1975). All the species are characterized by a terminal inflorescence which is generally an elongate spike-like raceme with sparsely/closely set flowers, with axillary flowers borne on pubescent pedicels (Dahlgren, 1975). Flowers are typical papillionaceous type, where the calyx is

generally small, thin and short-lobed with broad upper lobes and the corolla has quite long claws with petal colours ranging between bright/pale yellow to purple/rose (Dahlgren, 1975). The fruits of all species are indehiscent, stipulate and normally range from being single seeded to occasionally two seeded with closely pressed valves around the seeds (Dahlgren, 1975; Lewis *et al*., 2005).

Among the Crotalarieae, *Wiborgia* is characterized by the presence of wings on the walls of the pods with a typical samara-like appearance (Lewis *et al*., 2005). An exception is *W. leptoptera*, *W. obcordata*, and *W. humilis* (currently circumscribed as part of the recently described genus as *Wiborgiella humilis* (Thunb.) Boatwr. and B.-E.van Wyk) which have reduced or no wings on the pods hence have a nutlet-like appearance (Table 2.1; Dahlgren, 1975; Lewis *et al*., 2005). The morphology of the upper and lower wing is quite variable, usually the upper wing is a faintly veined (never thickened along the margin) flattened projection of the placental suture, and the lower wing is much broader and can be observed best in the fruits of *W. tenuifolia* (Dahlgren, 1975). Unlike the variable fruit morphology, the seeds have a less distinctive variation, are generally small and have a light-orange to salmon-brown colour (Dahlgren, 1975). Seeds are characterized by a prominent radicular lobe with a more or less circular hilum, with shape variable among species, e.g., ovoid and square on the broader side (*W. obcordata*) to laterally flattened with an almost rectangular (*W. mucronata*) (Table 2.1) (Dahlgren, 1975). Table 2.1 provides a summary of the morphological characteristics as well as habitat specialization, endemism and flowering periods, and other information explaining the morphology and taxonomy of *Wiborgia* species.

2.1.2 *Wiborgia* taxonomy

Wiborgia was most recently revised by Dahlgren (1975) who divided the genus into two subgenera, namely subgenus *Wiborgia* Thunb. and *Pterocarpia* R. Dahlgr., based on the presence or absence of tubercular surface on the hairs of the species. *Wiborgia* subgenus *Wiborgia* is characterized by erect ascending shrubs with sparsely pubescent branches which do not terminate in rigid thorns (Dahlgren, 1975). The leaves are borne on short pubescent petioles, the leaflets are pubescent on the lower midrib side and are narrowly obtriangular with a characteristic obcordate apex, and younger branches and leaves are covered with small hairs that have a warty covering on their surface (Dahlgren, 1975). Fruits of the species within the subgenus are elevated on both sides, are less compressed and stipulate with an irregular venation pattern, and are characterized by the absence of a distinct upper wing (Dahlgren, 1975). The species in this subgenus were regarded by Dahlgren (1975) as representative of an early evolutionary branching from *Lebeckia*-like ancestors. *Wiborgia obcordata* is the type species, and the subgenus is currently monotypic following recent taxonomic change moving *Wiborgia humilis* to the newly described genus *Wiborgiella* (Boatwright *et al*., 2008). Dahlgren (1975) recognized subgenus *Wiborgia* by the peculiar trichomes which are covered by small warty layers on

the surface of long apical cells, in contrast to the subgenus *Pterocarpia* (comprising rest of *Wiborgia*) characterized by trichomes with smooth non-warty apical cells. He further suggested a closer link between fruit specialization (the presence of wings on the fruit) with the presence of a warty layer on the trichomes of the species, whereby species with little fruit specialization (absence of wings on the fruit) also were characterized by those peculiar trichomes.

Wiborgia subgenus *Pterocarpia,* whose type species is *W. mucronata*, comprises of *W. fusca*, *W. incurvata, W. leptoptera*, *W. mucronata*, *W. monoptera*, *W. tenuifolia*, *W. tetraptera* and *W. sericea*, all characterized by erect (mainly rigid) shrubs with glabrous thorny branches which for some species may be pubescent, and covered with smooth surfaced sericeous hairs (Dahlgren, 1975). The leaves, similar to the branches, may be glabrous (e.g. *W. mucronata*) or pubescent (e.g. *W. sericea*), fruits are strongly compressed and have smooth or prominently veined walls as well as an upper wing with a size ranging between 1.5-7 mm in length (Dahlgren, 1975). The subgenus seems not entirely homogenous as variation in location and size of wings occurs (e.g. *W. tetraptera*, *W. tenuifolia*, and *W. sericea*), whilst others have fruits with an upper wing only. Dahlgren (1975) suggested that fruits lacking lateral wings represented a more primitive stage than those having lateral wings, and regarded the size of the upper wing as representing a more advanced character whilst those with narrower upper wings represented a less advanced feature.

In his taxonomic revision of *Wiborgia*, Dahlgren (1975: 66-68) came up with an evolutionary diagram (Appendix 1.1) which grouped *W. incurvata*, *W. fusca*, *W. monoptera* and *W. mucronata* together based on the absence of lateral ridges or wings on the fruits, and further *W. fusca* and *W. incurvata* were grouped together due to sharing fruits with smooth sides. Similarly, *W. sericea*, *W. tenuifolia* and *W. tetraptera* were grouped together based on the presence of lateral ridges or wings on the fruits, and further *W. tenuifolia* and *W. tetraptera* were further grouped together based on the glabrous branches. Dahlgren (1975) however pointed out that these groupings were merely speculative.

Table 2.1: Summary of the morphological and ecological characteristics of the nine *Wiborgia* species, adapted from Dahlgren (1975). Locality names sorted according to their respective subregions within the GCFR (CCR and ECR shown in bold) as defined by Manning & Goldblatt (2012) and Snijman (2013) respectively.

Species name	Morphological descriptions	Flowering period	Distribution	Habitat
***W. fusca* Thunb.**	Erect spreading greyish shrub 0.6-1.5 m, glabrous leaves and branches with weakly thorny ends. Pale greenish-yellow flowers. Fruits oval flat with a ~4mm wide dorsal wing.	August to October	**CCR**: Clanwilliam, Malmesbury; **ECR**: Calvinia, Namaqualand, Vanrhynsdorp	Mountain and lowland fynbos, clayey hills and flats on loamy sandy soils.
***W. incurvata* E. Mey.**	Low spreading well-branched shrublet 0.2-0.6 m, grey glabrous branches seldom with thorny tips, glabrous or seldom sparsely hairy leaves. Cream to pale lemon coloured glabrous flowers. Fruits papery with undulate walls and 4 mm broad wing.	June to August	**ECR**: Namaqualand, Witteberg	Granite derived soils in the renosterveld-fynbos scrub and arid fynbos at altitudes between 660-1330 m
***W. leptoptera* R. Dahlgr.**	Erect rigid thorny shrublet 0.3-1.2 m, pubescent rigid straight branches and silky-grey sericeous leaves. Light yellow flowers with pubescent pedicel, calyx and petals. Fruits with an upper crest, longitudinal wing and strongly reticulate veins on the sides.	July to mid-September	**CCR**: Cederberg, Citrusdal, Clanwilliam, Malmesbury, Piketberg	Clayey hills and foothills of the Cederberg Mountains in the fynbos- renosterveld scrub at altitudes between 130-250 m
***W. monoptera* E. Mey.**	Erect rigid thorny shrub 0.6-1 m, greyish thorny branches sparsely pubescent, glabrous or sparsely pubescent leaves. Pale yellow flowers. Fruits circular samara-like with reticulate veins and a 5 mm broad upper wing.	July to September	**CCR**: Clanwilliam; **ECR:** Calvinia, Namaqualand, Vanrhynsdorp	Karroid scrub, fynbos-renosterveld scrub, broken velds of granite hills, rocky or sandy slopes at altitudes between 500-1160 m
***W. mucronata* (L. Fil.) Druce.**	Rigid spreading thorny shrub 1-2.5 m, glabrous thorny branches and distinctly tipped leaflets. Light bright-yellow coloured flowers. Fruits flat compressed samara-like, winged and strongly	August to October	**CCR**: Clanwilliam, Ladismith, Malmesbury, Montagu, Swellendam, Paarl, Piketberg, Robertson, Tulbagh, Worcester; **ECR**: Calvinia, Namaqualand, Vanrhynsdorp	Mountain fynbos, renosterveld-fynbos scrub, as well as mixed renosterveld on clayey, sandy or gravelly soils at altitudes between 160-1260 m

	veined.			
***W. obcordata* (Berg.) Thunb.**	Erect slender willowy shrub 1.5-3 m, grey to white coloured pubescent pendulous branches. Leaves glabrous on upper sides and sparsely hairy on lower sides. Bright yellow flowers with beaked keel and pubescence along the midrib. Fruits are a small stalked nutlet with a raised narrow pointed upper ridge, with prominently veined walls.	August to October	**CCR**: Caledon, Cape Peninsula, Clanwilliam, Hopefield, Malmesbury, Mossel Bay, Paarl, Piketberg, Riversdale, Robertson, Stellenbosch, Swellendam, Tulbagh; **ECR**: Calvinia, Vanrhynsdorp	Sandy flats and slopes, sandy bushveld, sandveld, marine sand deposits at altitudes between 50-800 m
***W. sericea* Thunb.**	Erect rigid thorny shrub 0.3-1.5 m, pubescent rigid straight branches and silky-grey sericeous leaves. Light yellow flowers with pubescent pedicel, calyx and petals. Fruits strongly compressed with lateral and a 3 mm long dorsal wing.	May to September	**CCR**: Ceres, Clanwilliam, Laingsburg; **ECR**: Calvinia, Namaqualand,Vanrhynsdorp	Sandy flats and slopes, clayey or loamy soils on mountain slopes/plateaus in the fynbos-renosterveld transition at altitudes between 50-800 m
***W. tenuifolia* E. Mey.**	Erect multi-branched thorny shrub 0.4-1.5 m, glabrous branches and dull green leaves. Pink coloured flowers. Fruits papery samara-like, with dorsal, ventral and lateral flattened wings.	September to November	**CCR**: Bredasdorp, Ladismith, Riversdale, Swellendam, Worcester	Clayey soils in the fynbos-renosterveld fragments not exploited for crops, at altitudes between 160-1000 m
***W. tetraptera* E. Mey.**	Erect well-branched thorny shrub 0.4-1.5 m, straight rigid and grey glabrous branches, glabrous leaves with pubescent petioles. Cream or pale greenish-yellow flowers. Fruits circular, papery, samara-like, veined with dorsal, lateral and ventral wings.	September to November	**CCR**: Ceres, Clanwilliam, Malmesbury, Piketberg, Stellenbosch, Worcester; **ECR**: Calvinia, Vanrhynsdorp	Clayey soils, a mixture of clayey and sandy soils in the fynbos-renosterveld scrub at altitudes between 160-1000 m

2.1.3 Sources of data for phylogenetic inference

In plants, sources of DNA data normally used for phylogenetic inference are from the mitochondrial genome which is maternally inherited (Ankel-Simmons & Cummings, 1996; Mogensen, 1996), the nuclear genome which is biparentally inherited (Petit *et al*., 2005), and the plastid genome (mainly chloroplast) which is mainly maternally inherited for most angiosperms but has also been shown to be biparentally in a number of angiosperm species (Tilnet-Basset, 1976; Hu *et al*., 2008). Among these genomes, the chloroplast (within the plastid) is the smallest with a length ranging between 135 to 160 kilobasepairs (kbp) and it has received massive exploration and has been used for phylogenetic inference mainly due to its size as well as stability within cells and species; as well as the fact that rearrangements within this genome are rare enough in evolution to allow good utility of this genome in demarcating major groups (Palmer, 1987). For example, the *rbcL* gene which codes the subunit of the photosynthetic enzyme Rubisco has been used extensively to infer supra-generic relationships of angiosperms since the ground breaking publication by Chase *et al*. (1993). However, *rbcL* as a coding region is limited in its utility, as is the case with most coding regions, due to their slow rate of change, thus rendering them less informative in phylogenetic inference at lower taxonomic levels. Therefore more attention has been given to the non-coding gene regions of the chloroplast such as the *rps16* intron, trnL intron, and the trnL-F intergenic spacer, for utility in studying algae, bryophytes and vascular plants (Pennington *et al*., 2001; Klak *et al*., 2004; Kock *et al*., 2005). In addition to this, Shaw *et al*. (2005 & 2007) further reviewed and identified other non-coding regions such as trnS-trnG, trnT-trnL, rpl32-trnL, psbA-trnH (amongst others) as having good utility at generic and specific levels for various plant lineages. The use of the mitochondrial genome is not common in phylogenetic studies of plants, but has been used extensively in animal studies such as baboons (Newman, 2004), birds (Sturmbauer, 1998), and fish (Bargelloni, 2000). The reason for this may be due to most plant studies mainly focussing on recent speciation events, whilst the mitochondrial DNA (mtDNA) is suggested to be more useful in assessing ancient events due to mitochondrial genes evolving slowly (Crochet & Desmaria, 2000). Also mitochondrial genes are known to undergo frequent genomic rearrangements, as well as the incorporation of foreign DNA fragments from the other two genomes (nuclear and chloroplast), and the presence of introns and exons which disrupt the gene continuity (Knoop, 2004). Nonetheless, the mitochondrial genome also shows good utility in studying older plant lineages because the variable occurrence of introns/exons and the slow sequence evolution in plant mitochondrial DNA provides a reservoir of phylogenetic information. Several studies on plants have been carried out to infer and reconstruct the phylogeny of seed plants using the mitochondrial DNA; these include Gugerli *et al*. (2001), Soltis *et al*. (2002), Barkman *et al*. (2004) and Qui *et al*. (2005).

Nuclear genes encoding ribosomal RNA (rRNA) are arranged in tandem arrays of hundreds of thousands of copies, and are the only genes with a number of copies high enough to allow easy study

(Baldwin *et al*., 1995). These genes encode the small subunit 18S, the large subunit of the ribosome 26S, and these are both separated by the small 5.85 gene. In addition, the short internal transcribed spacer (*ITS*) lies in between the three genes, and in turn the sets of genes are separated by the large intergenic spacer (*IGS*) (Judd *et al*., 2008). Because these sequences are highly repetitive within the genome, they normally undergo a homogenization process known as concerted evolution; which is a process whereby the occurrence of a mutation in one copy sequence is either corrected by the changing of the mutated copy to match the non-mutated copies, or the non-mutated copies may be corrected to match the mutated sequence, thus resulting in nucleotide changes being propagated throughout the array (Elder *et al*., 1995). The internal transcribed spacer has been and is still used extensively in phylogenetic studies of various plant groups including legumes (e.g. Wojciechowski *et al*., 1999; Lavin *et al*., 2001; McMahon & Hufford, 2004; Edwards & Hawkins, 2007; Egan & Crandall, 2008; Boatwright *et al*., 2008; Dludlu *et al*., 2013). Recently the *ITS* was used to reconstruct phylogenetic relationships within the *Indigofera* (Indigofereae) which has ca. 750 species which have mostly diversified within the last 10 million years (my) (Schrire *et al*., 2009). The extensive use of *ITS* may be due to it evolving faster compared to the widely used chloroplast regions, thus making it very useful when exploring phylogenetic relationships of closely related genera/species (Alvarez & Wendel, 2003). The *ITS* is however subjected to some molecular genetic processes which may affect the sequences of this region and thus leading to inaccurate/confounded phylogenetic inferences (Alvarez & Wendel, 2003; Choi *et al*., 2006). The molecular genetic proceses affecting the ITS include the genetic harbouring of pseudo genes in their various states of decay as well as incomplete intra- or inter array homogenization, as well as difficulty in amplification or sequence alignment which may be caused by the high levels of variability of the *ITS* which is possibly a result of the existence of multiple copies of varying sizes and locations within the ribosomal DNA (Buckler *et al*., 1997; Choi *et al*., 2006). Despite these disadvantages of *ITS*, Feliner & Rosello (2007) still argued that although *ITS* has a number of drawbacks, it can still be used to produce insightful phylogenetic inference results provided that a set of recommendations are followed. The recommendations include careful laboratory protocols, representative sampling which follows prospective pilot studies, and mindful analysis which would make it possible to assist in reducing the inaccuracy of phylogenetic estimations when using the *ITS*. Apart from the *ITS*, another region which has also widely been used and is part of the nuclear genome is the external transcribed spacer (*ETS*), which has been used in studies (e.g. Baldwin & Marcos, 1998; Chandler *et al*., 2001; Chandler *et al*., 2003; Jousselin *et al*., 2003; Sanches-Baracaldo, 2004; Okuyama, 2005; Choi *et al*., 2006). The *ETS* is generally a more variable and relatively longer region (Bena *et al*., 1998) compared to *ITS*, thus making it possibly more suitable for use in interspecific and infraspecific phylogenetic studies. However, as discussed for *ITS* above, the *ETS* also has a similar rate of molecular evolution to the *ITS*, and is also subject to the same molecular genetic processes that affect *ITS* (Soltis *et al*., 2008). Thus the use of *ETS*, although it

is more variable and longer in length, also shares disadvantages as well as advantages similar to those of the *ITS*.

Recently, morphology as well as both the nuclear (*ITS*) and plastid (*rbcL*) markers have been used in studying the phylogenetic relationships within the Crotalarieae (Boatwright *et al*., 2008); as well as studying species relationships within several genera in the 'Cape clade' of the Crotalarieae. These include *Lebeckia* (Boatwright *et al*., 2009), *Wiborgiella* (Boatright *et al*., 2010), species identifications via DNA barcoding of *Aspalathus* (Edwards *et al*., 2008); and the most recent phylogenetic study on the Crotalarieae resulted in the monophyly of all Cape clade genera being resolved (Boatwright *et al*., 2008). Species relationships within *Wiborgia* are unknown as the most current phylogeny only inferred phylogenetic relationships using two DNA markers and included seven out of nine *Wiborgia* species (Boatwright *et al*., 2008) which may be the reasons why the produced tree was unresolved (not strongly supported). Therefore a study which includes all species within the genus, sampled multiple times from different populations is needed in order to first identify the phylogenetic relationships that exist within the genus and also to identify if all species are genetically uniform across their entire distribution range, as Dahlgren (1975) pointed out that the species are morphologically variable across their distribution range. This study aims to explore the phylogenetic relationships within the genus, with all the species represented by several accessions, and to test Dahlgren (1975) hypotheses on taxonomy and evolution.

Objectives

i. To test the monophyly of *Wiborgia* and infer phylogenetic relationships within the genus using multiple molecular markers.

ii. To test the support of Dahlgren's (1975) morphological subgeneric classification using molecular markers

Hypothesis

- *Wiborgia* is monophyletic and sister to *Wiborgiella*

2.2 Materials and Methods

A total of 33 samples covering all nine *Wiborgia* species (ingroup taxa) were studied, and multiple populations of most species were sampled across the distribution range of the species thus covering the taxonomic and morphological range. In addition, 14 outgroup taxa (*Aspalathus*, *Calobota*, *Rafnia*, *Lebeckia*, and *Wiborgiella)* were analysed covering representative sister lineages, as identified in previous studies (Boatwright *et al*., 2008). All outgroup tissues samples were obtained from an existing collection made as part of other legume studies in the GCFR, whereas all *Wiborgia* leaf

tissues were collected as part of this study, where different accessions across multiple populations were given different voucher numbers (Table 2.2) and dried in silica gel.

2.2.1 Plant DNA extraction, amplification and sequencing

2.2.1(a) Extraction

Plant DNA was extracted from silica gel dried leaf material using a modified Cetyltrimethylammonium Bromide (CTAB) protocol by Doyle & Doyle (1987) and Gawel & Jarret (1991). However extraction using the CTAB protocol proved ineffective for most samples possibly due to the plants having higher levels of secondary compounds which interfered with the extraction process. Therefore the DNA of most samples was extracted using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, Cape Town, South Africa) following the manufacturer's protocols. For those few samples extracted using the CTAB protocol, the method involved mixing the CTAB extraction buffer with mercapto-ethanol in the ratio 700:1 respectively, and then incubating the mixture at 65°C in a water bath. About 20 mg of the silica gel dried plant material was weighed and placed into a 2 ml Eppendorf™ tube, the material was ground into fine powder by placing two silver ball grinders in each tube and the tubes placed in the Retsch MM400 ball-grinder at 30 Hz/s for 30 minutes. After the grinding, 700 µL of the pre-heated CTAB extraction buffer was added to each of the ground samples, the mixture was then vortexed in order to obtain a homogenous mixture. The samples were then incubated at 65°C using a heating block for 60 minutes, and at every 15 minute interval the samples were gently shaken by inversion. Afterwards, 600 µL of a mixture made up of 24/1 v/v chloroform:isoamyl alcohol, was added to each of the samples and mixed by inversion for 5 minutes and then centrifuged for 5 minutes at 12 000 rpm. After centrifuging, the supernatant was then carefully pipetted out from the 2 ml Eppendorf™ tube and placed into a clean 1.5 ml tube and an equal volume of ice-cold isopropanol was added to the pipetted-out supernatant, these were then briefly mixed by inversion. The samples were then placed in the -20°C freezer for 24-48 hours in order to allow DNA precipitation. Post the precipitation stage, the DNA pellet could in most cases be observed as a visible white to brownish precipitate at the base of the tube; in order to recover the DNA pellet, the chilled samples were centrifuged for 5 minutes at 12 000 rpm. The isopropanol was then carefully discarded and the tubes were left open, inverted and placed on laboratory paper towel in order to allow drainage of excess residual liquid; during the discarding of the isopropanol, great care and attention was applied in order to ensure that the pellet was not lost. After leaving the tubes inverted for 10 minutes, residual droplets were wiped off the rim of the tube; then 250 µL of 75% ethanol was added to the tubes in order to wash the DNA pellets. The ethanol was then discarded and the DNA pellets were air dried by leaving the tubes open on the bench top for about 30 minutes. Once dried, the DNA pellet was then suspended in 50 µL of sterile PCR water, and stored in a fridge at 4°C to await subsequent amplification experiments.

Table 2.2 Taxa and voucher information of all accessions used in this study. All samples were amplified and sequenced as part of this study. Successfully amplified sequences are denoted by a + ; - denotes poor quality sequences or those samples which could not be amplified with several attempts. Collectors were AM Muasya (AMM), N Moiloa (NM) and CH Stirton (CHS). All herbarium specimens for the collections made as part of the study will be deposited at the Bolus Herbarium (BOL) as part of a manuscript to a peer reviewed journal for publication which is still in preparation.

Taxon name	**Voucher details**	**Locality**	**DNA loci sampled**				
			ITS	**rpl32-trnL**	**trnS-trnG**	***rps16***	**trnT-trnL**
Aspalathus pallidiflora Thunb.	AMM5100	Rawsonville valley	+	+	+	+	+
Aspalathus retroflexa L.	AMM6405	Rhodes Memorial	+	+	+	+	+
Aspalathus macrocarpa Eckl & Zeyh.	AMM5542	Barrydale	+	+	+	+	+
Aspalathus empetrifolia (Dahlg.) Dahlg.	CHS13201	Mitchel's Pass	+	+	+	+	+
Lebeckia cytisoides L.	AMM5379	Steenberg cave	+	+	+	+	+
Lebeckia sp.	AMM6927	Grootvlei	+	+	+	+	+
Lebeckia uniflora B-E van Wyk and M.M Le Roux	AMM6660	Kogelberg	+	+	+	+	+
Lebeckia wrightii (Harv.) Bolus.	AMM5677	Houwhoek mountains	+	-	+	+	+
Rafnia angulata Thunb. ssp *angulata*	AMM5486	Cape Point	+	+	+	-	-
Rafnia diffusa Thunb.	AMM4808	Clanwilliam	+	+	+	+	+
Rafnia triflora (L.) Thunb.	AMM5038	Stilbaai	+	+	+	+	+
Wiborgia fusca Thunb. ssp fusca	NM10	Elandsbaai	-	+	+	+	+
Wiborgia fusca Thunb. *ssp fusca*	AMM6920	Garies-Kamieskroon	+	+	+	+	+
Wiborgia fusca Thunb. *ssp fusca*	AMM6924	Grootvlei-Soebatsfontein	+	+	+	+	+
Wiborgia fusca Thunb. ssp fusca	NM11	Lambertsbaai	+	+	-	+	+
Wiborgia incurvata E. Mey	AMM6928	Leliefontein	+	+	+	+	+
Wiborgia leptoptera R. Dahlgren. *ssp leptoptera*	NM6	Darling	+	+	+	+	+
Wiborgia monoptera E. Mey	AMM6922	Grootvlei-Soebatsfontein	-	+	+	+	+
Wiborgia monoptera E. Mey.	AMM6937	Studer's Pass	+	+	+	+	+
Wiborgia monoptera E. Mey.	AMM6938	Studer's Pass	+	+	+	+	+

Wiborgia monoptera E. Mey.	AMM6941	Soebatsfontein	+	+	-	+	+
Wiborgia mucronata (L.F.) Druce	NM19	Brandvlei dam	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	NM14	Botterkloof Pass	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	NM15	Botterkloof Pass	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	AMM6932	Citrusdal	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	NM3	Darling reserve	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	NM5	Darling-Mamre	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	AMM6921	Garies-Kamieskroon	+	+	-	+	+
Wiborgia mucronata (L.F.) Druce	AMM6912	Khoisan’s kitchen	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	AMM6930	Studer’s Pass	+	+	+	+	+
Wiborgia mucronata (L.F.) Druce	NM1	Wellington	+	+	-	+	+
Wiborgia mucronata (L.F.) Druce	AMM6936	Worcester	+	+	+	+	+
Wiborgia obcordata (P.J. Bergius.) Thunb.	NM20	Brandvlei	+	+	+	+	+
Wiborgia obcordata (P.J. Bergius.) Thunb.	CHS13742	Gifberg plateau	+	+	+	+	-
Wiborgia obcordata (P.J. Bergius.) Thunb.	NM45	Kommetjie	+	-	-	+	-
Wiborgia obcordata (P.J. Bergius.) Thunb.	AMM6931	Vanrhynsdorp	+	+	+	+	+
Wiborgia obcordata (P.J. Bergius.) Thunb.	NM2	Vredenburg	-	+	+	+	+
Wiborgia sericea Thunb.	NM41	Ceres	-	-	-	+	-
Wiborgia sericea Thunb.	AMM6923	Grootvlei-Soebatsfontein	+	+	+	+	+
Wiborgia sericea Thunb.	AMM6942	Khoisan’s kitchen	+	+	+	+	+
Wiborgia tenuifolia E. Mey.	AMM5446	Brandvlei	+	+	+	+	+
Wiborgia tenuifolia E. Mey.	NM18	Brandvlei	+	+	+	+	+
Wiborgia tetraptera E. Mey.	NM12	Lambertsbaai	+	+	-	+	+
Wiborgia tetraptera E. Mey.	NM13	Lambertsbaai	+	+	-	+	+
Wiborgiella bowieana (Benth.) Boatwr and B.-E. van Wyk.	CHS13568	Uitvlugt farm	+	+	+	+	+
Wiborgiella humilis (Thunb.) Boatwr and B.-E. van Wyk	CHS13751	Gifberg plateau	+	+	+	+	+
Wiborgiella inflata (Bolus) Boatwr and B.-E. van Wyk	CHS13780	Kogelberg nature reserve	+	+	+	+	+

2.2.1(b) Screening of molecular markers and DNA amplification (PCR)

A number of markers were screened for successful amplification, sequence quality and the level of sequence variation between species. One nuclear and eight chloroplast regions (Table 2.3) were screened by performing PCR amplification reactions with 5 selected samples of *Wiborgia* for each of the abovementioned markers. Reaction volumes for all regions were made up to a total volume of 30 µL, each made up of 3 µL buffer; 3 µL $MgCl_2$; 1.2 µL dNTP's; 0.2 *Taq* polymerase; 1 µL forward primer; 1 µL reverse primer; 18.6 µL of sterile PCR water and 2 µL of template DNA. All PCR amplifications were run on Applied Biosystems GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR thermal profiles for all the regions are summarized below, a key to the shorthand code used to describe the thermal profiles is as follows: initial denaturation (temperature and time); number of cycles [denaturation temperature and time; annealing temperature and time; chain extension temperature and time], and final extension (temperature and time), then all reactions were held at a temperature of 4°C.

The primers for the different regions are given in Table 2.3 below, and thermal profiles are as follows:

- For psbA-trnH^{GUC} thermal parameters were 80°C for5 min; 35 cycles of (94°C for 30 s; 50–56°C for 30 s; 72°C for 1 min); 72°C for 10 min (Shaw *et al*., 2005).
- For trnS^{GCU}-trnG^{UUG} thermal parameters were 80°C for 5 min; 30 cycles of (95°C for 1 min; 66°C for 4 min); 66°C for 10 min (Shaw *et al*., 2005).
- For trnT^{GGU}-trnD^{GUC} thermal parameters were 80°C for 5 min; 30 cycles of (94°C for 45 s; 52-58°C for 30 s; 72°C for 1 min); 72°C for 5 min (Shaw *et al*., 2005)
- For trnF^{GAA}-trnL^{UAA} thermal parameters were 80°C for 5 min; 35 cycles of (94°C for 1 min; 50°C for 1 min; 72°C for 2 min); 72°C for 5 min (Shaw *et al*., 2005).
- For rps12-rpl20 thermal parameters were 96°C for 5 min; 35 cycles of (96°C for 1 min; 50–55°C for 1 min; 72°C for 1 min); 72°C for 5 min (Shaw *et al*., 2005).
- For rps16 thermal parameters were 80°C for 5 min; 35 cycles of (94°C for 30 s; 50–55°C for 30 s; 72°C for 1 min); 72°C for 5 min (Shaw *et al*., 2005).
- For trnT-trnL thermal parameters were 80°C for 5 min; 35 cycles of (94°C for 1 min; 50°C for 1 min; 72°C for 2 min); 72°C for 5 min (Shaw *et al*., 2005).
- For rpl32-trnL^{UAG} thermal parameters were 80°C for 5 min; 30 cycles of (95°C for1 min; 50°C for1 min; 65°C for 4 min); 65°C for 5 min (Shaw *et al*., 2007).
- For *ITS* thermal parameters were 94°C for 3 min; 45 cycles of (94°C for1 min; 55°C for 1 min; 72°C for 4 min); 72°C for 5 min (Varela *et al*., 2004)

Successful amplification of the target regions was determined by loading, through pipetting, 3-5 µL of each PCR product onto a 1% agarose gel stained with ethidium bromide. The loaded gel was then

placed in an electrophoresis tank containing 0.5x TBE buffer, and the electrophoresis was run at 100 volts (V) for 15-30 minutes. The gel was then viewed under ultraviolet (UV) light for visualization of the fluorescing DNA bands, a photo of the gel was taken at 0.200 seconds to visualise the samples which successfully amplified; this was followed by visual inspection of the DNA bands on the photo. All successful amplicons were sent unpurified to University of Stellenbosch DNA sequencing facility; or purified using a modified version of the Exo/Sap enzyme cleaning protocol (Werle *et al*., 1994; Lemaire *et al*., 2015) and sent to Macrogen in Netherlands (https://www.macrogen.com), for sequencing using the same primer pairs initially used for the PCR amplifications.

Table 2.3: Primers and their corresponding sequences used for amplification and sequencing of the different DNA regions as part of this study. F/R= forward and reverse respectively.

Region	Name(Code)	F/R	Sequence	Reference
ITS	ITS5 F	F	GGA AGT AAA AGT CGT AAC AAG G	White *et al*. (1990)
	ITS4 R	R	TCC TCC GCT TAT TGA TAT GC	White *et al*. (1990)
trnT-trnL	trnL^{UAA}(H5) F	F	CGA AAT CGG TAG ACG CTA CG	Taberlet *et al*. (1991)
	trnT^{UGU}(H6) R	R	CAT TAC AA TGC GAT GCT CT	Taberlet *et al*. (1991)
rpl32-trnL	trnL$^{(UAG)}$(V1) F	F	CTG CTT CCT AAG AGC AGC GT	Shaw *et al*. (2007)
	rpL32-F(V2) R	R	CAG TTC CAA AA A AAC GTA CTT C	Shaw *et al*. 2007)
rps16	rps16(B1) F	F	GTG GTA GAA AGC AAC GTG CGA CTT	Shaw *et al*. 2005)
	rps16(B2) R	R	TCG GGA TCG AAC ATC AAT TGC AAC	Shaw *et al*. 2005)
trnS-trnG	trnS^{GCU}(C4) F	F	GCC GCT TTA GTC CAC TCA GC	Hamilton (1999)
	3'trnG^{UUG}(C1) R	R	GAA CGA ATC ACA ATT TTA CCA C	Hamilton (1999)
trnL-trnF	trnF^{GAA}(H1) R	R	ATT TGA ACT GGT GAC ACG AG	Taberlet *et al*. (1991)
	5'trnL^{UAA}(H4) F	F	CGA AAT CGG TAG ACG CTA CG	Taberlet *et al*. (1991)
trnD-trnT	trnT^{GGU}(F1) R	R	CTA CCA CTG AGT TAA AAG GG	Demesure *et al*. (1995)
	trnD^{GUC}F(F4) F	F	ACC AAT TGA ACT ACA ATC CC	Demesure *et al*. (1995)
rps12-rpl20	5'rps12(I1) F	F	ATT AGA AA(CTAG) (AG)CA AGA CAG CCA AT	Shaw *et al.* (2005)
	rpl20(I2) R	R	CG(CT) (CT)A(CT) CGA GCT ATA TAT CC	Shaw *et al.* (2005)
psbA-trnH	psbA(A7) F	F	GTT ATG CAT GAA CGT AAT GCT C	Sang *et al*. (1997)
	trnH^{GUG}(A5) R	R	CGC GCA TGG TGG ATT CAC AA TC	Tate and Simpson (2003)

2.2.1 (C) Sequence alignment and phylogenetic analysis

Sequences were assembled and edited using Staden package version 2.0.0b8 (Staden *et al.,* 1998). The consensus sequences were then imported into Bioedit version 7.2.0 (Hall, 1999), and initially aligned electronically using the ClustalW multiple alignment tool on Bioedit version 7.2.0 (Hall, 1999) or

Mega version 6 (Tamura *et al*., 2013). The alignments were manually inspected and edited, where any remaining misaligned residues were aligned manually.

The gene regions which were ultimately selected for phylogenetic analyses in this study were *ITS*, rpl32-trnL, *rps16*, trnS-trnG, trnT-trnL. All regions were first analysed separately, then the plastid data were concatenated and analysed separate from the nuclear data. A test of data combinability, the incongruence length difference (ILD) test, was then carried out to determine if there was any incongruence between the individual data and also the chloroplast versus the nuclear data. Furthermore, the individual trees were also subjected to a comparison in order to test for well-supported conflict (Bergh, 2009) which is defined in terms of bootstrap value (75%) and posterior probability (0.99). These two methods of incongruence/conflict testing were done because the ILD test has been reported in literature to show high rates of type I error (Planet, 2006; Yoder *et al*., 2001; Barker & Luzoni, 2002) and is difficult to interpret when dealing with more than two data partitions (Planet, 2006; Berg, 2009). Results of the ILD test are shown in Table 2.4 below, and comparison of the individual trees show no conflict (congruency with *p*-vales above 0.05), therefore the five regions were then concatenated and analysed. Taxa which could not be amplified or sequenced for either one of the five data partitions and thus absent, were coded as missing.

Table: 2.4 Summary of ILD analyses testing for congruency between all five DNA regions used as part of this study. The numbers represent *p*-values.

ITS	rpl32	rps16	trnS-trnG	trnT-trnL	
-	0.141	0.441	0.344	0.086	**ITS**
	-	0.467	0.403	0.423	**rpl32**
		-	0.906	0.894	**rps16**
			-	0.681	**trnS-trnG**
				-	**trnT-trnL**

Phylogenetic analyses were then conducted using Maximum Likelihood (ML) and Bayesian Inference (BI) criteria, both carried out on the CIPRES web portal (https://www.phylo.org/). Model tests for all regions, including the concatenated chloroplast and concatenated nuclear-chloroplast, were conducted using Mega version 6 (Tamura *et al*., 2013) under the Akaike Information Criterion (AIC) (Akaike 1974). The model that best fit all matrices according to the AIC was the Tamura-3-parameter model (T92), with *ITS*, *rps16*, trnT-trnL, trnS-trnG, combined plastid and all combined markers having gamma-distributed rate variation across sites (+G). The T92 model was then specified for each of the regions during the BI and ML analyses, and also for the combined matrices. This best-fitting model of DNA substitution was applied for each separate dataset and in the combined analyses, the five-gene dataset was partitioned and the same models were assigned to separate unlinked partitions. The BI analyses were carried out using the MrBayes v.3.2.3 on XSEDE tool (Ronquist & Helsenbeck 2003);

running two simultaneous runs, where each run had four simultaneous Markov chains with one cold and three heated, with a temperature setting of 0.20. The Markov chain was run for five million generations, sampling a tree every 1000 generations. The first 25% of the trees sampled were regarded as 'burn-in' and thus discarded and not included in the analysis when posterior probabilities were calculated. ML analyses were carried out using the RAxML-VI-HPS v2.2.3, using the GTR-Gamma as the most complex model of substitution best fitting the data (Stamatakis, 2006). Evaluation of support was done based on analyses with a multi-parametric bootstrap resampling of 1000 replicates. The 50% majority rule consensus trees were then viewed in FigTree version 1.4.2 (Rambaut, 2014).

2.3 Results

2.3.1 Screening for molecular markers

From all the markers screened *ITS*, rpl32-trnL, rps16, trnS-trnG, and trnT-trnL amplified well for about 90% of the total number of accessions used in this study, and were the ones ultimately used for phylogenetic analyses. For the five regions selected to be utilized for phylogenetic analyses, some samples could not be amplified or resulted in poor quality sequences despite several attempts (Table 2.2). The other markers screened (trnL-F, trnD-trnT, rps12-rpl20, *ETS*, psbA-trnH) could not be used for phylogenetic analyses mainly due to the low sequence variability of the few accessions used for the screening, or in other cases the inability to amplify most of the samples despite several attempts with varied PCR thermal profiles (especially the annealing temperature) as well as template DNA concentrations. For *ETS*, none of the samples could be successfully amplified and sequenced, except for the sample of *W. fusca*_AMM6920 which showed unspecific binding resulting in multiple amplification bands which suggested that the primers used may have not match sequences of the Crotalarieae taxa studied.

2.3.2 Data matrices and models of sequence evolution

The aligned *ITS* matrix had 40 taxa (Table 2.5), 12 of which were outgroups whilst the remaining 28 taxa were different accessions belonging to the nine species of *Wiborgia*. The entire matrix had 585 characters, with 52 of those characters being parsimony informative (Table 2.5). Similarly, the rpl32-trnL matrix had 43 taxa (Table 2.5), 12 of which were outgroups whilst the remaining 31 were different accessions belonging to the nine species of *Wiborgia*. The entire rpl32-trnL matrix had 460 characters, with 30 of those characters being parsimony informative. The rps16 matrix on the other hand had 42 taxa, of which 11 were outgroups whilst the remaining 31 taxa were different accessions belonging to the 9 species of *Wiborgia*. The entire rps16 matrix had 692 characters, of which 31 of

those characters were parsimony informative (Table 2.5). The trnS-trnG had a total number of 45 taxa, of which 14 of those taxa were outgroups whilst the remaining 31 taxa were different accessions belonging to the 9 species of *Wiborgia* (Table 2.5). The entire trnS-trnG matrix had 763, of which 78 of those characters were parsimony informative (Table 2.5). And lastly, the trnT-trnL matrix had a total number of 39 taxa, of which 10 of those were outgroups whilst the remaining 29 taxa were different accessions belonging to the nine species of *Wiborgia* (Table 2.5). The entire trnT-trnL matrix had 729 characters, of which 84 of those characters were parsimony informative. Of the five data matrices corresponding with the five DNA loci used in this study, trnT-trnL had the highest number of parsimony informative characters and variable sites, whilst rpl32-trnL had the lowest (Table 2.5). In addition, trnS-trnG had the highest number of conserved characters whilst rpl32-trnL had the lowest. Moreover, rpl32-trnL also had the lowest number of singletons, whilst rps16 had the highest (Table 2.5).

The five DNA datasets were then concatenated as they were identified to be congruent based on the ILD test (Table 2.4). And the combined plastid matrix made up of four markers had a total number of 46 taxa, of which 14 of those taxa were outgroups and the remaining 32 accessions belonged to the ingroup (*Wiborgia*) The combined plastid matrix had a total of 2647 characters, of which 223 of those characters were parsimony informative (Table 2.5). On the other hand, the combined plastid and nuclear matrix also had 46 taxa, of which 14 of those taxa were outgroups and the remaining 32 accessions belonged to the 9 species of *Wiborgia*. The combined nuclear and plastid matrix had a total of 3232 characters, of which 275 of those characters were parsimony informative (Table 2.5). For all five DNA loci, the Tamura-3-parameter (T92+G) model was identified as the one that best explains the sequence evolution according to the Akaike Information Criterion (AIC).

Table 2.5: Summary of DNA data matrices.

Data description	DNA loci						
	ITS	rpl32-trnL	rps16	trnS-trnG	trnT-trnL	Concatenated plastid	Concatenated all
Number of taxa in matrix	40	43	42	45	39	46	46
Number of characters in the matrix	585	460	692	763	729	2647	3232
Number of conserved characters	512	366	639	651	615	2274	2786
Number of parsimony informative characters	52 (9%)	30 (7%)	31 (5%)	78 (10%)	84 (12%)	223 (8%)	275 (9%)
Number of singletons	17	13	22	17	21	73	90
Variable sites	69	43	53	95	105	296	365

2.3.4 Phylogenetic relationships

2.3.4 (a) Trees from individual DNA loci

(i) ITS

The *ITS* datas*et al*one produced similar ML and BI trees, with strong support for the deeper nodes, whilst in the terminal nodes there was formation of polytomies. The *ITS* tree showed the *Lebeckia*-*Rafnia* clade sister to a polytomy which includes *Aspalathus*, *Calobota* and *Wiborgia*-*Wiborgiella* clades. *Wiborgia* was strongly supported as monophyletic (PP=1; BS=91), and within the *Wiborgia* clade, two subclades could be observed; the first subclade was only made up of accessions of *W. obcordata*, which was weakly supported (PP=0.67; BS=61). The second subclade includes the rest of genus, and is further subdivided to smaller clades, where the one of those clades is made up of a polytomy between *W. fusca*, *W. mucronata* and *W. tetraptera* (strongly supported PP=0.97; BS=85); the second of these smaller clades was made up of *W. incurvata*, and W. *monoptera* (also strongly supported P=1; BS=99); and these smaller subclades however formed a polytomy with *W. sericea*, *W. leptoptera*, and *W. tenuifolia* [Figure 2.1 (a)].

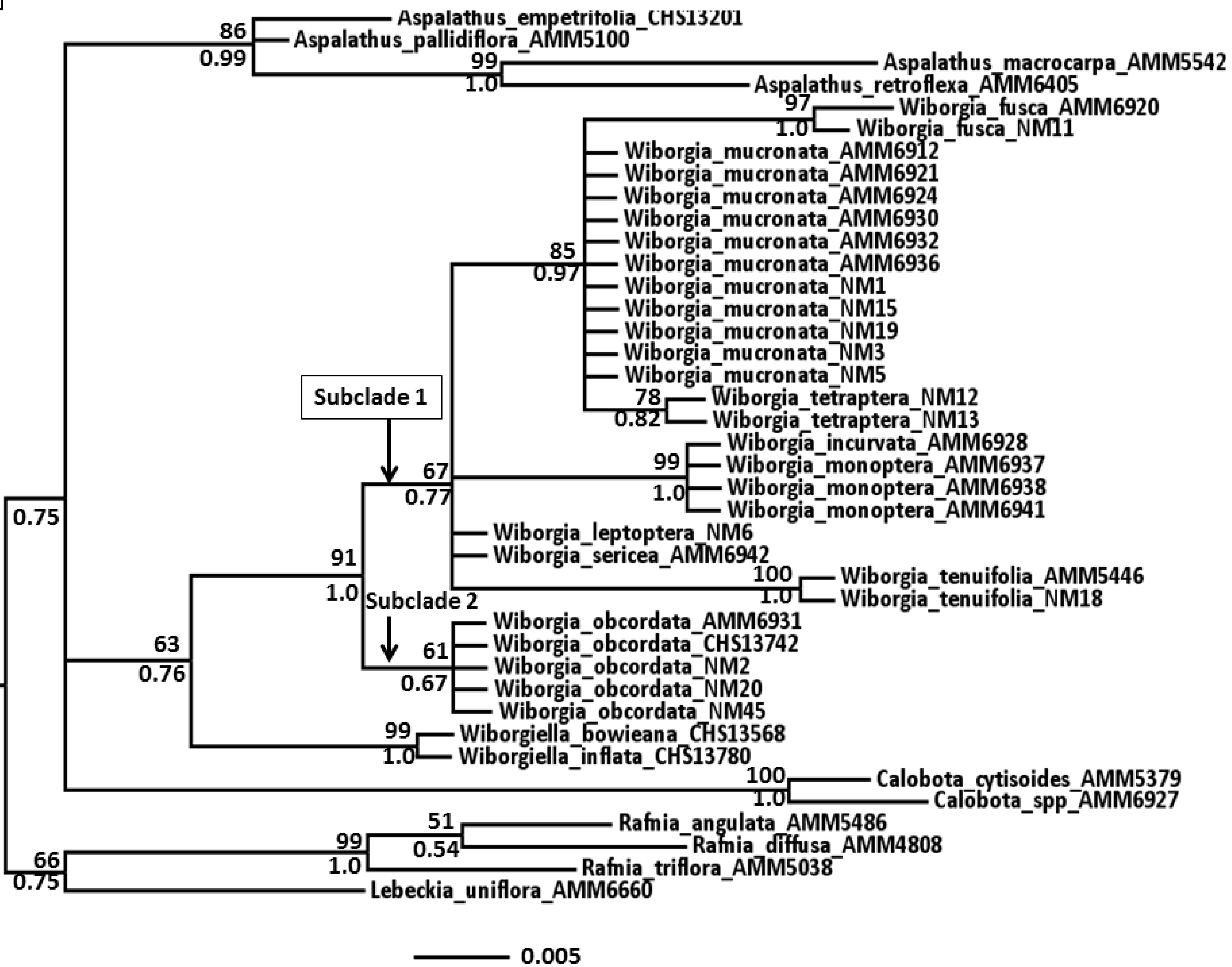


Figure 2.1 (a): 50% majority rule consensus tree from the analysis of the (i) ITS data set for the members of the "Cape clade" of the Crotalarieae. Numbers above branches are bootstrap percentages from maximum likelihood analysis and those below are posterior probabilities from the Bayesian analysis (only values above 50% and 0.50 are shown for the bootstrap and posterior probability respectively.

(ii) rpl32-trnL

The similar rpl32-trnL (BI and ML trees) showed the *Wiborgia* clade strongly supported as monophyletic (PP=1; BS=99), but differed from the *ITS* phylogeny as *W. obcordata* is not sister to rest of species. Two subclades could be identified where the first well-supported (PP=0.97; BS=89) subclade was made up of a polytomy between *W. fusca*, *W. incurvata*, *W. monoptera*, *W. tetraptera*, *W. sericea*, and *W. obcordata*. The second well-supported subclade (PP=1; BS=98) comprised several accessions of *W. mucronata* from Piketberg, Grootvlei, Studer's Pass, together with *W. tenuifolia* and other *W. mucronata* accessions. The rest of the other *W. mucronata* accessions from Citrusdal, Wellington, Brandvlei, as well as one accession of *W. sericea* from Cederberg all formed a at the base of the *Wiborgia* clade [Appendix 1.2(a)i].

(iii) rps16

The similar *rps16* BI and ML trees) showed a strongly supported monophyletic clade of *Wiborgia* species (PP=1; BS=96), and this clade further subdivided into two subclades where the first subclade was made up of a polytomy between multiple accessions of *W. sericea*, *W. fusca*, *W. incurvata*, *W. leptoptera*, *W. monoptera*, *W. obcordata*, and *W. tetraptera*; this entire clade was moderately supported (PP=0.82; BS=68). On the other hand the other smaller subclade revealed a well-supported sister relationship between *W. mucronata* and *W. tenuifolia* (BS=92; PP=1). The sister relationship between *Wiborgia* and the outgroup *Aspalathus*-*Wiborgiella* clade was weakly supported (PP=0.72; BS=63) [Appendix 1.2(a)ii].

(iv) trnS-trnG

The similar trnS-trnG (BI and ML trees) showed resolved deeper nodes, with *Wiborgia* being moderate to strongly supported as monophyletic (PP=1; BS=88). Within the *Wiborgia* clade, two well supported subclades could be identified; one subclade was made up of a polytomy formed by *W. tenuifolia* and *W. mucronata* (PP=1; BS=95), whilst the other subclade was also made up of a polytomy formed by *W. monoptera*, *W. incurvata*, *W. sericea*, *W. tetraptera*, *W. fusca*, and *W. obcordata* (BS=89; PP=0.99). The *Wiborgia* clade formed a polytomy with the *Aspalathus* and *Wiborgiella* clades, which were all strongly supported as monophyletic (PP=1; BS=97 for both genera) (PP=1; BS=100 for all three genera) [Appendix 1.2(b)iii].

(v) trnT-trnL

The trnT-trnL datas*et* produced a weakly supported, but similar BI and ML trees, especially for the terminal nodes; however the deeper nodes for most major clades were all strongly supported. The *Wiborgia* clade was strongly supported as monophyletic (PP=1; BS=100), and within this clade two subclades could be observed; the first subclade was made up of a moderately supported sister pair (PP=0.89; BS=70), *W. tenuifolia* and *W. mucronata*. Within the *W. mucronata* clade, accessions from

Darling-Mamre formed their own well-supported clade (PP=1; BS=90), and similarly the accessions from Grootvlei, and Piketberg also formed their own well supported clade (PP=0.98; BS=77). The second subclade within the *Wiborgia* clade had *W. sericea* sister to a polytomy made up of a well-supported clade of *W. obcordata* (PP=1; BS=83); a strongly supported sister relationship (PP=0.98; although weakly supported in the ML analysis with a BS of 66) between *W. fusca* and *W. tetraptera*; as well as another well supported sister relationship (BS=65; PP=0.97) between *W. incurvata* and *W. monoptera*; as well as *W. leptoptera* (PP=0.59; BS=63) [Appendix 1.2(b)iv].

2.3.4 (b) Concatenated datasets

(a) Plastid

Based on the combined plastid data, the ingroup clade is strongly supported from trees of both ML and BI analyses (BS=100; PP=1) thus showing that *Wiborgia* is monophyletic. Within the *Wiborgia* clade two strongly supported subclades [Bootstrap (BS) =93-100%; Posterior probability (PP) =1] could be identified, and subclade 2 shows *W. tenuifolia* as sister to *W. mucronata* and subclade 1 has *W. sericea* as sister to rest of taxa in the genus. The first subclade [subclade 1 on Figure 2.1 (b)] is made up of *W. fusca*, *W. tetraptera*, *W. incurvata*, *W. leptoptera*, *W. monoptera*, *W. sericea*, and *W. obcordata*. Within this first subclade, a strongly supported sister relationship between *W. fusca* and *W. tetraptera* all from the Elandsbaai-Lambertsbaai region was observed (PP=1; BS=94). The other accession of *W. fusca* from Piketberg did not form a clade with the rest of *W. fusca* accessions, thus resulting in *W. fusca* being paraphyletic. Another sister relationship between *W. incurvata* and *W. monoptera* is observed (PP=1; BS=69), whereby *W. incurvata* only formed a clade with accessions of *W. monoptera* from Grootvlei and Studer's Pass, whilst the other two accessions of *W. monoptera* came out at the base of the clade, thus presenting *W. monoptera* as paraphyletic. *Wiborgia sericea* is strongly supported as monophyletic and successively sister to the sister pair of *W. incurvata*-*W. monoptera*, *W. fusca*-*W. tetraptera* as well as *W. leptoptera* the *W. obcordata* clade [Figure 2.1 (b)]. The second subclade [subclade 2 Figure 2.1 (b)] observed was made up only of the strongly supported sister relationship between *W. tenuifolia* and *W. mucronata* (PP=1; BS=100). Within the *W. mucronata* clade, the accessions from the Darling-Mamre, Brandvlei, Citrusdal and Wellington area formed a well-supported distinct clade (PP=0.90; BS=69), with those accessions from Darling-Mamre further forming another smaller well-supported clade (PP=1; BS=98). Still within the *W. mucronata* clade, accessions from Studer's Pass, Piketberg, and Grootvlei similarly formed a smaller distinct clade within the bigger *W. mucronata* clade (BS=95; PP=1). The first outgroup clade is that made up of a moderately supported sister relationship between *Aspalathus* and *Wiborgiella* (PP=0.95; BS=86), with that clade successively being sister to the entire *Wiborgia* clade (PP=1; BS=99). Within the *Wiborgiella* clade, a sister relationship is observed between *Wiborgiella bowieana* and *Wiborgiella*

inflata which is weakly supported (PP=0.55; BS=81), and these were strongly supported as both sister to *Wiborgiella humilis* (PP=1; BS=100). The second outgroup clade was made up of *Lebeckia* (sensu stricto) which was strongly supported as sister to *Rafnia* (PP=0.98; BS=93), and these were successively sister to *Calobota* (PP=1; BS=99). Clades for all the genera used as outgroups were strongly supported as monophyletic (PP=1; BS=100 for each of the genera) [Figure 2.1 (b)]

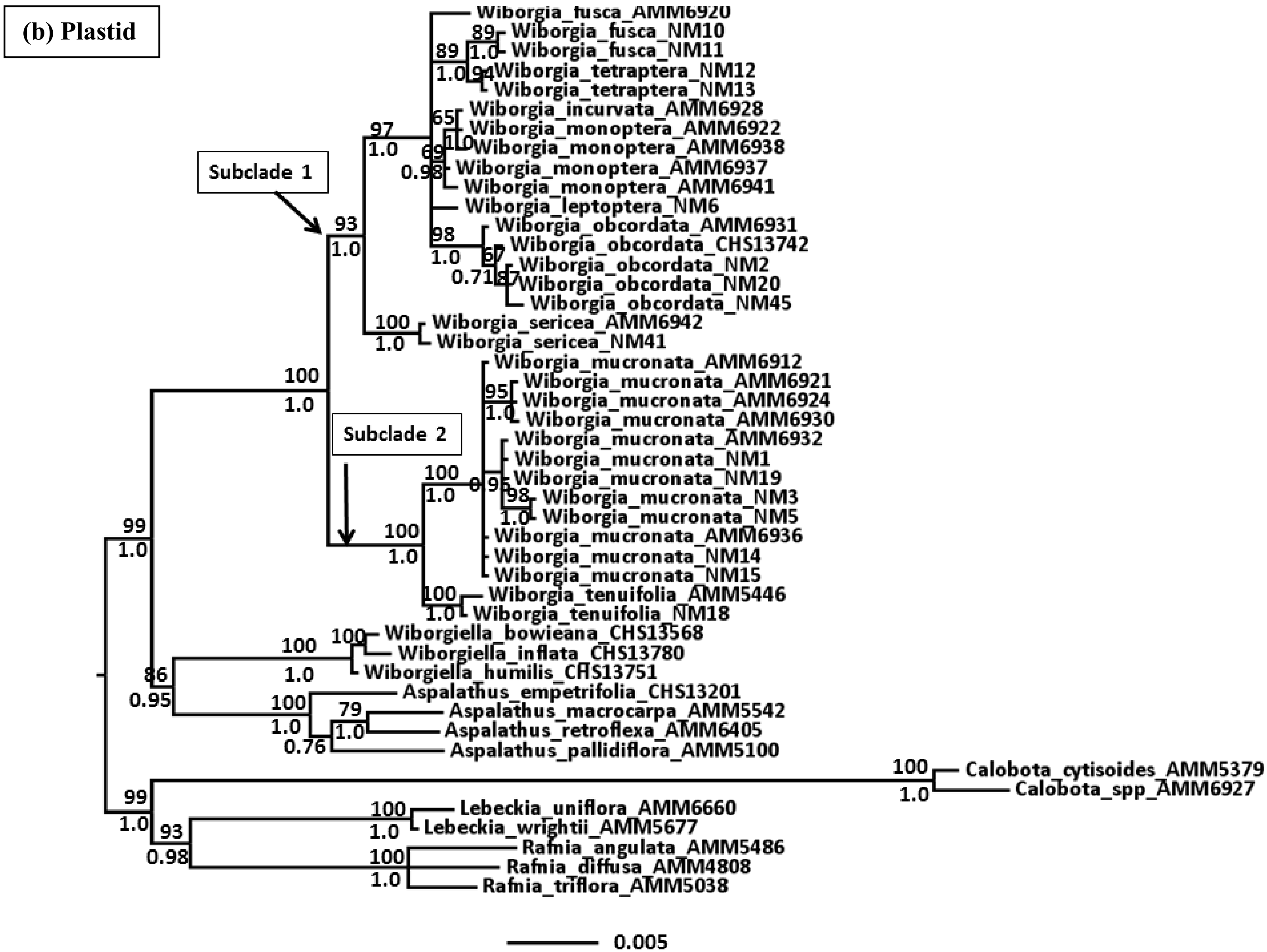


Figure 2.1 (b): 50% majority rule consensus tree from the analysis of the (a) combined plastid data sets for the members of the "Cape clade" of the Crotalarieae. Numbers above branches are bootstrap percentages from maximum likelihood analysis and those below are posterior probabilities from the Bayesian analysis (only values above 50% and 0.50 are shown for the bootstrap and posterior probability respectively.

(b) Nuclear and plastid

The combined nuclear and plastid data, from both the ML and BI analyses, shows *Wiborgia* is strongly supported as monophyletic (BS=100; PP=1) and sister to the *Wiborgiella*-*Aspalathus* alliance (PP=1; BS=99). Within the *Wiborgia* clade two strongly supported (BS=99-100%; PP=1) subclades could be identified, and these show *W. tenuifolia* as sister to *W. mucronata* (subclade 1), and *W. sericea* as sister to rest of taxa in the genus (subclade 2). Within the *Wiborgia* clade, the first subclade [subclade 1 Figure 2.1 (c)] was made up of *W. fusca*, *W. tetraptera*, *W. incurvata*, *W. monoptera*, *W. leptoptera*, and *W. obcordata*. Within this same clade a strongly supported sister relationship between *W. fusca* and *W. tetraptera* was observed (PP=1; although moderately BS=88). The accessions of *W. fusca* from Lambertsbaai-Elandsbaai region formed one well supported clade which is sister to that of the accession of *W. fusca* collected from Piketberg (PP=1; BS=88). *Wiborgia monoptera* was observed to be paraphyletic where several accessions formed part of a small clade with *W. incurvata* whilst the rest of the *W. monoptera* accessions remained at the base of the entire *W. incurvata*-*W. monoptera* alliance which was strongly supported (PP=1; BS=98). *Wiborgia obcordata* seems to be monophyletic and forms a well-supported smaller clade (PP=1; BS=100) within subclade 1 [Figure 2.1 (c)]. *Wiborgia sericea* is monophyletic and comes out at the base of subclade 1 and is sister to the smaller clades identified within subclade 1 (strongly supported PP=1; BS=91). The second subclade [subclade 2 Figure 2.1 (c)] within the *Wiborgia* clade shows a strongly supported sister relationship between *W. tenuifolia* and *W. mucronata* (BS=99; PP=1). Within the *W. mucronata* subclade, there is further separation where accessions from Citrusdal, Wellington, Brandvlei, Darling-Mamre all formed a weakly supported smaller clade (BS=57; PP=0.83), whilst accessions from Studer's Pass, Piketberg, and Grootvlei also formed their own strongly supported smaller clade (BS=95; PP=1). Looking at the first outgroup clade made up of the Aspalathus-*Wiborgiella* alliance, a moderately supported sister relationship between these genera is observed (PP=0.83; BS=70). *Wiborgiella bowieana* and *Wiborgiella inflata* showed a weakly supported sister relationship (PP=0.61; BS=89), with these successively sister to *Wiborgiella humilis*; the entire *Wiborgiella* clade was well supported (PP=1; BS=100). Similarly, the entire *Aspalathus* clade was well supported (PP=1; BS=100). Looking at the second outgroup clade, each of the three genera (*Lebeckia*, *Rafnia* and *Calobota*) were all supported as monophyletic (PP=1; BS=100). *Lebeckia* and *Rafnia* show a well-supported sister relationship (PP=0.99; BS=99), and these two successively being sister to *Calobota* (PP=1; BS=99) [Figure 2.1 (c)]

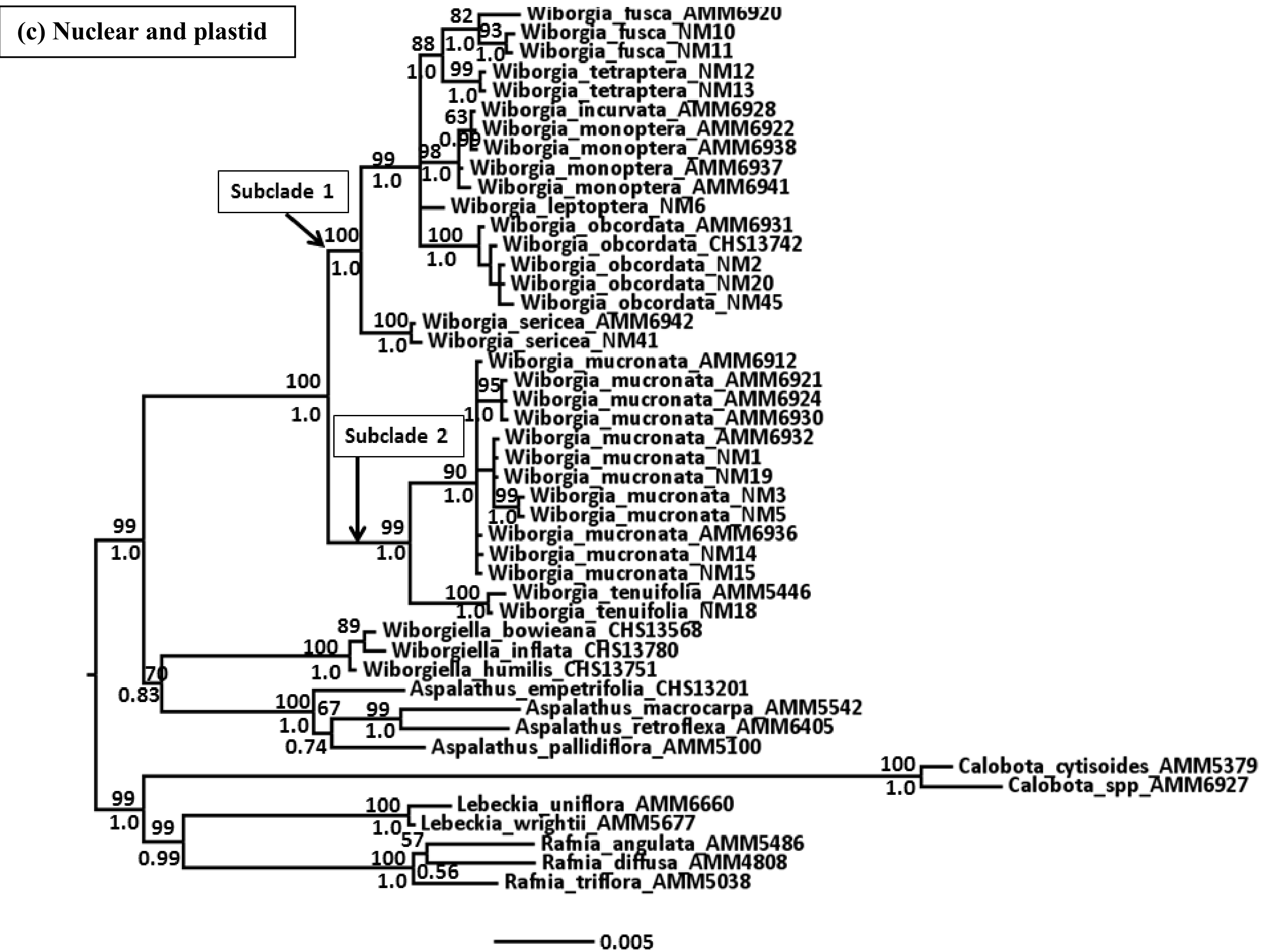


Figure 2.1 (c): 50% majority rule consensus tree from the analysis of the (b) combined nuclear and plastid data sets for the members of the "Cape clade" of the Crotalarieae. Numbers above branches are bootstrap percentages from maximum likelihood analysis and those below are posterior probabilities from the Bayesian analysis (only values above 50% and 0.50 are shown for the bootstrap and posterior probability respectively.

2.4 Discussion

2.4.1 Molecular markers

The first objective of this study was to test the monophyly of *Wiborgia,* given that previous studies on the Crotalarieae [e.g. Boatwright *et al*., (2008)] included only a few species in the genus. The results for all five loci sampled in this study all showed that *Wiborgia* is well supported as monophyletic, from both the BI and ML analyses; and also the genus is sister to the *Wiborgiella-Aspalathus* clade, consistent with the results of Boatwright *et al*. (2008). Another important finding which was also consistent with the finding of Boatwright *et al*. (2008), was the position of *Wiborgiella humilis* which was nested and well supported as being part of the *Wiborgiella* clade. This further supports the decision by Boatwright *et al*. (2008) to separate *Wiborgiella humilis* from *Wiborgia* as it is also strongly supported as not belonging to the *Wiborgia*. Similarly, the relationships within the outgroups were also well supported, which were also consistent with those recovered by Boatwright *et al*. (2008); where *Lebeckia* and *Rafnia* were weakly supported as sister in their study. In addition, *Calobota* is sister to both *Rafnia* and *Lebeckia*, a noteworthy finding as previously the deep nodes in Crotalarieae were poorly resolve in phylogenies reconstructed using *ITS* and *rbcL* data. The current study builds on the works of previous authors, adding four faster evolving plastid markers and multiple samples among species, to obtain a more robust dataset which resulted in trees with better resolution. And the strongly supported monophyly of *Wiborgia* as well as the other deeper nodes in this study shows that with increased sampling using fast evolving markers, more generic relationships within other unexplored genera of the Crotalarieae (e.g *Lotononis*) could be unambiguously resolved. This is an important finding because resolution of circumscriptions and relationships within the Crotalarieae have been reported to be complicated mainly due to rapid recent radiation (Linder, 2003; Linder, 2005), reticulation and convergence (Dahlgren 1970a & b; van Wyk *et al*., 2002; Moteetee & van Wyk, 2007; Boatwright *et al*., 2008).

The individual DNA loci sampled generally had low levels of sequence variation which resulted in poor node support (especially in the terminal nodes). From the individual trees of the different loci, *rps16* had lowest sequence variability and produced an unresolved tree with very low support values. From our study, *rps16* had an almost similar low PIC (5%) but this is comparable to that of Shaw *et al*. (2007) (4%), yet deeper node topology support is similar to rest of markers. Also rpl32-trnL and *ITS* had relatively low sequence variability (compared to trnS-trnG and trnT-trnL) yet both markers have been reported to be fast evolving and variable across a number of lineages [(*ITS;* Alvarez & Wendel, 2003) and (rpl32-trnL; Shaw *et al*., 2005, 2007)].Unlike the high PIC % reported by Shaw *et al*. (2007; 10%), our study show a lower PIC (7%) suggesting the low variability of the marker in the taxa studied. The other two markers, trnS-trnG and trnT-trnL, had highest and second highest levels

of sequence variability respectively, produced similar topology and most major clades were moderately supported. Both trnS-trnG and trnT-trnL were quite variable with PIC % values higher (10% and 12% respectively) than previously reported by Shaw *et al*. (2007) (4% and 5% respectively). The concatenated datasets showed higher levels of node support compared to the individual markers, for both the concatenated plastid as well as the concatenated nuclear and plastid datasets. Several well supported sister relationships between the species of *Wiborgia* could be identified, and some of those are consistent with those of Boatwright *et al*. (2008).

2.4.2 Phylogenetic relationships within *Wiborgia*

The most recent phylogenetic study of Crotalarieae (Boatwright *et al*., 2008) used one to two samples representing seven *Wiborgia* species and it produced a poorly resolved phylogeny. Hence the second part of the first objective was to infer relationships within the genus, testing hypothesis by Dahlgren (1975) on subgeneric classification and evolution of morphological characters. Dahlgren (1975) recognized two subgenera, with subgenus *Wiborgia* was made up of two species (*W. humilis* and *W. obcordata)* and characterized by fruits that lack wing. However, the shared morphology is likely due to convergence, as Boatwright *et al*. (2008, 2009) demonstrated that *Wiborgiella humilis* is part of *Lebeckia* sect *Viborgoides* clade which they recognized as new genus *Wiborgiella. Wiborgia obcordata* remained the only species in the subgenus *Wiborgia* and in this study as well as that of Boatwrght *et al* (2008), is found to be embedded within subgenus *Pterocarpia* [Table 2.1; Figure 2.1 (c)]. This is an interesting relationship because it does not give support to the subgenera classification of Dahlgren (1975), in a sense that *W. obcordata* which belongs to the subgenus *Wiborgia* was embedded in a strongly supported clade (subclade 2) made up of a polytomy with species belonging to the subgenus *Pterocarpia* [Figures 2.1 (a) and (b)], and therefore based on both the findings of Boatwright *et al* (2008) and the results in this study, Dahlgren's (1975) subgeneric concept cannot be upheld. It is therefore hypothesized that the absence of wing observed in *W. obcordata* to be derived from a pod with well-developed wings, and that wings were independently lost (or vestigial) in *W. leptoptera.*

Similar to the findings of Boatwright *et al*. (2008), our study recovered the same relationships where *W. tetraptera* was strongly supported as sister to *W. fusca* according to both the BI and ML analyses, and likewise *W. monoptera* was strongly supported as sister to *W. incurvata*. *Wiborgia* is resolved into two strongly supported subclades in all our analyses, a novel result. Furthermore a noteworthy well-supported sister relationship between *W. mucronata* and *W. tenuifolia* was identified; this relationship has not been previously reported in literature. The rest of the other species of *Wiborgia* did not show any resolved relationships, with *W. obcordata* and *W. leptoptera* were both part of the subclade with the *W. fusca*-*W. tetraptera* sister pair.

Dahlgren (1975) presented an evolutionary diagram mainly based on several morphological characters which included flower colour, presence/absence of hairs on branches/leaves, fruit morphology and presence/absence of wings on the fruits. Using these characters he then divided *Wiborgia* into two subgenera as well as further subdividing these subgenera, with the *Pterocarpia* group further being subdivided to an extent where *W. sericea* was grouped together with *W. tetraptera* and *W. tenuifolia* mainly because they have fruits with lateral ridges or wings and that of *W. sericea* and *W. monoptera* based on the presence of pubescence on young branches. Although *W. sericea* forms part of the same subclade with *W. monoptera*, it is still distant to *W. monoptera* which is in a smaller well supported clade with *W. obcordata*, *W. incurvata*, *W. fusca* and *W. tetraptera* thus the latter grouping is not supported by our analyses. Based on the smoothness of the fruit walls groups *W. fusca* and *W. incurvata,* taxa which formed a part of a smaller well supported clade within subclade 1 [Figures 2.1 (a) and (b)]. Our study therefore brings out the point that morphological characters in *Wiborgia,* such as wing size and pubescence, are not suitable for delineating between subgenera or even smaller groupings within those subgenera because they are quite variable characters even within a single species. Although Dahlgren (1975) did point out that the groupings were merely speculative (as indicated by Dahlgren, 1975 pages 66-68), the morphological characters he used to develop those speculations still form an integral part of delineating species of *Wiborgia* presently, thus further showing the need for a much a more recent taxonomic revision of *Wiborgia* which incorporates both morphological data such as those identified by Dahlgren (1975) as well as those used in the cladistic analysis of Boatwright *et al* (2008), and also molecular data from fast evolving markers which have currently been identified to have good utility when working at lower taxonomic levels [e.g those identified by Dong *et al*. (2012)].

Despite *Wiborgia* being scattered within a specific area in the CCR and ECR, some species are widespread (e.g. *W. mucronata*) whereas two (*W. incurvata* and *W. tenuifolia)* have narrow ranges. It was found that sister species (*W. incurvata* and *W. monoptera; W. fusca* and *W. tetraptera; W. tenuifolia* and *W. mucronata)* have overlapping distribution ranges. Given that sister species have all been identified to co-occur or have overlapping geographical ranges, one may speculate that speciation has involved subtle shifts in their niches. Some level of niche differentiation occurs among *W. incurvata* and *W. monoptera*, whereby *W. incurvata* mainly occupies clayey flat habitats whilst *W. monoptera* is mainly found occurring on rocky outcrops, the spatial distribution of these taxa is however sufficiently close to one another to an extent that it may be possible to suspect that geographically these taxa may have overlapping ranges whilst occupying a variety of niches. Also the widespread *W. mucronata* has been found to co-occur with its sister species *W. tenuifolia* in Brandvlei where they are found occupying the same area but with the former common on sandy flats whereas the later occurs on steep slope with more clayey soils. In addition, *W. fusca* has also been identified to co-occur with *W. tetraptera* in the sandy soils of the Elandsbaai-Lambertsbaai area, and it was

hypothesized that speciation did not involve a shift in the sister species occupying different soil types. It was also found that a number of widespread species are sister to narrowly distributed species, and further investigate their edaphic regimes by analysing nutrients in Chapter 3. Dahlgren (1975) identified that there were several different forms of *W. mucronata* which varied across the geographic range of this species, and pointed out that in the future, with there being more material from the entire distribution range available to study, the forms of *W. mucronata* would possibly be split and recognised as different entities. This allusion, is further supported by our analyses because within the *W. mucronata* clade, where the accessions two smaller clades in CCR (Darling-Mamre, Citrusdal, Wellington, Brandvlei areas) and predominantly ECR (Studer's Pass, Soebatsfontein, Piketberg), thus suggesting genetic pools separating between populations [Figures 2.1 (a) and (b)]. These *W. mucronata* subclades occur in quite distant localities with intervening areas not occupied by *Wiborgia* and could be occupying distinct soil type and microclimates. Similarly, accessions of *W. fusca* and *W. obcordata* also showed genetic structuring, even though there is no obvious accompanying morphological differentiation.

Flowering in *Wiborgia* starts in autumn and winter (*W. incurvata*, *W. leptoptera*, *W. monoptera*, *W. sericea*) into spring and early summer (*W. fusca*, *W. mucronata, W. tenuifolia*, *W. tetraptrera*) or may be nearly year round (*W. obcordata*). Therefore species in a clade may overlap in their flowering, for example *W. tenuifolia* (September to November) and *W. mucronata* (August to October). Although there is overlap between the flowering times of these taxa, there was however a shift in flower colour where *W. tenuifolia* has pink flowers whilst *W. mucronata* has bright-yellow flowers [Table 2.1; Figure 2.1 (c)]. This finding is consistent with findings by van der Niet & Johnson (2009) that strong pollinator specialization selection in Cape plant lineage was strongly associated with floral features. In addition to this, *W. monoptera* and *W. incurvata* show a similar pattern of floral differentiation where the former species has pale yellow flowers whilst the latter species has creamy to pale-lemon coloured glabrous flowers [Table 2.1; Figure 2.1 (c)]. On the other hand *W. fusca* and *W. tetraptera* do not show a differentiation in floral features whereby both species seem to have similar pale-greenish yellow coloured flowers and overlap in flowering times [Table 2.1; Figure 2.1 (c)].

2.5 Conclusion

This study has identified that *Wiborgia* is a monophyletic genus, sister to both *Aspalathus* and *Wiborgiella*. Detailed studies of character variation and evolution still need to be undertaken in order to evaluate whether there will be strong congruence between morphological patterns in relation to the patterns from our molecular analyses. It is clear from the results that *W. fusca*, *W. monoptera*, *W. obcordata*, *W. sericea*, and *W. mucronata* are all undergoing some process of population differentiation/isolation which over time could lead to speciation. In order to investigate the extent and implications of these molecular sequence differences, a population study concentrating mainly on

the species mentioned above, needs to be undertaken where genetic diversity between populations could be explored. The co-occurrence of all sister species pairs which are morphologically and genotypically distinct indicates presence of reproductive isolating barriers, probably pollinator separation linked with the shift in floral colour between *W. tenuifolia* and *W. mucronata*. Although the results showed that there is no geographical separation of taxa, especially sister taxa, it is highly probable that these sister taxa are occupying distinct niches, and I further investigate (Chapters 3 and 4) the role of edaphic parameters in the biogeography of *Wiborgia*. Dahlgren's (1975) subgenera classification was not supported by our study or previous studies (Boatwright *et al*., 2008); because some of the morphological characters like wing size or pubescence of leaves/branches, seem to be quite variable characters which are not suitable for classifications due to their instability as they are able to be variable even intraspecifically. Other aspects within the genus also need to be comprehensively explored e.g. character evolution, biogeography, dispersal mechanisms as well as divergence date estimations.

CHAPTER 3

3.0 DISTRIBUTION PATTERNS IN THE GENUS *WIBORGIA*: EXPLORING THE ROLE OF EDAPHIC HETEROGENEITY

3.1 Introduction

The Greater Cape Floristic Region (GCFR) is a megadiverse region made up of the Extra Cape Subregion (ECR; Snijman, 2013) and the Core Cape Subregion (CCR, formerly known as CFR; Goldblatt & Manning, 2000; Manning & Goldblatt, 2012), and these two subregions encompass two major (Succulent Karoo and Fynbos) plus one minor (Desert) biomes characterised by a variety of edaphic characteristics. The CCR is made up of a mosaic of various sharply delimited edaphic habitats (Linder, 1985), where the montane habitats are characterised by sandy soils which are acidic, nutrient poor, coarse grained and shallow with a longitudinal gradient of increasing clay and silt content when moving from west to east (Campbell, 1986). The lowland habitats are more clay rich with deeper fine textured soils mainly derived from shales and granites, which have a higher pH compared to the soils of the montane habitats (Campbell, 1986). The CCR habitats generally show a longitudinal gradient of increasing soil fertility between soils derived from different systems when moving from west to east (Campbell, 1983). The ECR on the other hand has edaphic habitats which are characterized by a great diversity of soil types which can broadly be grouped in three major categories: i) the weakly structured sands of the sandveld derived from marine deposits, low in clay content and underlain mostly by silica or calcium rich hardpans, ii) the variably grained sandy loam soils of the Kamiesberg and Richtersveld which are mostly shallow, freely drained with a red to yellow colour, and iii) the clay rich hardeveld soils with higher levels of nutrients especially in those areas which are associated with heuweltjies; these are termite derived mirma-moulds with deeper, finer textured alkaline soils with a high water holding capacity mostly prominent in the Namaqualand ecosystems, mainly found on base-rich colluvial sands at low elevations (Desmet, 2007). Similar to the CCR, the geology of the ECR is a complex system made up of a varied sequence of pre-Gondwanian rocks characterising the mountainous desert, with the peaks of the granitic derived hardeveld landscape in the southern parts ranging about 1200 to 1700 m in the Kamiesberg Mountains (Snijman, 2012; Cowling *et al*., 1997). The diverse ecological characteristics create a mosaic of edaphic habitats available for plants in the GCFR, and are thought to drive the high species turnover along habitat gradients (Cowling, 1990, Cowling *et al*., 1992).

There is growing evidence that speciation in the GCFR involves ecological shifts (van der Niet & Johnson, 2009). A study by Goldblatt (1979) looking at the genus *Galaxia* (Iridaceae), showed the

differentiation of species pairs on soil type, where one species occupied clayey fine textured fertile and neutral soils, whilst the sister species occupied a more sandy coarser textured infertile and acidic soils. The same study also found evidence of edaphic differentiation where hybrids in the genus *Freesia* (Iridaceae) occupied the ecotone region between the different soil types not similar to those occupied by the parents. Another study by Ellis *et al*. (2006) showed that geographically separated populations of the species in the genus *Argyroderma* (Aizoaceae) diverged phenotypically due to the effects of divergent habitat selection, whereby this morphological divergence triggered a correlated response of evolution of reproductive isolation by means of differentiation in flowering periods. These examples illustrate that adaptation to different soil types is one of the main factors differentiating between species, where there is an absence of a pre- or post-zygotic isolating mechanism as suggested by Linder (2003). Apart from studies looking at specific Cape lineages, it is also generally known that certain vegetation types are predominant in specific soil types with certain characteristics (e.g. fynbos vegetation is generally found occurring on sandy nutrient poor soils whilst renosterveld vegetation is generally found occurring on shale derived clay rich soils which are more fertile) (Cowling *et al*., 1997; Manning & Goldblatt, 2012; Mucina & Rutherford, 2006). There are several studies which show ecological shifts between closely related plant species; these include *Aquilegia* (Whittall & Hodges, 2007), *Mimulus* (Macnair &Christie, 1983; Schemske & Bradshaw, 1999) and *Rhodocoma* (Hardy & Linder, 2007). Currently, the most exhaustive study showing the direct link between soil nutrition and speciation in the CCR is by van der Niet & Johnson (2009). Several studies have documented evidence showing that the distribution of some CCR species may strongly be related with chemical and physical characteristics of the soil types they occur on. For example, a study by Richards *et al*. (1997a) which compared how competition and soil factors influenced the distribution of six Proteaceae species in the fynbos, found that soil factors played an important role in the distribution of the species as opposed to species competitively excluding on another from their ranges. In another study, Richards *et al*. (1995) found that five communities in the Soetanysberg hills area were associated with distinct soil types and identified two main compositional gradients which were associated with soil texture, pH, and depth. Furthermore, Richards *et al*. (1997b) showed that soil nutrient characteristics such as total phosphorus, nitrogen, organic carbon, cations such as calcium, magnesium, sodium and potassium differed significantly between 18 sites in Soetanysberg, which were of different soil and vegetation types; which led to their conclusion that species distributions and community compositions of nutrient-poor Mediterranean-climate ecosystems could importantly be explained by spatial variation in soil nutrient availability. Fynbos species' distribution have been strongly linked with edaphic factors such as soil nutrient availability and also nutrient acquisition strategies which are most of the time in response to infertile soils with low levels of nutrients (Cowling, 1990; Cowling *et al*., 1992; Richards *et al*., 1995, 1997a & b). Other examples in the CCR suggested that closely related species occurring on different soil types are differentially adapted (Verboom *et al*., 2004), or those occurring in close proximity occupy distinct climatic niches

(Linder & Vlok, 1991). In general most plants are known to exhibit strategies which enable them to be able to cope with a shortage of nutrients or water (Lambers *et al*., 2007). In addition to this, it has been reported that in the CCR there are some species within the plant lineages from the Proteaceae, Fabaceae, and Cyperaceae that have evolved different strategies of acquiring nutrients as a response to the characteristic poor soil nutrition of the Cape. These strategies include amongst others, cluster roots, which enhance the uptake of phosphorus by the plants (Lambers *et al*., 2007).

Phosphorus has been reported to play an essential role in processes involving the genetic, metabolic, structural and regulatory macromolecules (White & Hammond, 2008), thus it is regarded as one of the most limiting elements needed for plant growth (Vance *et al*., 2003). The infertile sandstone derived soils of the CCR which mainly support the fynbos vegetation (Rebelo *et al*., 2006) have been reported to be particularly deficient in N and P (Witkowski & Mitchell, 1987). For example, a study by Shane *et al*. (2008) investigated how phosphorus availability influences the uptake and growth of three species from the family Proteaceae. They found that *Protea compacta* which is endemic to nutrient poor colluvial sands was unable to down-regulate its P uptake when grown in soils of *Protea obtusifolia* and *Leucadendron meridianum* which are comparatively fertile limestone derived soils. This inability to down-regulate P uptake resulted in toxicity in the tissues and affected growth of the species. The authors thus concluded that the inability to down-regulate P uptake was a trait associated with the limited distribution of *P. compacta* to its nutrient poor sandy habitat.

General distribution patterns and habitats of *Wiborgia*

Wiborgia is a GCFR endemic genus mainly distributed from the lowlands north of the Cape Peninsula extending all the way to the northern parts of Namaqualand (Fig 3.1). The genus generally occurs on a variety of habitats characterised by different vegetation types as well as with soils derived from the Malmesbury and Cape System (Dahlgren, 1975). The distribution of the genus closely overlaps with arid types of the fynbos, however the genus is ecologically different to most sclerophyllous fynbos groups because of the characteristic mesomorphous deciduous leaves (Dahlgren, 1975). In addition, several species such as *W. fusca*, *W. tetraptera*, *W. tenuifolia*, *W. leptoptera* and *W. sericea* are known to occur mostly on clayey soils characterized by a vegetation of thorny sclerophyllous scrub known as the renosterveld that is dominated by *Elytropappus* species (Dahlgren, 1975; Lewis *et al*., 2005). A summary of habitat types for each *Wiborgia* species is presented in Table 2.1 (Chapter 2).

Wiborgia obcordata occurs in the southern divisions is mainly distributed in the Mossel Bay to the Cape Peninsula and extends along the western divisions and up to the northern divisions mainly in Vanrhysdorp and Calvinia; occurring mainly on marine sand deposits covered by the strandveld vegetation (sensu Acocks as referred to in Dahlgren, 1975) in both the western and southern coastal

regions. *Wiborgia mucronata* is a wide-spread species occurring both in sandy and clayey soils in mixed renosterveld vegetation. According to Dahlgren (1975), *W. mucronata* is generally a western species, having atypical forms distributed in the Swellendam, Montagu and Paarl Divisions, as well as the more typical forms (characterised by strong thorny branches) mainly distributed in the Worcester, Malmesbury, and Clanwilliam Divisions extending all the way up to Little Namaqualand (Table 2.1). *Wiborgia sericea* is mainly distributed in areas with clayey soils on the slopes of mountain ranges ranging between 600-1200 metres, mainly in the transitional fynbos-renosterveld vegetation, with a wide distribution range from the Witterberg to the mountains north-east of Ceres extending up north to the Cederberg, Calvinia, Vanrhynsdorp and Little Namaqualand. Similar to *W. sericea*, *W. tetraptera* is confined mainly to clayey soils or a mixture of sandy and clayey soil, mainly on the transitional fynbos-renosterveld vegetation, ranging from Stellenbosch to Malmesbury Divisions and extending up to the Calvinia Division (Dahlgren, 1975). *Wiborgia fusca* also grows mainly on clayey lowlands in the southern parts whilst in the central parts its mostly found occurring in mountainous areas; the species has a distribution that ranges from Malmesbury in the south extending all the way up to the central parts of Little Namaqualand in the north. The two subspecies (*W. fusca* ssp *fusca* and *W. fusca* ssp *macrocarpa*) seem to occupy different habitats whereby *W. fusca* ssp *fusca* is mainly distributed in the southern parts of Malmesbury extending to Little Namaqualand, but absent in Saldanha Bay where *W. fusca* ssp. *macrocarpa* occurs and is restricted to mainly to the coastal hills near Vredenburg mainly composed of granite derived soils or generally sandy ground (Table 2.1). *Wiborgia monoptera* occurs on rocky slopes as well as hills with granite derived soils, with distribution ranging from Clanwilliam and extending to Namaqualand mainly occurring in the transitional fynbos-renosterveld and the broken veld for the different regions respectively (Table 2.1). Although the species seem to be distributed both in the CCR and the ECR, Dahlgren (1975) suggests that the species is more common and has a concentrated distribution in the Little Namaqualand (ECR). However, he pointed out that distribution and habitat is not sufficiently known because of limited number of collections. *Wiborgia leptoptera* generally occurs on clayey soils in the Malmesbury, Cederberg and Clanwilliam Divisions; where *W. leptoptera* ssp. *leptoptera* is mainly restricted and common in the clayey hills between Mamre and Darling in the Malmesbury Division, whilst *W. leptoptera* ssp. *cedabergensis* is restricted to clay rich soils in the western sides of the Cederberg Mountains mainly at low altitudes. *Wiborgia tenuifolia* is a CCR endemic species with a distribution that is mainly concentrated on transitional fragments of renosterveld-fynbos vegetation in regions not exploited for crop farming; whilst *W. incurvata* is an ECR endemic species mainly restricted to the Little Namaqualand division (Table 2.1) (Dahlgren, 1975; Lewis *et al*., 2005).

Species within the genus mainly occur in the transitional zone between fynbos-renosterveld and in some instances they occur in several kinds of the renosterveld vegetation (Dahlgren 1975) [of which fynbos is generally associated with infertile sandy soils whilst the renosterveld is generally associated

with relatively nutrient rich clayey soils (Cowling *et al*., 1997)]. Most species occupy clayey soils, for example subspecies of *W. leptoptera* (*W. leptoptera ssp leptoptera* and *W. leptoptera ssp cedabergensis*) remained undifferentiated in terms of soil type (both occupy clayey soils), but rather differentiating geographically as well as in aspect and altitude where one occurs on the western sides of the Cedaberg mountains at low altitudes (*W. leptoptera ssp cedabergensis*), whilst the other (*W. leptoptera ssp leptoptera*) occurs on the clayey hills in Darling and Mamre (Dahlgren, 1975). In contrast to this, the subspecies of *W. fusca* show a differentiation geographically as well as in soil types they occupy whereby *W. fusca ssp fusca* mainly occurs on clayey soils in the southern parts of Malmesbury going up to Little Namaqualand, whilst *W. fusca ssp macrocarpa* is restricted to the Vredenburg hills occurring mainly on granite derived sandy soils, there seems to be partitioning and no overlap in the niches occupied by these subspecies (Dahlgren, 1975). Given these examples in the patterns of distribution of *Wiborgia* species, as well as the studies highlighted above focussing on how edaphic factors may have an important role in driving lineage diversification; these evidence from literature thus show the importance of including edaphic factors when studying the evolution and diversification of *Wiborgia* in the GCFR. However, there is little known about the extent to which edaphic factors could be linked with the distribution of *Wiborgia* species, thus these unexplored grounds and gaps in the knowledge of *Wiborgia* inspired the focus of this Chapter.

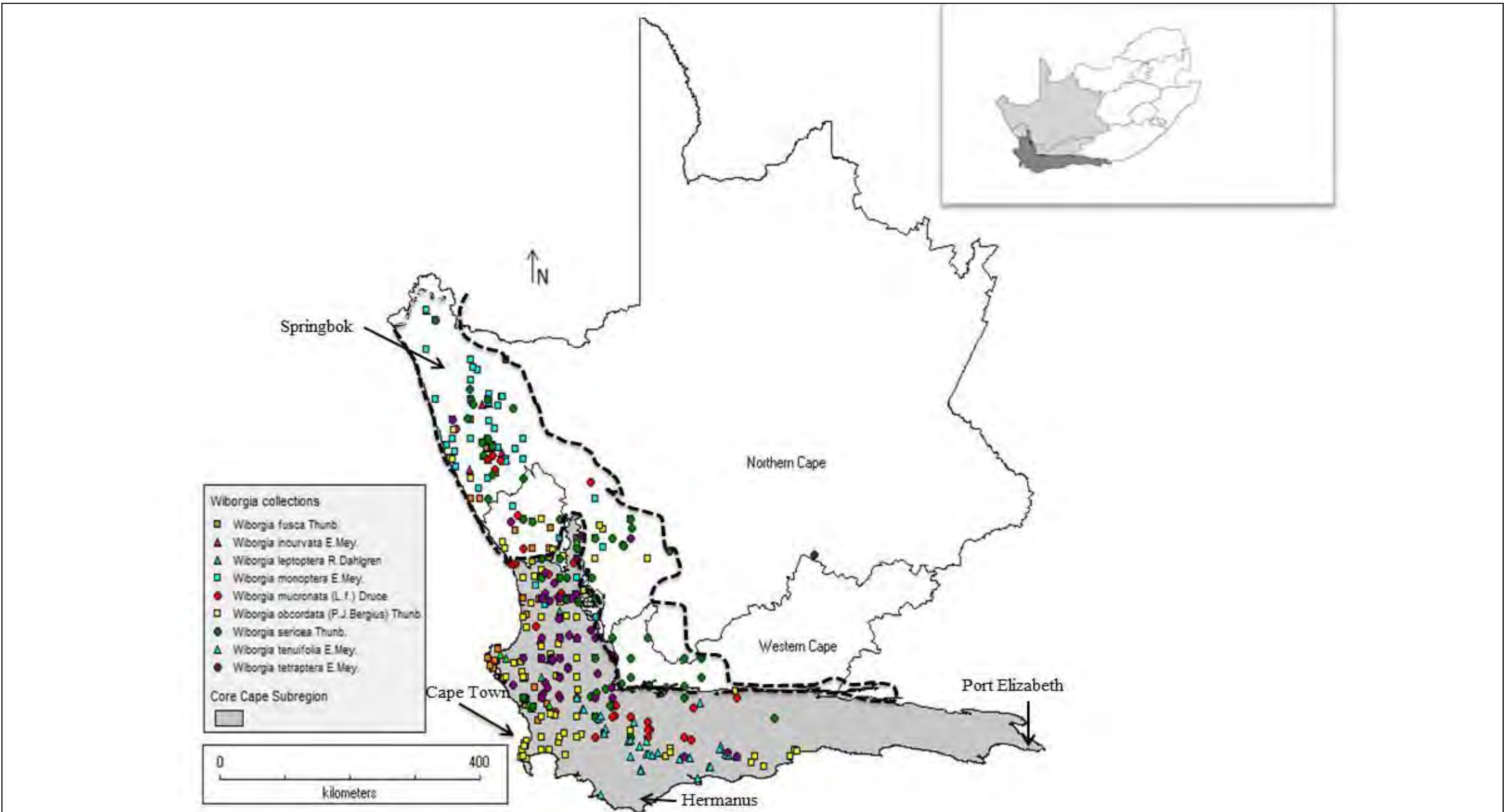


Figure 3.1: Distribution of *Wiborgia* species in the GCFR (Based on data from the SANBI PRECIS database). The CCR is shown in light grey, whilst the South African portion of the ECR is demarcated by dashed lines.

Objective and hypothesis of the Chapter

The objective of this Chapter is to determine if there is a difference in nutritional characteristics of soils occupied by different *Wiborgia* species and to compare with sites where *Wiborgia* species have never been recorded to occur. This Chapter tests the hypotheses that:

(a) *Wiborgia* species occupy soils of similar nutritional levels across their entire distribution range
(b) There are soil nutritional differences between *Wiborgia* and non-*Wiborgia* sites
(c) Narrowly distributed species (*W. incurvata* and *W. tenuifolia*) in the genus are edaphic specialists whilst widely distributed species (e.g. *W. mucronata* and *W. obcordata*) are edaphic generalists

3.2 Materials and Methods

3.2.1 Soil collections and analysis

Soil samples were collected from a total of 30 sites (Table 3.1) representative of the distribution range of all *Wiborgia* species where atleast one or more species was reported to occur based on georeferenced herbarium specimen data from the PRECIS database of the South African National Biodiversity Institute (SANBI). At each of the sites, three replicates of soil samples were randomly collected within a population stand of *Wiborgia* species ensuring that the collection points are evenly spread from each other in order to have a good representation of soils for each locality. The soil sampling for nutrient analysis involved taking a uniform slice of soil to a depth of 10-15 cm below ground level using a soil corer or a trowel. The soil samples were placed into individual plastic Ziploc bags and given the same voucher number as that of the corresponding voucher specimen and adding a letter next to the voucher number in order to differentiate between the three replicates. The global positioning system (GPS) was used to record the coordinates of the localities of each sample. In the laboratory, the samples were then left open on top of the laboratory-benches in the University of Cape Town' soils laboratory for about to three weeks in order to allow them to air dry, followed by sieving using a 2 mm sieve in order to separate off any plant residues and stones. The sieved soils were placed in 50 mm Ziploc bags, labelled and then sent to two different labs for nutrient analysis: BemLab Private Laboratory in Somerset (Western Cape, South Africa) and Elsenburg Plant Sciences Laboratory, Department of Agriculture, (Stellenbosch, South Africa).

At the BemLab Private Laboratory, the soil samples were analysed for total nitrogen (N), total phosphorus (P) and available phosphorus (Bray II P). The total P in the soil was determined using a method adapted from Sommers and Nelson (1972) where the phosphorus was first extracted using

acid digestion by a 1:1 mixture of 1N nitric acid ($HN0_3$) and hydrochloric acid (HCl) at 80°C for 30 minutes. The concentration of P within the extract was then determined using and optical emission spectrophotometer (ICP-OES, Varian, United States). Determination of available P on the other hand was done by extracting the P from the soil using the extractant, Bray II solution by Bray and Kurtz (1945), and the extractant was then colourimetrically measured using the reaction with ammonium molybdate, with a molybdenum-blue colour change as an indicator. Total soil N was determined by combustion method using a LECO FP-528 CN analyser (Leco Corporation, St. Joseph, USA).

At the Elsenburg Plant Sciences Laboratory, both physical and chemical parameters were analysed. The pH was measured using the potassium chloride (KCl) extraction method where soil samples were added to 20 ml of 1M KCl and mixed by shaking at 180 rpm for 60 minutes, then centrifuged at 4000 g for 20 minutes. The supernatant was then collected and used for pH measurements. Ammonium was determined colourimetrically by first extracting the ammonium from the soil with 1N KCl and then reacting the extractant with solution made up of sodium salicyclate, sodium nitroprusside and sodium hypoclorite all buffered at a pH of 13.0 (AgriLASA, 2004). The rest of the nutrients including potassium (K), magnesium (Mg), calcium (Ca) and sodium (Na), and carbon (C) were determined using the protocols from the Handbook of Standard Soil Testing Methods for Advisory Purposes (1990); where carbon concentration was determined using the Walkey-Black method which involved the oxidation of organic C using acidic dichromate followed by titration with ferrous sulphate. Carbon concentration was then determined by calculating the difference between the total added dichromate and the unreacted dichromate after oxidation. The concentrations of K, Mg, Ca, and Na were all determined using the 1% citric acid extraction and the concentrations in the sample determined using ICP-OES (Thermo Fischer Scientific Inc., NYSE:TMO). Soil texture was assessed by chemical dispersion using sodium hexametaphosphate where three sand fractions were determined through sieving for the determination of sand content as described in The Non-affiliated Soil Analyses Work Committee (1990); whilst silt and clay content were assessed through the sedimentation rates at 20°C using a hydrometer (ASTM E100). The methods described above are adapted from the methods and specifications handbooks of the respective labs.

Table 3.2: Soil collections made as part of the current study representing habitat as well as non-habitat sites of *Wiborgia*. Divisions are based on Dahlgren (1975)

Locality (voucher no.)	Divisions(subregion)	Habitat species	Latitude	Longitude
Bainskloof Pass (8)	(CCR)	Non-*Wiborgia* site	S33°35'35.02"	E19°07'22.74"
Barrydale (6934)	Montagu (CCR)	*W. tenuifolia*	S33°54'52.2"	E20°40'55.8"
Brandvlei dam slope (18)	Worcester (CCR)	*W. tenuifolia* and *W. mucronata*	S33°40'39.0"	E19°23'20.7"
Brandvlei dam banks (19)	Worcester (CCR)	*W. mucronata* and *W. obcordata*	S33°40'40.4"	E19°23'20.2"
Calvinia Rd (6914)	Cederberg (CCR)	*W. obcordata*	S32°4'17.9"	E19°5'59.6"
Cape Point (6953)	Cape Peninsula (CCR)	Non-*Wiborgia* site	S34°18'30.3"	E18°23'04.8"
Ceres (41)	Ceres (CCR)	*W. sericea*	S33°19'4.72"	E19°50'4.37"
Darling (3)	Malmesbury (CCR)	*W. mucronata* and *W. leptoptera*	S33°22'59"	E18°99'53.7"
Darling-Mamre (5)	Malmesbury (CCR)	*W. mucronata*	S33°22'59"	E18°99'53.7"
Elandsbaai (9)	Piketberg (CCR)	*W. tetraptera* and *W. fusca*	S32°35'41.3"	E18°41'26.7"
Khoisan's kitchen (6912)	Cederberg (CCR)	*W. mucronata* and *W. sericea*	S32°4'18.5"	E19°4'30.9"
Klipbok reserve (32)	Worcester (CCR)	*W. mucronata*	S33°49'32"	E19°24'11.5"
Lambertsbaai (11)	Piketberg (CCR)	*W. fusca, W. tetraptera*	S32°18'30.1"	E18°24'3.4"
PiketbergA (26)	Piketberg (CCR)	*W. obcordata* and *W. mucronata*	S32°22'20.9"	E18°42'52.1"
PiketbergB (29)	Piketberg (CCR)	*W. leptoptera*	S32°36'56.8"	E18°46'13.2"
Piketberg-Citrusdal (28)	Piketberg (CCR)	*W. tetraptera* and *W.obcordata*	S32°27'24.1"	E18°43'24.5"
Piketberg-ElandsbaaiA (22)	Piketberg (CCR)	*W. fusca* and *W. tetraptera*	S32°35'42"	E18°41'26.5"
Piketberg-ElandsbaaiB (21)	Piketbeg (CCR)	*W. mucronata* and *W. fusca*	S32°41'19.4"	E18°48'0.4"
Rawsonville (33)	Worcester (CCR)	*W. obcordata* and *W. mucronata*	S33°43'29.9"	E19°27'15.3"
Rhodes Memorial (7)	Cape Peninsula (CCR)	Non-*Wiborgia* site	S33°57'6.73"	E18°27'24.58"
Wellington (1)	Malmesbury (CCR)	*W. mucronata*	S33°30'18.5"	E18°56'8.3"
Worcester (6936)	Worcester (CCR)	*W. mucronata*	S33°21'58.6"	E19°57'35.8"
Botterkloof PassA (14)	Calvinia (ECR)	*W. mucronata*	S31°49'07.9"	E19°15'40.7"
Botterkloof PassB (15)	Calvinia (ECR)	*W. mucronata*	S31°26'16.9"	E19°08'37.4"
Garies-Kamieskroon (6920)	Little Namaqualand (ECR)	*W. fusca* and *W. mucronata*	S30°18'12.2"	E17°53'38.4"
Grootvlei-Soebatsfontein (6922)	Little Namaqualand (ECR)	*W. sericea, W. monoptera, W. fusca* and *W. mucronata*	S30°13'27.2"	E17°45'50.1"
Leliefontein (6928)	Little Namaqualand (ECR)	*W. incurvata*	S30°16'4.7"	E18°3'18"
Studer's PassA (6930)	Little Namaqualand (ECR)	*W. mucronata*	S30°13'54.5"	E18°3'38.6"
Studer's passB (6937)	Little Namaqualand (ECR)	*W. monoptera*	S30°23'59.3"	E18°3'44.9"
Vanrhynsdorp (6931)	Vanrhynsdorp (ECR)	*W. obcordata*	S31°31'9.7"	E18°43'10.4"

3.2.2 Statistical analyses

The soil nutrient data were first normalised by the use of log transformation before multivariate and univariate analyses using Statistica software version 12 (Statsoft, Tulsa, Oklahoma, USA). All the variables measured showed to be high skewed when using untransformed data, however after transformation the data were approximately normally distributed. The data were first explored by cluster analysis where a hierarchical dendogram based on the agglomerative algorithm was performed with the use of the unweighted pair-group method using arithmetic means (UPGMA), and because the data were continuous the linkage distance measure used was the Euclidian distance. The clusters identified from the hierarchical dendogram plot were then subjected to a Discriminant Function Analysis (DFA) to test the significance of the separated clusters from the cluster analysis in multivariate space. This separation was also used to identify which nutrients contributed to such separation. The nested ANOVA (where sites were nested in the clusters) were performed to test for the similarities of individual nutrient concentrations between the different sites and clusters. Tukey's pair-wise multiple comparison tests were used to show the separation of significantly different means at $p<0.05$.

3.3 Results

3.3.1 Cluster analysis

Cluster analysis results revealed ten distinct clusters when the cut-off point was set at a Euclidian distance of 1.8 (Fig. 3.2). The first cluster was made up of soils from the sites Khoisan's kitchens, Studer's PassB, Wellington, Darling, Brandvlei dam slope, Brandvlei dam banks, Klipbok reserve, PiketbergB, and Ceres. The second cluster was made up of soils from Calvinia Rd, Piketberg-ElandsbaaiA, Rawsonville, Piketberg-Citrusdal, and Cape point. The third cluster was made up of from Garies-Kamieskroon, Grootvlei-Soebatsfontein, PiketbergA, Studer's PassA, Elandbaai, Botterkloof PassA. The fourth cluster made up of soils from Leliefontein, Darling-Mamre, and Barrydale. These four clusters were the largest in the dataset in terms of number of sites within each cluster, whilst the last six clusters were made up of one or two sites. Cluster five, six and seven were made up of soils from Botterkloof PassB, Rhodes memorial, and Bainskloof Pass respectively. Cluster eight was made up of soils from Vanrhynsdorp and Worcester, whilst cluster nine and ten were made up of soils from Lambertsbaai and Piketberg-ElandsbaaiB respectively.

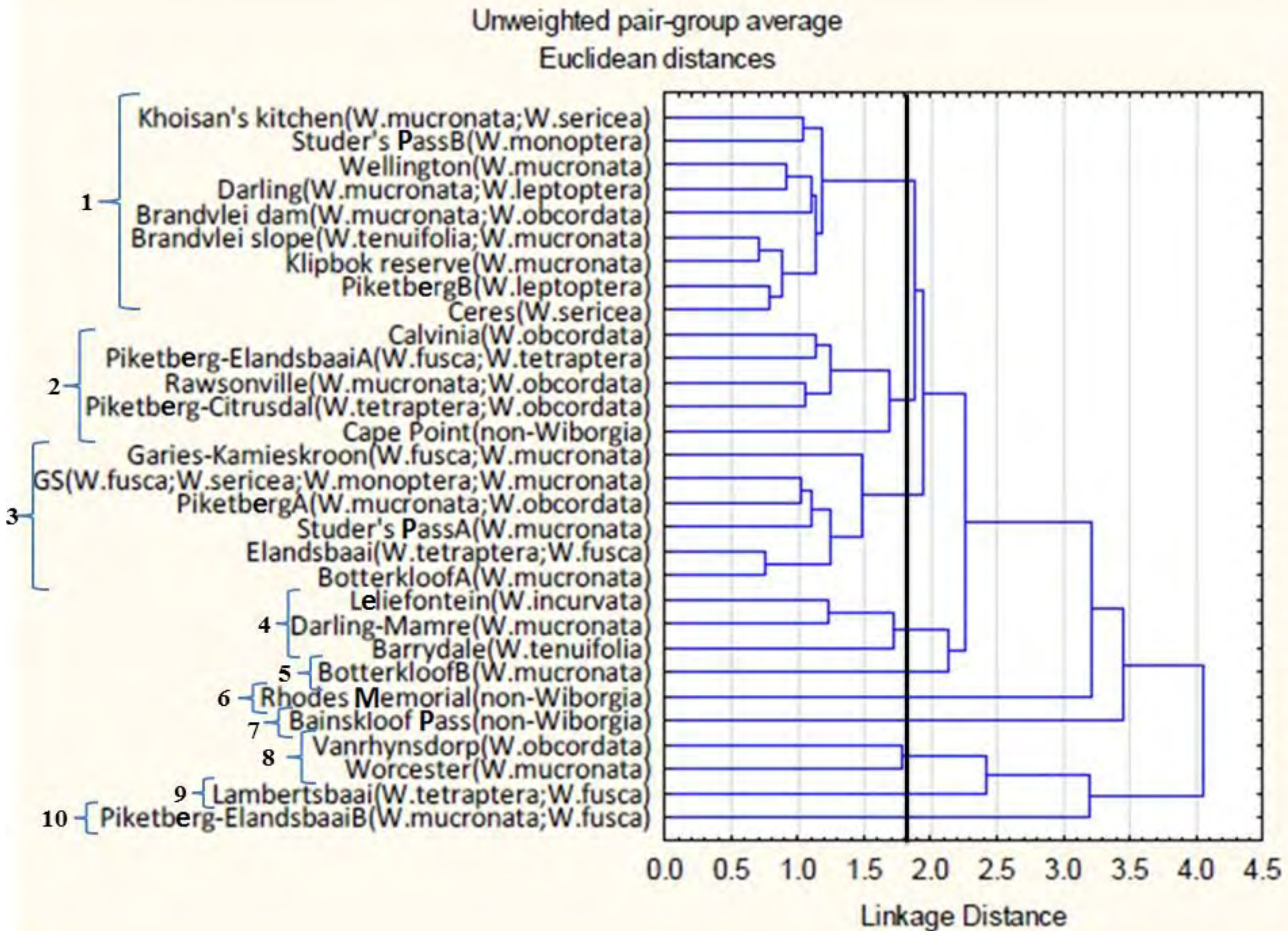


Figure 3.2: Dendogram from the cluster analysis of *Wiborgia* as well as non-*Wiborgia* sites sampled as part of this study. Clusters numbered 1-10 at a cut-off point of 1.8.

3.3.2 Assessment of statistical support, using multivariate analyses, for the groups identified from the Cluster analysis

The Discriminant Function Analysis (DFA) of the clusters results in nine Canonical Variates (CVs), of which 97% of the total variance is accounted for by the first six CVs which were identified as the only significant ones based on the Chi Square tests. Table 3.2 shows six of the significant CVs and the corresponding values, eigenvalues and cumulative proportions of contribution to the variance for all the thirteen soil parameters analysed. The first CV (CV 1) accounted for 50% of the total variance and the two parameters: C and Sand contributed the highest to the separation of the clusters [Fig. 3.2]. The second CV (CV 2) accounted for 23% of the total variance and the three parameters: pH, Ca and total P contributed the highest to the cluster separation. The third CV (CV 3) accounted for 11% of the total variance and the two parameters: Mg and Ca contributed the highest to the cluster separation. The fourth CV (CV 4) accounted for 5.6% of the total variance and the three parameters: C, Ca, and Mg contributed highest to the cluster separation. The fifth CV (CV 5) accounted for 4.2% of the total variance and the four parameters: Bray II P, C, Silt and Clay contributed highest to the cluster separation. And lastly, the sixth CV (CV 6) accounted for 3.1% of the total variance and the two parameters: Silt and Clay contributed highest to the cluster separation. On the other hand, nutrients such as N, K, NH4, and Na showed insignificant contribution to the separation and grouping of the sites as shown by multivariate analysis [Fig. 3.3 and Table 3.2]. The DFA scatterplot (Fig. 3.3) shows that only clusters 6 (Rhodes memorial) and 7 (Bainsklof Pass) consisting of non-*Wiborgia* sites clearly separated from the rest of the clusters, and that most *Wiborgia* sites are similar to one non-*Wiborgia* site (Cape point, in cluster 2).

Table 3.2: Standardized coefficient for all canonical variates of the soil nutrient data analysed (of which only six were significant). Parameters contributing highest to the cluster separation are shown in bold print.

Variable	CV 1	CV 2	CV 3	CV 4	CV 5	CV 6	CV 7	CV 8	CV 9
P Bray II mg/kg	-0.03	0.22	-0.23	-0.39	**-1.22**	0.32	-0.11	0.21	0.27
P mg/kg	-0.09	**-0.78**	0.62	-0.51	0.64	-0.53	0.21	0.04	-0.31
N %	0.68	-0.15	-0.09	0.45	-0.50	-0.37	0.04	0.07	-0.13
C %	**0.78**	0.03	-0.26	**-1.12**	**0.87**	0.41	0.26	-0.99	-0.08
Ca cmol(+)/kg	-0.18	**0.87**	**0.99**	**1.30**	-0.39	0.41	1.77	-0.48	0.10
K mg/kg	0.34	0.21	-0.23	0.12	0.16	0.69	-0.39	-0.14	0.09
Mg cmol(+)/kg	-0.33	-0.35	**-1.23**	**-0.77**	0.33	0.12	-0.99	1.41	0.07
NH4+ %	0.06	-0.16	0.001	0.42	-0.40	-0.24	-0.33	0.49	-0.06
Na mg/kg	-0.01	-0.10	0.57	0.53	0.21	-0.15	-0.29	-0.07	0.65
Sand %	**0.76**	-0.66	-0.13	-0.04	-0.74	0.72	0.27	-0.81	1.90
Clay %	0.13	-0.57	0.40	0.37	**-0.81**	**0.82**	0.007	-0.75	0.95
Silt %	0.17	-0.50	0.17	0.18	**-0.87**	**0.92**	0.40	-0.15	1.50
pH (KCl)	-0.23	**-1.12**	-0.41	-0.12	0.25	-0.41	-0.30	-0.56	-0.18
Eigenval	14.45	6.63	3.23	1.64	1.21	0.91	0.52	0.20	0.07
Cum.Prop	**0.500**	**0.729**	**0.84**	**0.89**	**0.94**	**0.97**	**0.99**	**0.99**	**1.00**

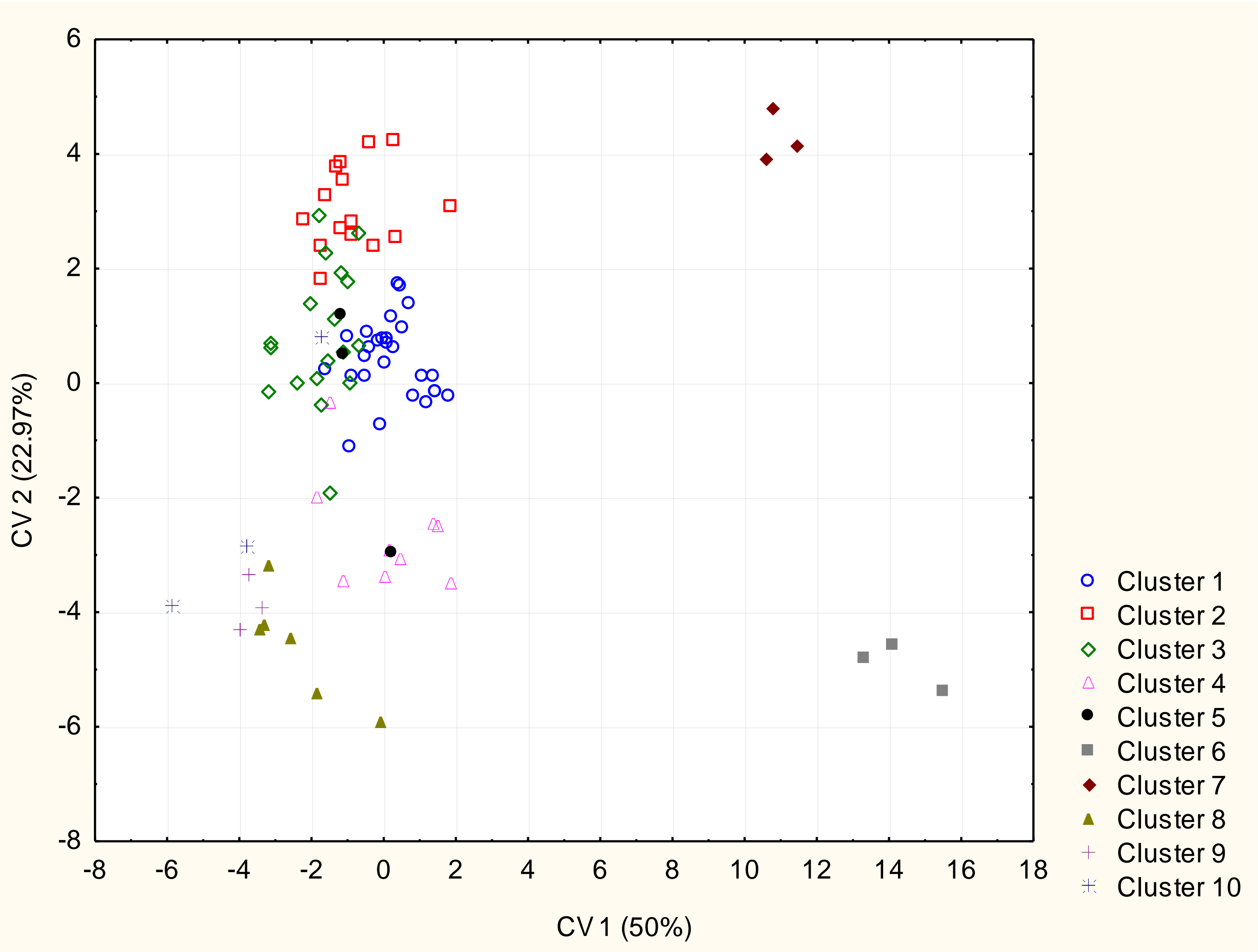


Figure 3.3: Ordination of groupings of the clusters obtained from the cluster analysis (made up of both *Wiborgia* and non-*Wiborgia* sites), along the first two canonical variates of the soil dataset with all clusters included

3.3.3 Soil nutrients in *Wiborgia* sites

In this section, the nested ANOVA for the nine parameters presented are selected from the DFA analysis and are those which were shown to contribute largely to the separation of the clusters into different groups and these include: total P, Bray II P, C, Ca, Mg, Sand, Clay, Silt and pH. The descriptions of the results from the nested ANOVA analyses for each of the nine parameters are shown and described below. Due to the interaction between clusters and sites for all parameters measured, nutrient data for all the sites were presented. A summary of the means and standard errors for the four remaining soil nutrient parameters [nitrogen (N), potassium (K), ammonium (NH_4^+), sodium (Na)], which showed insignificant contribution to the site separation by the multivariate analysis presented in Appendix 2.

Total P levels in *Wiborgia* sites were highly variable with a very wide range of 10 mg/kg to 234.5 mg/kg. Barrydale, Lambertsbaai and Darling-Mamre with total P levels of 234.5 mg/kg, 173 mg/kg and 150 mg/kg respectively, are the sites with the highest total P levels amongst all *Wiborgia* sites [Fig. 3.4(a) A]. Meanwhile, Grootvlei-Soebatsfontein and PiketbergA with total P levels of 10.2 mg/kg and 10.7 mg/kg respectively, show the lowest levels, thus making them the most P poor soils within the marked *Wiborgia* range soils. Other sites such as Khoisan's kitchen, Studer's PassB, Wellington, Brandvlei dam slope, Klipbok reserve, Leliefontein, Botterkloof PassB show intermediate levels of total P ranging between 60 mg/kg to 86.7 mg/kg. These sites, in the cluster analysis, were grouped in different clusters [Fig. 3.4(a) A]. In terms of non-*Wiborgia* sites, Cape Point with a total P level of 6 mg/kg seems to be even lower than that of any of the *Wiborgia* sites, thus making it the most P poor soil in the dataset, whilst Bainskloof Pass with a P level of 32 mg/kg show an intermediate level which is similar to that of most *Wiborgia* sites, and lastly Rhodes memorial with a P level of 245 mg/kg is within the highest total P of *Wiborgia* sites[Fig. 3.4(a) A].

Bray II P levels in *Wiborgia* sites were also highly variable ranging from about 3-41 mg/kg. Rawsonville, Piketberg-Citrusdal, Grootvlei-Soebatsfonein, Studer's PassA, PiketbergA, Leliefontein and Piketberg-ElandsbaaiA, have the lowest level of available P amongst all sites, whilst Lambertsbaai, Botterkloof PassB, and Khoisan's Kitchen have the highest available P levels [Fig. 3.4(a) B]. Other sites such as Khoisan's Kitchen, Studer's PassB, Wellington, Garies-Kamieskroon, Botterkloof PassA, Barrydale, Brandvlei dam banks, Botterkloof PassB, Vanrhynsdorp and Worcester show relatively intermediate levels of available P ranging between 10-26 mg/kg. In addition to this there are sites showing relatively low levels of available P ranging from 3-9 mg/kg, although not as low as the Rawsonville site. The sites include Darling, Brandvlei dam slope, PiketbergB, Klipbok reserve, Ceres, Calvinia Rd, Elandsbaai, and Darling-Mamre. Moreover, with regards to the non-*Wiborgia* sites, Cape Point (4.6 mg/kg) was among the low levels of available P whilst Rhodes memorial (15.6 mg/kg) was among the highest levels of P, while Bainskloof Pass (8.7 mg/kg) was

among the intermediate levels. This shows that the available P levels of non-*Wiborgia* sites are within the range of the *Wiborgia* sites [Fig. 3.4(a) B].

With regards to calcium concentration in *Wiborgia* sites, the variability across the sites ranged between 0.22 cmol/kg to 8.7 cmol/kg. Worcester and Piketberg-ElandsbaaiB sites showed Ca levels of 7.9 cmol/kg and 8.7 cmol/kg respectively and were the sites with the highest concentrations, whilst Calvinia Rd, Rawsonville, Piketberg-Citrusdal with Ca levels of 0.36 cmol/kg, 0.30 cmol/kg and 0.22 cmol/kg, respectively, were the lowest. In addition to this, Barrydale and Darling-Mamre with Ca concentrations of 2.8 cmol/kg and 1.8 cmol/kg respectively, were intermediate levels. Moreover, the rest of the *Wiborgia* sites, which includes quite a number of them, seem to show quite similar levels of Ca which lie between low and intermediate levels compared to the rest of the abovementioned sites. With regards to non-*Wiborgia* sites, Cape Point and Bainskloof Pass show intermediate Ca levels of 0.9 cmol/kg and 0.89 cmol/kg which are similar to those of most of the *Wiborgia* sites; whilst Rhodes memorial has a high Ca level of 6 cmol/kg which is however still similar to that of Piketberg-ElandsbaaiB and Worcester [Fig. 3.4(b) A].

The pattern of variation in magnesium in both *Wiborgia* and non-*Wiborgia* sites is similar to that of the Ca levels in terms of comparison of sites and which sites have highest or lowest Mg levels. Barrydale, Worcester and Piketberg-ElandsbaaiB are the sites with the highest levels of Mg concentrations amongs all *Wiborgia* sites, 1.5 cmol/kg, 3.0, cmol/kg and 1.9 cmol/kg for these sites respectively. Whilst Piketberg-ElandsbaaiA has the lowest Mg level compared to *Wiborgia* sites with a concentration of 0.12 cmol/kg. Moreover, the rest of the *Wiborgia* sites seem to show quite similar levels of Mg which lie between low and intermediate levels compared to the rest of the abovementioned sites, ranging between 0.4 cmol/kg and 0.88 cmol/kg. On the other hand, non-*Wiborgia* sites such as Cape Point and Bainskloof Pass show intermediate Mg levels of 0.41 cmol/kg and 0.71 cmol/kg, whilst Rhodes memorial shows a high level of Mg, 2.37 cmol/kg, which is also similar to that of Worcester and Barrydale [Fig. 3.4(b) B].

The concentration of C in *Wiborgia* sites in overall seem to be low with a somewhat narrowed range. The sites with the lowest levels of C include Calvinia Rd, Brandvlei dam banks, Piketberg-Citrusdal, Garies-Kamieskroon, Grootvlei-Soebatsfontein, Studer's PassA, Elandsbaai, Leliefontein, PiketbergA, Vanrhynsdorp, Lambertsbaai, Botterkloof PassA, Piketberg-ElandsbaaiA and Rawsonville, Piketberg-ElandsbaaiB, all with C levels ranging between 0.1 to 0.34%. Whilst the rest of the *Wiborgia* sites show relatively higher C levels ranging between 0.49% and 1.3%, this includes sites such as Khoisan's kitchens, Studer's Pass, Wellington, Darling, Brandvlei dam slope, PiketbergB, Klipbok reserve, Barrydale, Darling-Mamre, Botterkloof PassB, and Worcester. For the non-*Wiborgia* sites C levels were the highest in soils from Rhodes memorial and Bainskloof at 5.06% and 3.65% respectively, levels higher than any of the *Wiborgia* sites. In contrast to these non-

Wiborgia sites, soils from Cape Point with a C concentration of 0.6%, was within the range of the *Wiborgia* sites [Fig. 3.4(b) C].

Wiborgia species occur in acidic and close to neutral pH soils. The pH ranged from 3.9 to 6.9 across the entire *Wiborgia* sites; with Studer's PassB, Brandvlei dam slope, Rawsonville, Piketberg-Citrusdal being the most acidic soils (ranging between 3.9 and 4.1), whilst Worcester, Lambertsbaai, Botterkloof PassA and Piketberg-ElandsbaaiB, with pH levels between 5.3 and 6.9, are the sites with relatively high pH compared to the rest of the other *Wiborgia* sites. In addition to this, the rest of the *Wiborgia* sites seem to show a similar intermediate pH level range of about 3.9 to 5.2. Looking at non-*Wiborgia* sites, Bainskloof was the most acidic soil with a pH of 2.9, indicating 1.5 times less than the pH level of Piketberg-Citrusdal which is the most acidic *Wiborgia* habitat soil in the dataset. On the other hand, Rhodes memorial and Cape Point soils with pH levels of 5.1 and 4.4 respectively, lie within the range of the *Wiborgia* sites [Fig. 3.4(b) D].

Most of the *Wiborgia* sites seem to be having high levels of sand % lying mainly above 80% for the entire collection. Also within these high levels of sand composition in these soils, there is a relatively stretched range of the amount of sand available across all the *Wiborgia* sites. *Wiborgia* sites with the lowest sand percentage ranged between 80 to 84% include Wellington, Brandvlei dam slope and Barrydale. On the other hand, sites with the highest levels of sand percentage ranging between 90 to 93% include Khoisan's kitchens, Studer's PassB, Calvinia Rd, Piketberg-ElandsbaaiA, Rawsonville, Pikeburg-Citrusdal, Garies-Kamieskroon, Grootvlei-Soebatsfontein, Elandsbaai, Botterkloof PassA, PiketbergA, Vanrhynsdorp, and Lambertsbaai. Moreover, other *Wiborgia* sites which seem to have an intermediate sand percentage ranging between 85 to 89% include Piketberg-ElandsbaaiB, Worcester, Botterkloof PassB, Darling-Mamre, Barrydale, Leliefontein, Studer's PassA, Ceres, Klipbok reserve, PiketbergB, Brandvlei dam banks, and Darling. With regard to non-*Wiborgia* sites, Rhodes memorial with a sand composition of 86% was similar to the rest of the *Wiborgia* sites and within the range 85 to 89%, whilst Cape Point and Bainskloof were among the highest sand composition percentage at 94% for both, however they were still similar (p<0.05) with the highest *Wiborgia* sites [Fig. 3.4(c) A].

Wiborgia habitat sites are generally clay poor, with varying levels across the entire range with a maximum and minimum of 12% and 4.6% respectively. Sites with higher levels of clay include Wellington, Darling, Brandvlei dam slope, Brandvlei dam banks, PiketbergB, Klipbok reserve, Ceres, Garies-Kamieskroon, Leliefontein, Barrydale, Botterkloof PassB and Worcester, all ranging between 8 and 12%. The sites with lower clay concentration include Piketberg-ElandsbaaiA, Rawsonville, Piketberg-Citrusdal, Elandsbaai, Botterkloof PassA, Vanrhynsdorp, Lambertsbaai and PiketbergA with clay levels of 4% for each of the sites. On the other hand, most of the rest of *Wiborgia* habitat soils seem to have intermediate levels of clay lying between 5.3-7.6%, these include Khoisan's kitchens, Studer's PassB, Calvinia Rd, Grootvlei-Soebatsfontein, Studer's PassA, Elandsbaai,

Piketberg-ElandsbaaiB; all ranging between 5.3 and 7.6%. For the non-*Wiborgia* sites, Cape Point soil seems to have the lowest clay content at 4%, which is, however, similar to that of the *Wiborgia* sites. On the other hand, Rhodes memorial soil seems to be the most clay rich of the non-*Wiborgia* soils, with a clay content of 8.6%, it is, however, still similar to the level of those *Wiborgia* sites with a high clay content such as Brandvlei dam slope Wellington and Ceres. The soil from Bainskloof Pass has a low level of clay content at 5.3%, which is similar to most of the rest of the *Wiborgia* sites which lie in the clay levels [Fig. 3.4(c) B].

Silt percentage levels in soils from *Wiborgia* sites are in overall poor and seem to show a less steep range, lying between 1 to 8% in overall. Most of the *Wiborgia* sites have low silt levels, where the most silt poor *Wiborgia* sites include Khoisan's kitchen, Studer's PassB, PiketbergB, Calvinia Rd, Rawsonville, Garies-Kamieskroon, Grootvlei-Soebatsfontein, Elandsbaai, Leliefontein, Lambertsbaai Botterkloof PassA, and Vanrhynsdorp; all ranging between 1 and 3.3%. On the other hand, *Wiborgia* sites showing the highest silt content levels include Wellington, Darling, Brandvlei dam slope, Brandvlei dam banks, Ceres, Piketberg-ElandsbaaiA, Piketberg-Citrusdal, Studer's PassA, PiketbergA, Barrydale, Darling-Mamre, Botterkloof PassB, Worcester, Klipbok reserve, Piketberg-ElandsbaaiB; all ranging between 6 and 8%. The rest of the *Wiborgia* sites not mentioned above have a somewhat intermediate level of silt ranging between 4 and 5%. Looking at non-*Wiborgia* sites, Bainskloof Pass and Cape Point at 1% and 2% silt levels respectively, seem to show low levels of silt, but similar to those of most *Wiborgia* sites. In addition, Rhodes memorial soils at 4.6% silt were similar to the intermediate levels of a number of *Wiborgia* sites [Fig. 3.4(c) C].

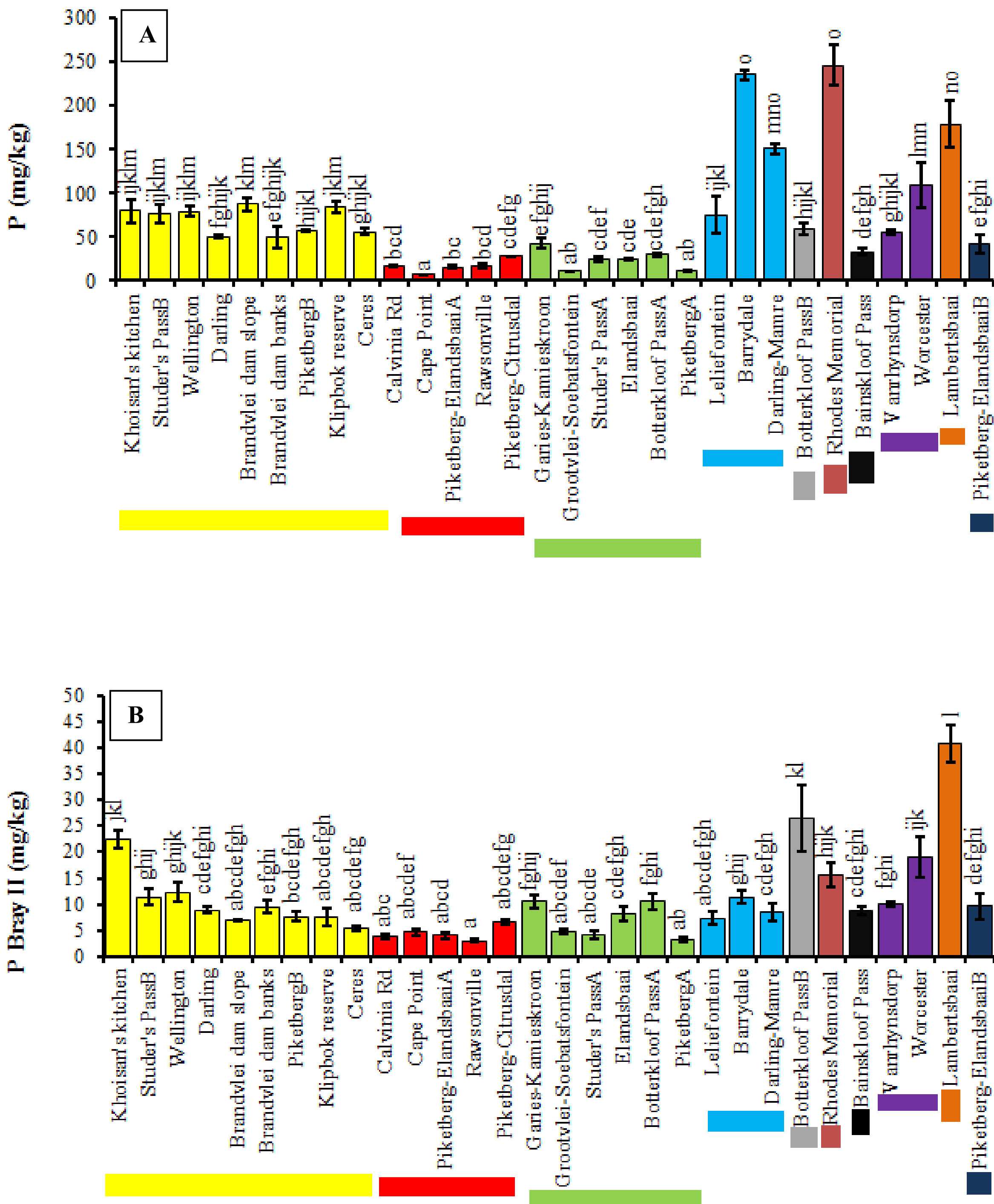


Figure 3.4(a): Mean±SE from nested ANOVA results for the concentration total P (A) and Bray II P (B) of the different *Wiborgia* and non-*Wiborgia* sites sampled. Different letters above the bars indicate significant differences at p<0.05. Yellow=cluster 1, red=cluster 2, green=cluster 3, blue=cluster 4, grey=cluster 5, brown=cluster 6, black=cluster 7, purple=cluster 8, orange=cluster 9, navy-blue=cluster 10

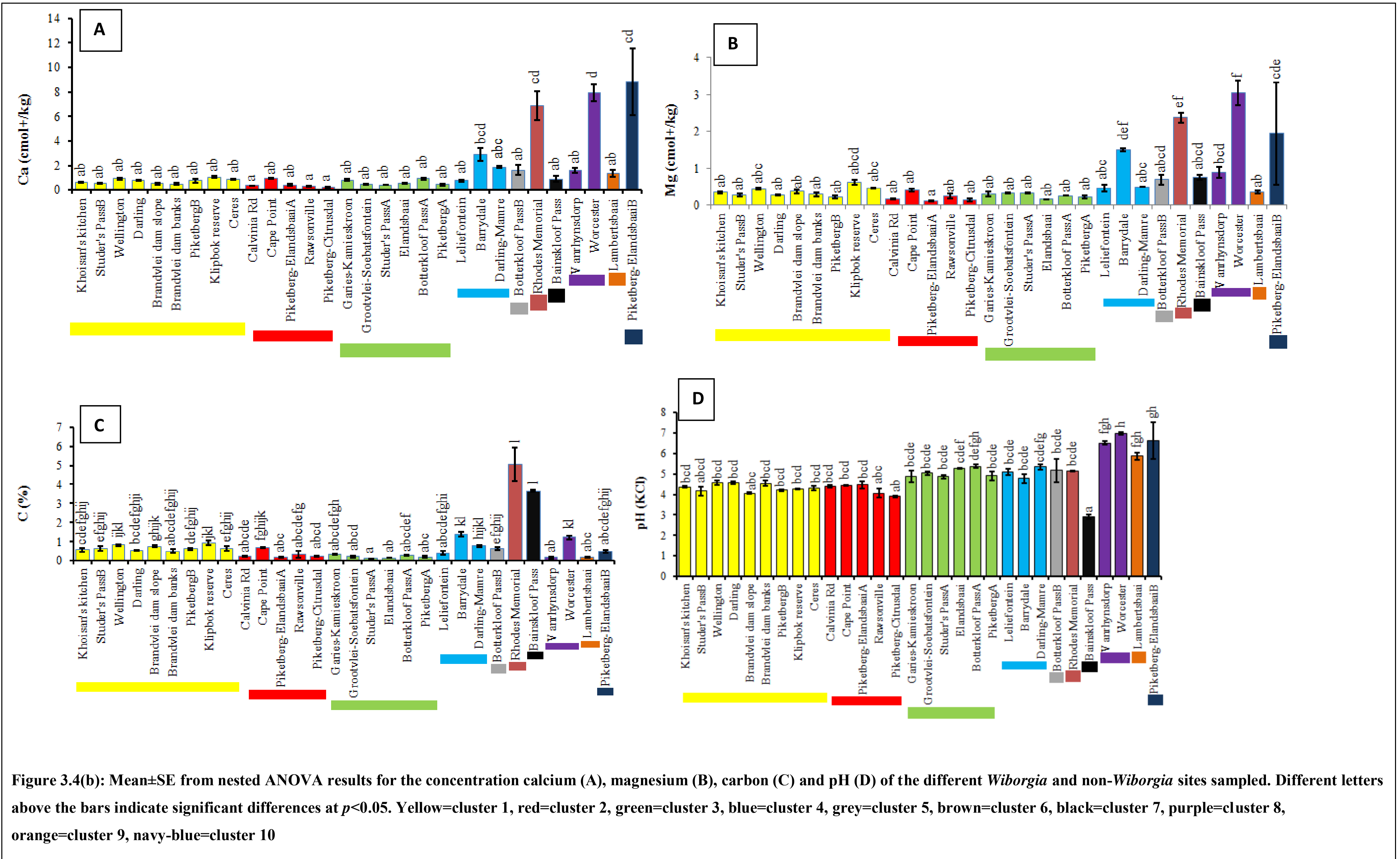


Figure 3.4(b): Mean±SE from nested ANOVA results for the concentration calcium (A), magnesium (B), carbon (C) and pH (D) of the different *Wiborgia* and non-*Wiborgia* sites sampled. Different letters above the bars indicate significant differences at $p<0.05$. Yellow=cluster 1, red=cluster 2, green=cluster 3, blue=cluster 4, grey=cluster 5, brown=cluster 6, black=cluster 7, purple=cluster 8, orange=cluster 9, navy-blue=cluster 10

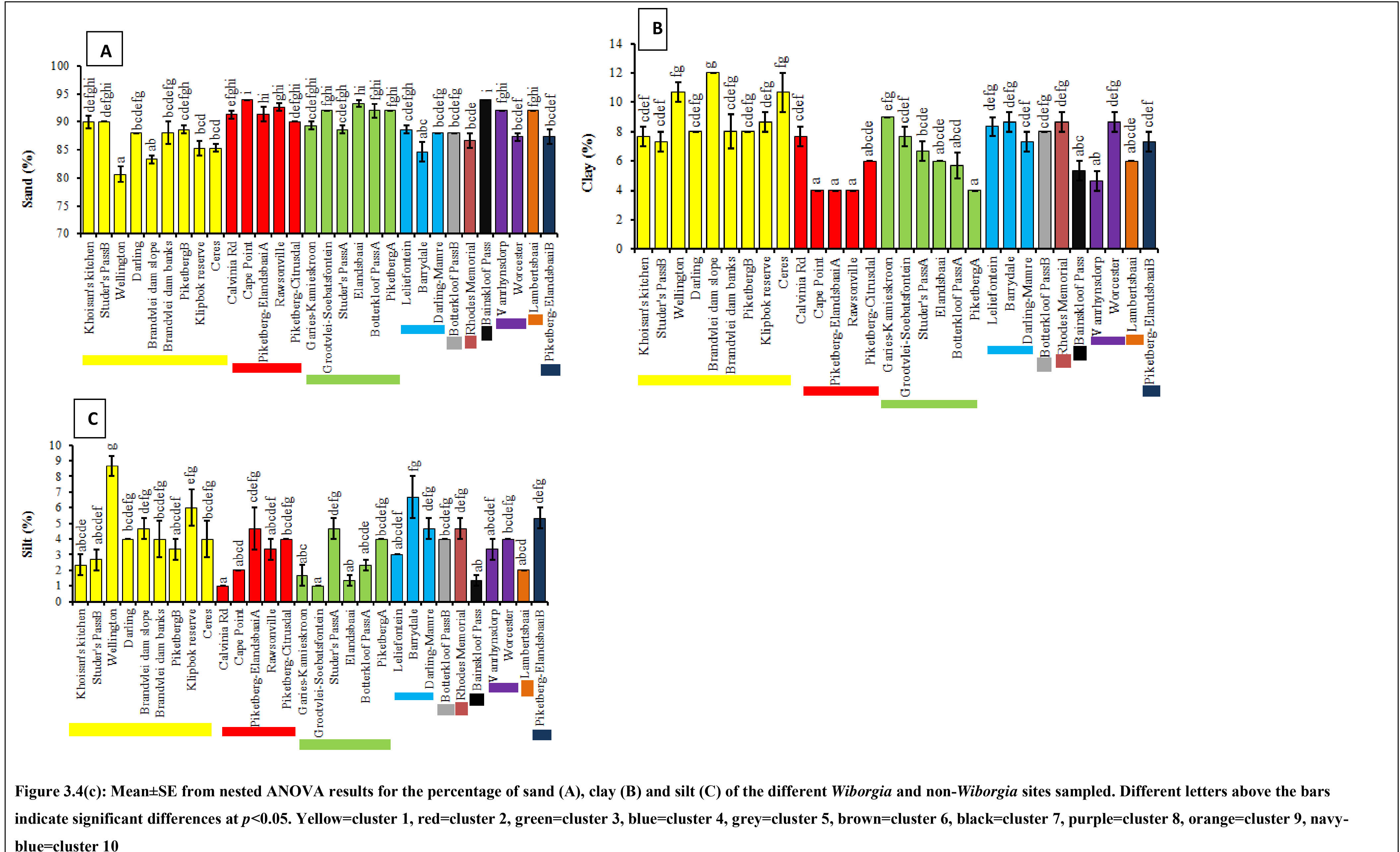


Figure 3.4(c): Mean±SE from nested ANOVA results for the percentage of sand (A), clay (B) and silt (C) of the different *Wiborgia* and non-*Wiborgia* sites sampled. Different letters above the bars indicate significant differences at $p<0.05$. Yellow=cluster 1, red=cluster 2, green=cluster 3, blue=cluster 4, grey=cluster 5, brown=cluster 6, black=cluster 7, purple=cluster 8, orange=cluster 9, navy-blue=cluster 10

3.4 Discussion

3.4.1 Do *Wiborgia* species occupy soils of similar nutritional characteristics across their entire distribution range?

The objective of this Chapter was to investigate whether there were any nutritional differences between soils within *Wiborgia* habitat sites and compared to non-habitat sites.It was hypothesized that *Wiborgia* species occupy soils of similar nutritional levels across their entire distribution range. And from our results it is clear that nutritionally, soils habitat to *Wiborgia* seems to be indifferent and that the species in the genus occupy soils of similar nutritional characteristics across their entire distribution range. Our initial exploration of the data (through cluster analysis) suggested that *Wiborgia* species occupied soils of different nutritional characteristics as evidenced by the formation of ten different clusters at a cut-off point of 1.8 (Fig. 3.2), these clusters were however not supported by the DFA analyses (Fig. 3.3). However, a closer look at the individual concentrations of the different *Wiborgia* sites (through nested ANOVA) show that P levels of the different *Wiborgia* sites were quite variable with sites such as Barrydale, Darling-Mamre, and Lambertsbaai all having quite high levels, whilst Grootvlei-Soebatsfontein and PiketbergA had the lowest levels; therefore considering these total P results in conjunction with available P results (Bray II P) [Fig. 3.4(a) B] a different picture is painted where Botterkloof PassB, Khoisan's Kitchen and Lambertsbaai have the highest Bray II P levels whilst Calvinia, Rawsonville, and PiketbergA all have the lowest Bray II P levels and therefore the implication of these two results together show that although soils of *Wiborgia* species seem to be in overall similar nutritionally, there are some sites which have surprisingly very high total P and available P levels as well as those which have quite low levels. This suggests that the genus has a wide tolerance range for P and other nutrients measured including Ca or Mg. Furthermore, it seems that the site variation in the minima and maxima of these nine nutrients is randomly spread across sites from both the ECR and the CCR, since there was no general pattern (subregion wise) for the existence of the higher or lower nutrient *Wiborgia* sites. Our first hypothesis was that *Wiborgia* occupies soils of similar nutritional levels across their entire distribution range, and so based on the results of the multivariate analysis can be accepted. Similarly the second hypothesis that there are nutritional differences between *Wiborgia* and non-*Wiborgia* sites was supported because the non-*Wiborgia* sites Rhodes Memorial (cluster 6) and Bainskloof (cluster 7) were clearly separated from the other sites (Fig 3.3). The soil factors identified as being the most significant in the separation of the non-*Wiborgia* sites from the *Wiborgia* sites included concentration of C, sand, Ca, total P and pH, which contribute highest values based coefficient of variations (CVs) 1 and 2 (Table 3.2). These observations are in agreement with the findings of Richards *et al*. (1997b), which identified that species distribution in the Soetanysberg was explained by the variation in soil nutrient levels.

3.4.2 Do soil nutritional characteristics between *Wiborgia* habitat and non-habitat soils differ?

This study tested the hypothesis that soils from *Wiborgia* sites are nutritionally different from the soils of non-*Wiborgia* sites. Based on the results of the cluster analysis and the DFA, it was observed that some soils of non-*Wiborgia* sites were distinct and incomparable to soils from *Wiborgia* sites and this was supported both by the cluster as well as the DFA [Figs. 3.2 and 3.3] where soils from Rhodes Memorial and Bainskloof Pass were undoubtedly quite different from the habitat soils of *Wiborgia*. From the nine nutrients identified to contribute most to the grouping where Rhodes Memorial and Bainskloof Pass were distinct from each other as well as *Wiborgia* habitat sites, the nested ANOVA results showed that Rhodes Memorial is set apart from the other sites by uniquely having the highest levels of P, Ca and C which were also among the nutrients identified to have high CVs, and thus responsible for separating the site from *Wiborgia* habitat sites. The involvement of P in the separation of *Wiborgia* habitat and non-habitat soil was consistent with the results of Shane *et al*. (2008) who identified *Protea compacta* to be restricted to low P sandy soils and is unable to occur in the soils occupied by *Protea obtusifolia* and *Leucadendron meridianum* which occur in limestone derived soils with 3.5 times higher in P concentration. In addition, the findings of Richards *et al*. (1997b) in their study where they identified, amongst other nutrients, total P, organic C and Ca to play a role in landscape-level species distribution, is in agreement with our findings.

Apart from C, soils from Bainskloof also had high sand percentage levels which were significantly similar to only that of the non-*Wiborgia* site, Cape Point; and considering the study by Goldblatt (1979) which showed that sister species from the genus *Galaxia* (Iridaceae) separated on soil type and pH; findings of the current study also show a similar pattern whereby soils from Bainskloof are unique from *Wiborgia* habitat soils by having a high sand composition and the lowest pH which may therefore be regarded among the main nutrients which set Bainskloof soils apart from the rest and thus limiting the occurrence of *Wiborgia* species in Bainskloof. Apart from these two non-*Wiborgia* sites (Rhodes Memorial and Bainskloof), there is also one non-*Wiborgia* site (Cape Point) which however did not form a distinct isolated group but rather was shown to be quite similar/comparable in terms of nutritional characteristics to *Wiborgia* sites as was revealed by both the cluster and DFA. From the results, Cape Point soils are embedded within the large group made up of only *Wiborgia* sites, thus effectively suggesting that the interaction of nutrients from soils of Cape Point in multivariate space result in a nutrient profile which is quite comparable to that of all soils from the *Wiborgia* sites [Fig. 3.3]. This is because Cape Point soils seem to have all nutrients (Bray II P, sand, clay, silt Mg, Ca, C, and pH) except total P falling within ranges similar to the soils from *Wiborgia* sites and it thus does not come as a surprise that Cape Point soils were classified as nutritionally similar to *Wiborgia* sites. The implications of our results concerning Cape Point in relation to other *Wiborgia* sites, is therefore

that the lack of *Wiborgia* in the area may not be attaributed to soil nutrient contents, but rather to other factors such as climate, altitude, pollinator availability and frequency of fire. Therefore, to address the issue of why *Wiborgia* does not occur in Cape Point, an ecological and climatic niche modelling which would incorporate all of the other factors not explored in our study would be appropriate, and then more robust statements addressing the absence of *Wiborgia* in Cape Point may then be drawn. Thus, our second hypothesis is partially accepted that soils from *Wiborgia* sites are sometimes nutritionally different from the soils of non-*Wiborgia* sites.

3.5 Conclusion

The results of this study showed that *Wiborgia* occupies soils with similar nutritional characteristics that cover a wide range of individual levels of nutrients, thus effectively showing that soil type and nutrient availability may be less important in the distribution of species within the *Wiborgia* habitat areas. Secondly, soil nutrients may partially be associated with the absence of *Wiborgia* species in some areas. The distribution of *Wiborgia* species is very interesting and further studies mainly involving ecological modelling using GIS tools, and incorporating a phylogenetic framework, regeneration strategies, as well as pollination and dispersal aspects need to be undertaken in order to explore and understand the drivers of the distribution of *Wiborgia* species, but for now it can be concluded that *Wiborgia* species are edaphic generalists within their distribution range.

CHAPTER 4

4.0 ARE *WIBORGIA* SPECIES ABLE TO GROW AND NODULATE IN SOILS OUTSIDE THEIR DISTRIBUTION RANGE?

4.1 INTRODUCTION

The GCFR is a hyperdiverse, highly distinctive phytogeographical region characterized generally by acidic soils that are low in nitrogen and phosphorus (Mitchell *et al*., 1984; Stock & Lewis, 1986; Witkowski & Mitchell, 1987; Cowling & Holmes, 1992). In the region, soil factors have also been reported to influence plant distribution, where for example, the boundaries of the five main vegetation types of the CCR have been suggested to be driven and determined by the underlying parent material of the soil (Cowling & Holmes, 1992; Richards *et al*., 1995; Mucina & Rutherford, 2006). The GCFR landscape (especially the CCR) is mainly characterized by a mosaic of soils derived mainly from sandstone and shale substrates, giving rise to a variety of soil types which include sandstone, aeolian sands, shale, granite, limestone, thus giving rise to a wide range of heterogeneous edaphic niches (Bond & Goldblatt 1984; Mucina & Rutherford 2006; Manning & Goldblatt, 2012). The soil type plays an important role in influencing plant distribution, becoming more pronounced when in conjunction with precipitation levels (Fraser, 1988; Linder *et al*., 2003). In the CCR, forest vegetation frequently occurs in areas characterized by deep soils and high levels of year-round precipitation, fynbos occurs in areas with sandy soils derived from sandstone and varying rainfall, whilst renosterveld is found in more clay rich, shale derived soils (Bond & Goldblatt 1984; Dean *et al*., 1995; Linder 2003; Manning & Goldblatt 2012). Edaphic heterogeneity is considered as a major driver of the high beta diversity characterizing the Cape flora (Cowling, 1990; Cowling *et al*., 1992; McDonald *et al*., 1996; Cowling *et al*., 1997; Cowling *et al*., 1996; Cowling & Lombard 2002). A study by Richards *et al* (1995 & 1997a) found that fynbos plant community boundaries of were mainly determined by soil depth, pH and availability of nitrogen and phosphorus, and particular species (*Protea* and *Leucadendron)* showed greater growth and survival when grown on their native soils as compared to other neighbouring soil types (Mustart & Cowling 1993; Richards *et al*., 1997b).

In addition to edaphic and climatic factors, abiotic factors also play a role in influencing plant distribution. Fire has been identified as one of the most important factors affecting plant distribution in the CCR, causing temporary fluctuations in the nutrient status of the soils post-fire (van Wilgen & Le Maitre, 1981; Brown & Mitchell, 1986; Stock & Lewis, 1986). Such fluctuations have been suggested to mainly increase the concentration of nitrogen and phosphorus (as well as other nutrient elements), and this increase would in return thus favour the growth of species with high nutrient

demands (Specht, 1979; van Wilgen & Le Maitre, 1981; Kruger *et al*., 1983; Brown & Mitchell 1986; Stock & Lewis 1986; Arroyo *et al*., 1995; Bond & van Wilgen, 1996; Cowling *et al*., 1996; Power *et al*., 2010; Rebelo *et al*., 2006). In the CCR context, legumes are known to be common in the early post-fire succession, and largely absent in mature Fynbos ecosystems (Kruger, 1979; Shea *et al*., 1979; Bell & Koch, 1980; Westman, 1981; Hoffman *et al*., 1987; Cocks, 1994; Kazanis & Arianoustou, 1996, 2004).

Understanding the factors influencing plant growth and distribution has interested scientists worldwide. Thus uncovering the factors driving plant growth and distribution forms an integral step into further understanding where and why certain plant species occur only in certain areas and not others (Salisbury, 1926; Kuper *et al*., 2006). Pearson and Dawson (2003) have suggested that the different environmental factors act differently at different scales, whereby climate has most influence at a global and regional scale, soil type influences at a site and local scale, whilst biotic interaction have influence at a micro level. Several other authors have thus pointed out that variations in chemical, physical and biological properties of soil may be favourable for the growth of one plant, whilst on the other hand being unfavourable to another plant and thus resulting in a soil driven structuring of plant distribution patterns (Billings, 1952; William & Palmanis, 1998; Rajakaruna, 2004). More recent studies have also reported that the heterogeneity in edaphic factors results in a variation in plant distribution, diversity and abundance (Clark *et al*., 1998; Rajakaruna, 2004; Arshad *et al*., 2008; Gregoire, 2010; Toledo *et al*., 2012). For example, Arshad *et al*. (2008) found that edaphic factors like salinity, organic matter content, and ionic concentration (mainly sodium, phosphorus, and potassium) seemed to be the most important factors responsible for plant distribution in the Cholistan desert. Other studies that have focussed on this topic have suggested that the interaction of factors ranging from climatic, biotic, edaphic, topographic, seem to be the drivers of plant distribution as opposed to the effect of one single factor acting alone (Salisbury, 1926; Billings, 1952; Grace, 1987; William & Pilmanis, 1998; Essl *et al*., 2009; Reed *et al*., 2009). Although the interaction of factors has been suggested to be more important, other studies have ranked some factors in their roles in influencing plant distribution. For example Cain (1944) ranked climatic and edaphic factors, first and second respectively, as the two factors which play a major role as well as strongly influencing plant distribution.

The South Afican legume flora comprises over 1600 species, mostly in Papilionoideae (92%; Mimosoideae (5%), Caesalpinoideae (3%) (Germishuizen, 2000), can be broadly portioned into three assemblages based on whether they occur in the Temperate, Succulent or Grassland areas (Lewis *et al*., 2005). The Temperate assemblage (coinciding with the GCFR) is nearly exclusively the home for the Hypocalyteae and Podalyrieae and there is nearly no legume species that occurs in both Temperate and Grassland areas, and this pattern appears to hold even at tribal level where genera are portioned

between the areas. Such partitioning of legume species into biomes or even within vegetation types is puzzling, and it was hypothesized that edaphic and biotic factors may be driving the process. In addition to edaphic and climatic factors, biotic factors (such as presence of compatible rhizobial symbionts) also play a role in influencing plant distribution. One of the pioneering studies on rhizobia on South African soils was by Deschodt & Strijdom (1976), who isolated *Bradyrhizobium* species from the nodules of *Aspalathus* and *Rafnia* species which are indigenous to the Core Cape Subregion (CCR). Following this study, a number of studies have focussed on investigating the rhizobia diversity associated with indigenous legumes, mainly *Cyclopia*, *Lotononis*, *Lebeckia,* as well as exotic *Acacia* species (Kock, 2004; Spriggs, 2004; Le Roux, 2003; Phalane, *et al*., 2008; Joubert, 2002) and most recently the studies of Lemaire *et al* (2015a & b) focussing on a number of GCFR tribes. This number of the studied legumes in the Cape is quite low considering that Fabaceae form a large component of the Cape flora, ranked second and fifth most speciose family in the Core Cape Subregion (CCR) (Manning & Goldblatt, 2012) and Extra Cape Subregion (ECR) (Snijman, 2013) respectively. Most legume species which have been studied in the Cape are thus far known to be able to nodulate and form a symbiosis with nitrogen fixing bacteria, thus highlighting the important ecological role played by rhizobia in this region (Sprent, 2009; Sprent & Gehlot 2010; Sprent *et al*., 2010; Sprent *et al*., 2013; Lemaire *et al*., 2015a & b). Emerging from a number of studies, rhizobia diversity in the Cape region (mainly CCR) includes *Burkholderia*, *Herbasprillum*, *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*, which were mainly isolated from genera of papilionoid legumes (Le Roux, 2003; Phalane *et al*., 2008; Hassen *et al*., 2011; Elliot *et al*., 2007; Gerding *et al*., 2012; Lemaire *et al*., 2015a & b).

Most recent studies on Papilionid tribes such as Crotalarieae (*Aspalathus* and *Lebeckia*), Hypocalypteae (*Hypocalyptus*), Indigofereae (*Indigofera*), Phaseoleae (*Bolusafra*, *Dipogon*, *Rhynchosia*), Podalyrieae (*Cyclopia*, *Podalyria*, and *Virgilia*), Psoraleeae (*Psoralea* and *Otholobium*) revealed the diversity of rhizobia species belonging to the Alpha- and Beta-rhizobia subclass (Kock, 2004; Elliot *et al*., 2007; Garau *et al*., 2009; Gyaneshwar *et al*., 2011; Gerding *et al*., 2012; Hassen *et al*., 2011; Kanu & Dakora, 2012; Beukes *et al*., 2013; De Meyer *et al*., 2013a & b; Howieson *et al*., 2013; De Meyer *et al*., 2014; Lemaire *et al*., 2015a & b). Some authors have suggested that *Burkholderia* species are dominant in the Cape mainly due to their adaptations to infertile acidic soils (Bontemps *et al*., 2010; dos Reis Junior *et al*., 2010; Mishra *et al*., 2012). In their recent study, Lemaire *et al* (2015a) pointed out that the focus of previous studies in the Cape explored very few legume genera, which may only be a fraction of the total rhizobia diversity, therefore suggesting that a large portion of the rhizobial diversity in the Cape could still be unexplored. In that study, Lemaire *et al*. (2015), isolated rhizobia from nodules of species from 14 genera belonging to 9 tribes, which included *Aspalathus*, *Crotalaria*, and *Rafnia* from the Crotalarieae. Their study found that *Mesorhizobium* symbionts showed general preference to Psoraleeae hosts, whilst *Burkholderia*

generally showed preference for Podalyrieae hosts, and in general both the Crotalarieae and Indigofereae showed a degree of symbiotic promiscuity where they nodulated with symbionts from both alpha- and beta-rhizobia lineages. The Crotalarieae is one of the 33 Cape floral clades as identified by Linder (2003), who pointed out that although most genera within the tribe have species in the CCR (formerly CFR), *Wiborgia* is the only genus which has most of its species in the Cape. Species within the genus mainly occur in the transitional zone between fynbos-renosterveld and in some instances they occur in several forms of the renosterveld vegetation (Dahlgren, 1975). Although some genera in the Crotalarieae have been fairly explored, little is known about the rhizobia nodulating *Wiborgia* species and whether their influence on the geographical distribution of *Wiborgia* species. It is also puzzling why *Wiborgia* mainly occurs in the dry fynbos-renosterveld transition (Dahlgren, 1975) and hardly overlaps with its sister taxa (e.g. *Aspalathus*) in the CCR (based field observations). Using field collected data and common garden experiments; I tested the ability of *Wiborgia* species to grow outside their current range and evaluated their ability nodulate in diverse soils.

The objectives of the study were:

- To evaluate potential of *Wiborgia* species to grow and nodulate in soils from within and outside distribution range.
- To identify the diversity, characterize and infer phylogenetic relationships of rhizobia nodulating *Wiborgia* species

Hypotheses tested in this Chapter were that:

- The distribution of *Wiborgia* species is determined by the presence of compatible rhizobia
- *Wiborgia* species occupy habitats with similar nutrient concentrations in the soil and will thus have similar nutrient concentration in the tissues
- *Wiborgia* species are nodulated by unique and closely related rhizobia species.

4.2 Materials and Methods

4.2.1 Field seed, soil and nodule collection

Pods and soils were collected from representative plants of each *Wiborgia* species along the distribution range in the CCR and ECR (Figure 3.1, Chapter 3). In total 48 different sites were visited twice and in some cases three times, and collections were made at different life stages of plant growth including flowering and seed formatting stages. For every visit, a new herbarium specimen collection was made under new voucher numbers in order to differentiate the specimens collected during different life stages to allow for future phenology studies. At each of the sites, mature pods were

collected from the different species and placed in brown paper bags and labelled accordingly. The pods were placed in -20°C for 48 hours to kill off any seed eating insects which may have been collected with the pods. In addition, nodules were collected from a total of 17 sites representing seven of the nine species of *Wiborgia* (excluding *W. monoptera* and *W. incurvata*). The nodules were placed in plastic vials filled partially with silica gel beads, and a piece of cotton separating the nodules and silica gel.

Soil samples for isolation of rhizobia (i.e. trapping experiment) were collected by taking soil from the rhizosphere of the species using a sterilized spatula. Three soil samples were taken for the trapping experiment per site. The soil samples were placed in labelled Ziploc bags, and placed in a cooler box and transported to the lab and stored in the 10°C constant temperature room. Furthermore, large amounts of soil samples were collected in 50 kg bags, labelled and transported to the lab for plant growth experiment to assess how *Wiborgia* species grows in soils collected from habitat and non-habitat areas. In the lab, the soils were passed through a 2 mm sieve to remove debris and stones, and then stored in the 10°C to await the plant growth experiment (section 4.2.3.2). A herbarium voucher specimen was taken every time pods, soils, and nodules were collected.

4.2.2 Scarification and surface sterilization of the seeds

The scarification of *Wiborgia* seeds was done following the protocol from the Master-class in Rhizobial Technology Manual (Tiwari *et al*., 2012). For all *Wiborgia* seeds used the glasshouse experiments, the process involved placing seeds on a sheet of coarse sandpaper and another sheet of sandpaper was used to firmly rub on the seeds until a visible scar was observed by the naked eye or a dissecting microscope. The manually scarified seeds were then surface sterilized following the modified protocol obtained from the Master-class in Rhizobial Technology Manual (Tiwari *et al*., 2012). The scarified seeds were placed in a tea strainer, dipped in 70% (v/v) ethanol for 1 minute, and then in 4% (v/v) hypochlorite solution for 3 minutes and rinsed in a series of 6 changes of sterile distilled water. An alternative chemical scarification using sulphuric acid (H_2SO_4) was also tested, but was unsuccessful for *Wiborgia*. For seeds of *Podalyria calyptrata* and *Macroptilium atropurpureum* (Siratro) which were used for the authentication experiment, scarification and surface sterilization was done by placing them in sterilized boiled water overnight.

The seeds were then placed onto plates of 1.5% (w/v) water agar with the use of a sterile applicator stick, and placed in a germination room maintained at 25°C. Upon the emergence of the radicle, the plates were then covered with aluminium foil and turned upside down in order to avoid the radicle

growing into the agar medium. The seeds were checked daily and the seeds which successfully developed a radicle were sown into the pots as described below (sections 4.2.3.1 and 4.2.3.2a).

4.2.3.1 Rhizobia trapping experiments

The preparation of pots for rhizobia trapping was done following a modified version of the protocol from the Master-class in Rhizobial Technology Manual (Tiwari *et al*., 2012). Two approaches were adopted for (i) the rhizobia trapping experiment using the O'Hara closed vial method (Tiwari *et al*., 2012), and (ii) the plant growth experiment (section 4.2.3.2a) where the plants were grown also under aseptic conditions. For the O'Hara closed system, the preparation of pots involved placing acid washed sand to fill approximately 100 mL of the O'Hara vial (the vial is of 500 mL volume). The vials containing the acid washed sand were autoclaved and cooled to room temperature. An amount of 20 mL of prepared nitrogen-free Centre for Rhizobial Studies (CRS) nutrient growth solution was added. The trapping soil, previously stored at 10°C, was added to the vials to make a layer of 3 cm, and a 5 mm layer of sterile sand was added to cover the soil. An amount of 10 ml of sterile distilled water was added at this point to moisten the sand and soil. Then, two 4 mm deep holes were drilled using a sterile orange applicator stick around the perimeter of the vial and two seedlings were then placed into the hole using sterile tweezers. Both positive and negative controls were included to the experimental set-up and used only sterile sand (i.e. without soil). For the positive controls, 20 mL of sterile KNO_3^- solution was added to the vials together with 20 mL of sterile CRS nutrient growth solution whilst for negative controls, only the CRS Nutrient Growth Solution (i.e. without N) was added to the vials. All prepared vials were covered with loose lids, labelled accordingly and the seedlings were allowed to grow for 8 weeks in a glasshouse at the University of Cape Town which has an average temperature of 20°C and a range of 6-35°C. During the growth period, the moisture levels within the pots were monitored and where there was need, sterile distilled water was added.

At harvest, the open end of the pot was covered with one hand and then tilted with another hand to loosen the soil layers. The pot was then held upside-down to allow both the plants and the soil to be removed from the pot and held on the covering hand; the soil and plants were then placed onto a 0.5 mm mesh sieve which was then placed into a bucket filled with tap-water in order to disentangle the roots from the soil particles. The plants were then washed in another bucket filled with tap water paying attention to avoid losing any root and nodules. The nodules were separated from the root by cutting a piece of root 2 mm close to the nodule using scalpel blade and tweezers. After all nodules were removed from the roots, they were placed in small plastic tubes containing a layer of silica beads at the bottom and cotton wool on top. Nodules from each plant were placed in a single tube and

labelled accordingly. The vials were then stored in a 10°C constant temperature for further rhizobial isolation as described in section 4.2.3.3 (a) below.

4.2.3.2(a) Plant growth on soils from different sites

For this experiment, 18 cm pots were sterilized by soaked in 3.5% sodium hypochlorite [(NaOCl) in the form of bleach] solution overnight and then rinsed with sterile distilled water, and allowed to dry under the laminar flow cabinet. Acid-washed sand was added to the pots to form a 10 mm layer and autoclaved then cooled under the laminar flow cabinet. Preparation of pots was done following the experimental setup shown on Table 4.1, where each of the five species shown were grown in soils from eight localities and two pots representing the positive and negative controls. The controls for this experiment were similar to those used in the trapping experiment where pots were filled with sand and autoclaved; positive controls were fed KNO_3 plus N-free CRS nutrient solution, whilst negative controls were only fed the CRS nutrient solution (Table 4.1). Of the eight localities (Table 4.1), five localities are habitat sites for *Wiborgia* species where Vanrhynsdorp is habitat to *W. obcordata* (Muasya_6931); Darling is habitat to *W. mucronata* (Moiloa_3); Grootvlei-Soebatsfontein is habitat to *W. sericea* (Muasya_6923); Leliefontein is habitat to *W. incurvata* (Muasya_6928); and Brandvlei dam slope is habitat *W. tenuifolia* (Moiloa_18), respectively. The selected species were identified as either widespread or narrowly distributed based on the extent of geographical range they cover, thus *W. mucronata*, *W. obcordata*, and *W. sericea* were regarded as widespread species endemic to the GCFR. *Wiborgia incurvata* was regarded as a narrowly distributed species endemic to the ECR and mainly restricted to the Namaqualand area and also the Kamiesberg Mountains (Snijman 2013); similarly *W. tenuifolia* was regarded as narrowly distributed species endemic to the CCR (Manning & Goldblatt, 2012; Snijman, 2013). The remaining three sites (Table 4.1) were made up of three different soils were from localities not representative to any *Wiborgia* species, these include Cape Point (Muasya_6953), Rhodes Memorial (Moiloa_7), Bainskloof pass (Moiloa_8), but these localities are habitat to other legume genera such *Aspalathus*, *Indigofera* and *Psoralea*. Rhodes Memorial, Cape Point and Bainskloof are regarded as the non-habitat soils for *Wiborgia* based on field observation and absence data from herbarium specimen data sourced from the SANBI PRECIS database as well as the taxonomic revision by Dahlgren (1975).

Table 4.3: Experimental setup for the plant growth experiment using five *Wiborgia* species grown in both habitat and non-habitat soils as shown. Habitat soils for the species used are highlighted in bold. For each species, the entire treatment row was relicated four times.

Species	**Voucher number for each soil treatment**									
	Wiborgia habitat soils					non-*Wiborgia* soils			Controls	
W. incurvata	**6928**	3	6931	6923	18	6953	7	8	positive	negative
W. mucronata	**3**	6928	6931	6923	18	6953	7	8	positive	negative
W. obcordata	**6931**	6928	3	6923	18	6953	7	8	positive	negative
W. sericea	**6923**	6928	3	6931	18	6953	7	8	positive	negative
W. tenuifolia	**18**	6928	3	6931	6923	6953	7	8	positive	negative

After filling the pots with soil, a 7 cm long sterile watering tube made from polyvinyl pipes with a 24 mm diameter was placed 5 mm deep in the centre of the pot for watering. Two germinated seedlings were planted into 10 mm deep holes using a sterile orange applicator stick, and then covered with sterile nylon beads. The pots were then labelled accordingly and allowed to grow for 6 months in a well-ventilated glasshouse of the University of Cape Town which had an average temperature of 20°C with a range of 6-35°C. The watering regime of the pots involved adding through the watering tube, 50 ml of CRS Nutrient Growth Solution per pot weekly, and sterile distilled water on every third day or as required depending on weather conditions. For positive controls, 50 ml of potassium nitrate (KNO_3) was also added to the pots weekly.

At harvest, the nylon beads were removed from the pots by hand and recycled by soaking in 70% ethanol overnight for later use. The above-ground plant matter (shoot system) was excised using a scalpel blade, separated into leaves and stem then weighed separately for determination of the fresh-weight. The root system was removed from the mesh and nodules were separated from the roots using the same protocol as described for the closed system trapping experiment above. The roots and the nodules were mopped dry with laboratory paper towel and weighed to determine their fresh weight biomass. The leaves, stem and roots were then placed in labelled paper bags, and oven-dried at 60°C for 72 hours before being weighed again to determine for dry-weight biomass. The samples were prepared for nutrient analysis by separately milling the shoot, with leaves and stem mixed due to inadequate amounts to analyse each organ separately, and root tissues using a Hammer Mill (United Scientific Pty Ltd, South Africa). The nodules on the other hand were placed in plastic vials as was done for the nodules obtained from the trapping experiment above to await subsequent rhizobia cultivation. All the nodules obtained in the above glass house experiment were used in the rhizobia isolation for the determination of diversity of rhizobia nodulating *Wiborgia* species.

4.2.3.2(b) Nutrient analyses in plant tissue

Plant tissue nutrient concentrations of samples with adequate biomass amounts (at least a minimum of 0.8 grams of plant sample in order to analyse for all nutrients as per laboratory protocol) were analysed at the BemLab Private Laboratory in Somerset (Western Cape, South Africa); where concentrations of Na, Mn, Fe, Zn, Cu, B, K, Ca, Mg, and P were determined by first digesting the shoot material in a 50:50 hydrochloric acid (HCl) solution and then the extract was measured using the optical emission spectrophotometer (ICP-OES, Varian, United States). Because most plants were too small and did not have enough biomass levels to analyse for nitrogen which needed larger quantities of tissue samples for analysis through the Bemlab methodology, nitrogen was therefore not analysed.

4.2.3.2(c) Statistical analyses

The data were then normalised by the use of log transformation before statistical analyses were conducted in Statistica v12 (StatSoft, Inc., Tulsa, USA). Both plant biomass and plant nutrient data were analysed using one-way analysis of variance (ANOVA) to test for equality of means for species grown in soil collected from different sites and different species grown on soil collected from one site. The analyses were done for each species or site because of variations of species germination and plant growth in soil from different sites. The Tukey HSD test was then used to evaluate for significantly different means at $p<0.05$.

4.2.3.3(a) Rhizobia cultivation/isolation from Root Nodules

The cultivation of rhizobia from root nodules from plants grown aseptically in the glasshouse as well as those collected from the field (Table 4.2) was done following the procedure by Vincent (1970). Silica gel dried nodules were initially rehydrated using sterile distilled water for 2 hours, then washed in sterile distilled water and dipped in 95% ethanol for 1 to 2 minutes; the nodules were then inundated in 1% acidified mercuric chloride ($HgCl_2$) or 3.5% sodium hypochlorite (NaOCl) in the form of bleach, for 4 minutes. The nodules were then rinsed in a series of six changes of sterile distilled water. The cultivation and isolation of rhizobia was achieved by aseptically crushing a single nodule on a plate with a drop of sterile sodium chloride ($NaCl_2$) solution. A flame-sterilized wire loop was then used to streak the contents of the nodule squash onto an agar plate containing Yeast Extract Mannitol Agar (YEMA), and streaked in a manner which gradually diluted the suspension in order to obtain single pure colonies. The plates were incubated at $28^{\circ}C$ in a dark constant temperature room for up to 10 days, with daily observation, in order to allow the bacteria culture sufficient time to grow. The plates were observed daily. The cultures were purified further by repeating a series of multiple consecutive streaks of a single colony. The purified cultures were stored in 20% autoclaved glycerol solution and placed at $-80^{\circ}C$ for long term storage.

Table 4.4: Voucher information of legume species whose nodules were collected and their locality and elevation. The entries in bold are those which were successfully isolated from the plant growth experiment (G), rhizobia trapping experiment (T), as well as from field collected nodules (F); and these were all used in the phylogenetic study in this study.

Host plant	Treatment/Voucher	Locality	Latitude	Longitude	Altitude(m)
Wiborgia fusca(T)	6924_6922	Grootvlei-Soebatsfontein	S30°13'27.2"	E17°45'50.1"	690
***Wiborgia fusca*(F)**	**23**	**Lambertsbaai**	**S32°18'30.2"**	**E18°24' 3"**	**96.2**
Wiborgia fusca(F)	22	Piketberg-ElandsbaiB	S32°41'19.4"	E18°48'0.4"	145.3
Wiborgia incurvata(T)	6928_6953	Cape Point	S34°18'30.3"	E18°23'4.8"	25
***Wiborgia incurvata*(G)**	**6928_3_1**	**Darling**	**S33°22'58.9"**	**E18°22'55.3"**	**170**
***Wiborgia incurvata*(G)**	**6928_6928**	**Leliefontein**	**S30°16'4.7"**	**E18°3'18"**	**1177**
***Wiborgia incurvata*(T)**	**6928_6928**	**Leliefontein**	**S30°16'4.7"**	**E18°3'18"**	**1177**
***Wiborgia leptoptera*(F)**	**31**	**Darling**	**S33°22'58.9"**	**E18°22'55.3"**	**170**
Wiborgia leptoptera(F)	29	PiketbergB	S32°36'56.8"	E18°46'13.2"	142.2
***Wiborgia mucronata*(G)**	**6930_18**	**Brandvlei dam slope**	**S33°40'39.0"**	**E19°23'20"**	**384.2**
Wiborgia mucronata(F)	34	Brandvlei dam slope	S33°40'39.0"	E19°23'20.7"	384.2
Wiborgia mucronata(F)	36	Brandvlei dam banks	S33°14'40.4"	E19°23'20.11"	211
***Wiborgia mucronata*(G)**	**6930_6953**	**Cape Point**	**S34°18'30.3"**	**E18°23'4.8"**	**25**
***Wiborgia mucronata*(F)**	**30**	**Darling**	**S33°22'58.9"**	**E18°22'55.3"**	**170**
***Wiborgia mucronata*(G)**	**6930_3_01**	**Darling**	**S33°22'58.9"**	**E18°22'55.3"**	**170**
***Wiborgia mucronata*(G)**	**6930_6922_2**	**Grootvlei-Soebatsfontein**	**S30°13'27.2"**	**E17°45'50.1"**	**690**
***Wiborgia mucronata*(F)**	**32**	**Klipbokkop**	**S33°49'32"**	**E19°24'11.5"**	**228.1**
***Wiborgia mucronata*(G)**	**6930_6928**	**Leliefontein**	**S30°16'4.7"**	**E18°3'18"**	**1177**
***Wiborgia mucronata*(F)**	**21**	**Piketberg-ElandsbaiB**	**S32°41'19.4"**	**E18°48'0.4"**	**145.3**
Wiborgia mucronata(F)	26	PiketbergA	S32°22'20.9"	E18°42'52.1"	212.9

***Wiborgia mucronata*(G)**	**6930_7_2**	**Rhodes Memorial**	**S33°57'6.73"**	**E18°27'24.58"**	
***Wiborgia mucronata*(T)**	**6930_6930**	**Studer's passA**	**S30°13'54.5"**	**E18°3'38.6"**	**878.2**
***Wiborgia mucronata*(G)**	**6930_6931**	**Vanrhynsdorp**	**S31°31'9.7"**	**E18°43'10.4"**	**143.5**
Wiborgia mucronata(T)	6930_6931	Vanrhynsdorp	S31°31'9.7"	E18°43'10.4"	143.5
Wiborgia mucronata(G)	6930_6931_4	Vanrhynsdorp	S31°31'9.7"	E18°43'10.4"	143.5
***Wiborgia obcordata*(G)**	**6931_18**	**Brandvlei dam slope**	**S33°40'39"**	**E19°23'20.7"**	**384.2**
***Wiborgia obcordata*(G)**	**6931_3_1**	**Darling**	**S33°22'58.9"**	**E18°22'55.3"**	**170**
***Wiborgia obcordata*(G)**	**6931_6928_4**	**Leliefontein**	**S30°16'4.7"**	**E18°3'18"**	**1177**
***Wiborgia obcordata*(F)**	**27**	**PiketbergA**	**S32°22'20.9"**	**E18°42'52.1"**	
***Wiborgia obcordata*(T)**	**6931_6931**	**Vanrhynsdorp**	**S31°31'9.7"**	**E18°43'10.4"**	**143.5**
Wiborgia obcordata(G)	6931_6931	Vanrhynsdorp	S31°31'9.7"	E18°43'10.4"	143.5
***Wiborgia sericea*(F)**	**41**	**Ceres**	**S33°19'2.5"**	**E19°49'32.7"**	**856.8**
***Wiborgia sericea*(G)**	**6923_3**	**Darling**	**S33°22'58.9"**	**E18°22'55.3"**	**170**
***Wiborgia sericea*(G)**	**6923_6922**	**Grootvlei-Soebatsfontein**	**S30°13'27.2"**	**E17°45'50.1"**	**690**
Wiborgia sericea(T)	6923_6922	Grootvlei-Soebatsfontein	S30°13'27.2"	E17°45'50.1"	690
***Wiborgia sericea*(T)**	**6942_6912**	**Khoisan's Kitchen**	**S32°41'8.5"**	**E19°4'30.9"**	**314**
***Wiborgia sericea*(G)**	**6923_6931**	**Vanrhynsdorp**	**S31°31'9.7"**	**E18°43'10.4"**	**143.5**
Wiborgia sericea(T)	6923_6931	Vanrhynsdorp	S31°31'9.7"	E18°43'10.4"	143.5
***Wiborgia tetraptera*(F)**	**24**	**Lambertsbaai**	**S32°18'30.2"**	**E18°24'3.0"**	**96.2**
Wiborgia tetraptera(F)	28	Piketberg-Citrusdal	S32°22'20.9"	E18°42'52.1"	163.3
***Wiborgia tenuifolia*(F)**	**38**	**Brandvlei dam slope**	**S33°40'39.0"**	**E19°23'20.7"**	**384.2**

4.2.3.3(b) Rhizobial genomic DNA Extraction

A modified CTAB extraction protocol was used. The bacterial total genomic DNA was extracted from freshly grown isolates on Yeast Extract Mannitol Agar (YEMA) using the modified Cetyltrimethylammonium Bromide (CTAB) protocol by Doyle & Doyle (1987) and Gawel & Jarret (1991). As described in Chapter 2 (section 2.2.1), the CTAB extraction buffer was mixed with mercapto-ethanol in the ratio 700:1 and heated using the heating block at 65°C. Then a sterilised spatula was used to collect a single pure colony from the agar plate and was suspended in 700 µL of the pre-heated CTAB extraction buffer, the mixture was then vortexed in order to obtain a homogenous mixture; which was then followed by incubation of the samples in the heating block at 65°C for 60 minutes and at every 20 minute interval the samples were gently shaken and mixed by inversion. From this point on the DNA extraction method followed that described in Chapter 2.

4.2.3.3(c) PCR amplifications and Sequencing

Amplifications of housekeeping genes (*16S* rRNA, and *recA*), and symbiosis genes (*nodA*, *nodC*, and *nifH*) were performed using a total reaction volume of 30 µL, which was made up 3 µL buffer; 3 µL $MgCl_2$; 1.2 µL dNTP's; 0.2 *Taq* polymerase; 1 µL each for forward and reverse primer; 18.6 µL of sterile distilled water and 2 µL of template DNA. The PCR amplification was run on Applied Biosystems GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR thermal profiles for all the regions are summarized below, a key to the shorthand code used to describe the thermal profiles is as follows: initial denaturation (temperature and time); number of cycles [denaturation temperature and time; annealing temperature and time; chain extension temperature and time], and final extension (temperature and time), all reactions were then held at a temperature of 4°C.

The primers for the different regions are given in Table 4.2, and thermal profiles are as follows:

- For *nifH* thermal parameters were 95°C for5 min; [3 cycles of (95°C for 1 min; 60°C for 135 s; 72°C for 75 s), followed by 30 cycles of (95°C for 35 s, 60°C for 75 s, 74°C for 75 s)]; and a final extension at72°C for 5min (De Meyer *et al*., 2011).
- For *nodC* thermal parameters were 95°C for 5 min; [3 cycles of (95°C for 1 min; 50°C for 135 s, 72°C for 75 s), followed by 30 cycles of (95°C for 35 s, 50°C for 75 s, 74°C for 75 s)] and final extension at 72°C for 5min (De Meyer *et al*., 2011).
- For *nodA* thermal parameters were 94°C for 2 min; 30 cycles of (93°C for 45 s, 62°C for 45 s, 72°C for 2 min); and 72°C for 5min (Haukka *et al*., 1998).
- For *recA* thermal parameters were 95°C for 5 min; 30 cycles of (94°C for 45 s; 50°C for 60 s; 74°C for 90 s); 74°C for 7 min (Gaunt *et al*., 2001).

- For *16S* thermal parameters were 94°C for 2 min; 30 cycles of (94°C for 1 min; 55°C for 1 min; 72°C for 1 min); and 72°C for 7 min (Lane, 1991; Weisburg *et al*., 1991)

After running the PCR, agarose gel electrophoresis, UV light visualization, and enzyme purification protocols were as those described in Chapter 2. Sequencing was performed using the same labs mentioned in Chapter 2, using the same primers as those used for amplification.

Table 4.5: DNA sequences for primers used for the different markers used in this study

Region	Primer(F/R)	Sequence	Reference
nifH	*nifH* (F1)	TAY GGN AAR GGN GGN ATY GGN AAR TC	Boulygina *et al*. (2002)
	nifH 439(R)	GGC ATN GCR AAN CCDCCR CA	De Meyer *et al*. (2011)
nodC	*nodC* 504(F)	TGA TYG AYA TGG ART AYT GGC T	Sarita *et al*. (2005)
	*nodC*1164(R)	GAY ARC CAR TCG CTR TTG	De Meyer *et al*. (2011)
nodA	*nodA*-1(F)	TGC RGT GGA ARN TRN NCT GGG AAA	Haukka *et al*. (1998)
	nodA-2(R)	GGN CCG TCR TCR AAW GTC ARG TA	
recA	*recA*(F)	CGK CTS GTA GAG GAY AAA TCG GTG GA	Gaunt *et al*. (2001)
	recA(R)	ATC GAG CGG TCG TTC GGC AAG GG	
recA	*recA* 63(F)	ATC GAG CGG TCG TTC GGC AAG GG	Gaunt *et al*. (2001)
	*recA*504(R)	TTG CGC AGC GCC TGG CTC AT	
16S	16f27(F)	AGA GTT TGA TCC TGG CTC AG	Lane (1991)
	16r1485(R)	TAC CTT GTT ACG ACT TCA CCC CA	

4.2.3.3(d) Sequence alignment and phylogenetic analyses of rhizobia isolates

Sequences amplified (*16S* rRNA and *recA*) as part of this study were assembled and edited using Staden package version 2.0.0b8 (Staden *et al.,* 1998). The consensus sequences, together with the sequences obtained from Genbank as well as the *16S* rRNA and *recA* matrices of Lemaire *et al*. (2015), were then imported into Bioedit version 7.2.0 (Hall, 1999) and electronically aligned using the ClustalW multiple alignment then manually inspected and edited for any misaligned residues. Phylogenetic analyses were conducted using Bayesian Inference (BI) criteria, which was carried out on the CIPRES web portal (https://www.phylo.org/). Model test for both loci (*16S* rRNA and *recA*) was conducted using Mega version 6 (Tamura *et al*., 2013) under the Akaike Information Criterion

(AIC). The model that best fit the matrices according to the AIC for *16S* rRNA and *recA* respectively, were the Tamura-Nei (TN93+G) and the Kimura 2-parameter (K2+G) models both with gamma-distributed rate variation across sites, and these model were specified during the analyses. Because the rhizobia sequences generated in this study were uncomplimentary across the two loci, the data were only analysed separately and not concatenated. The BI analysis for both loci was carried out using the MrBayes 3.2.3 on XSEDE tool, running four simultaneous Markov chains with one cold and three heated for five million generations with a temperature setting of 0.20, sampling a tree every 1000 generations. The first 25% of the trees sampled were regarded as 'burn-in' and thus discarded and not included in the analysis when posterior probabilities (PP) were calculated. The trees were then viewed in FigTree version 1.4.2 (Rambaut, 2014), then edited on Adobe Illustrator.

4.2.3.4 Rhizobia authentication experiment

Strains which were successfully isolated were authenticated in order to confirm their nodulation capabilities. A total of 22 strains out of 30 were authenticated based on successful growth of the strains in Yeast Mannitol Broth (YMB) at 28°C. The authentication was done using four secondary host species, *Aspalathus carnosa*, *Lebeckia ambigua*, *Macroptilium atropurpureum* (DC.) Urb. (Siratro) and *Podalyria calyptrata* (Retz.) Wild.. Isolates could not be authenticated using original host species because of the unavailability of seeds and also germination failure for most field-collected *Wiborgia* seeds. All four species were germinated as described above (section 4.2.2), with seeds of *Lebeckia ambigua* and *Aspalathus carnosa* manually scarified whilst those of *P. calyptrata* and Siratro were soaked in boiled water overnight. The seeds were germinated as described above and then sown onto autoclaved 18 cm pots filled with acid washed sand. This was done by drilling three 5mm holes along the perimeter of the pot and transferring the seedlings using sterilized tweezers. After sowing, the pots were covered with a plastic bag for a week to allow establishment of the seedlings, and then inoculated with 15-20 ml of inoculum. After inoculation, the pots were covered with sterile plastic beads; and the plants were watered using a sterile watering tube with both sterile distilled water and a nitrogen free watering solution. The plants were grown for eight weeks and at harvest nodulation was recorded and authentication was confirmed if inoculated plants nodulated and controls were nodule free.

4.3 Results

4.3.1 Plant Biomass

4.3.1(a) Species biomass in soil from different soil sites

(i) Wiborgia obcordata

Wiborgia obcordata was successfully grown in soil from six sites which include Brandvlei dam slope, Darling, Grootvlei-Soebatsfontein, Leliefontein, Rhodes Memorial, and Vanrhynsdorp. Of which Darling and Vanrhynsdorp are the habitat soils whilst the rest are the non-habitat soils. Plants grown in soils from Darling and Leliefontein accumulated the highest levels of biomass (3-4.9g) with a biomass five times more than those grown in Brandvlei, Grootvlei-Soebatsfontein, Rhodes Memorial, and Vanrhynsdorp which accumulated the least biomass (0.1-0.76g) [Figure 4.1(a) i]. These differences are significant with a *p*-value of 0.0001. Although plants from Brandvlei accumulated a slightly higher biomass compared to plants from Rhodes Memorial, the two treatments are still however not significantly different from each other.

(ii)Wiborgia incurvata

W. incurvata was successfully grown in soils from three sites namely Brandvlei, Darling and Leliefontein. Of which Leliefontein is the only habitat soil, whilst Brandvlei and Darling are regarded as non-habitat soils. Total biomass results show that plants grown on soils from Darling accumulated the highest biomass, whilst those from Brandvlei and Leliefontein accumulated the lowest biomass levels. These differences were significant with a *p*-value of 0.006 [Figure 4.1(a) ii]. Comparison of the allocation of biomass across leaves stem and roots show that the pattern of total biomass is also reflected in the leaf, stem and root biomass accumulation, with significant differences (*p*-values 0.002 and 0.009 respectively).

(iii) Wiborgia mucronata

W. mucronata was grown in soils from eight different sites which include Brandvlei dam slope, Bainskloof, Cape Point, Darling, Grootvlei-Soebatsfontein, Leliefontein, Rhodes Memorial, and Vanrhynsdorp. Of which, Brandvlei, Darling, Grootvlei-Soebatsfontein are regarded as habitat soils, whilst Leliefontein, Vanrhynsdorp, Rhodes Memorial, Cape Point and Bainskloof are regarded as the non-habitat soils. Looking at total biomass accumulation, plants grown on soils from Leliefontein and Grootvlei-Soebatsfontein show the greatest accumulation of total biomass (1.8g); whilst plants grown in soils from Brandvlei, Darling and Rhodes Memorial accumulated significantly similar biomass levels (0.4-0.8g), and those grown on Bainskloof and Cape Point soil show the least accumulation of biomass (0.06-0.2g) [Figure 4.1(b) iii]. These differences are significant with a *p*-value of 0.00001.

Comparing the allocation of biomass to the leaf, roots and stem, a slightly altered pattern is observed. Leaf biomass accumulation data show that plants grown in soils from Brandvlei, Darling, Grootvlei-Soebatsfontein, Vanrhynsdorp and Rhodes Memorial all showed similar biomass accumulation. Whilst plants from Bainskloof accumulated the lowest leaf biomass, followed by those from Cape Point. [Figure 4.1(b) iii]. Root biomass on the other hand was accumulated highest in plants grown on soils from Grootvlei-Soebatsfontein, followed by those from Brandvlei and Leliefontein. Stem biomass accumulation of plants grown on soils from Leliefontein accumulated the highest stem biomass, followed by those grown on Brandvlei, Darling and Grootvlei-Soebatsfontein soils. On the other hand, plants grown on Bainskloof and Cape Point soils accumulated the lowest stem biomass, followed by plants from Vanrhynsdorp and Rhodes Memorial soils which showed intermediate biomass accumulation levels [Figure 4.1(b) iii]. All the differences for leaf root and stem biomass accumulation mentioned above were significant with *p*-values of 0.001, 0.00006 and 0.00001 respectively.

(iv) Wiborgia sericea

W. sericea was grown in soils from three different sites which include Darling, Grootvlei-Soebatsfontein, and Vanrhynsdorp; the first two sites are regarded as the habitat sites based on the fact that seeds used for this experiment were collected in Grootvlei-Soebatsfontein whilst herbarium specimen data from SANBI PRECIS and Dahlgren's (1975) taxonomic revision confirm the occurrence of the species in Vanrhynsdorp. In addition, based on a single specimen collection done by Acocks (1948) suggests that the species might also occur in Darling, this is however contradicted by distribution information from Dahlgren's (1975) taxonomic revision. Therefore the Darling locality is regarded as a non-habitat site for *Wiborgia sericea*. Total biomass results show that plants grown on soils from Vanrhynsdorp show the highest accumulation of total biomass (3.3g), whilst those grown in Grootvlei-Soebatsfontein soils accumulated an intermediate biomass (1.7g), and plants grown in soils from Darling accumulated the lowest total biomass (0.9g) [Figure 4.1(b) iv]. The allocation of biomass across leaves, roots and stem show a pattern similar to that of the total biomass results. These differences were significant with a *p*-value of 0.017. Stem biomass follows the same pattern as that of the leaves, however with no significant differences (*p*-value 0.103).

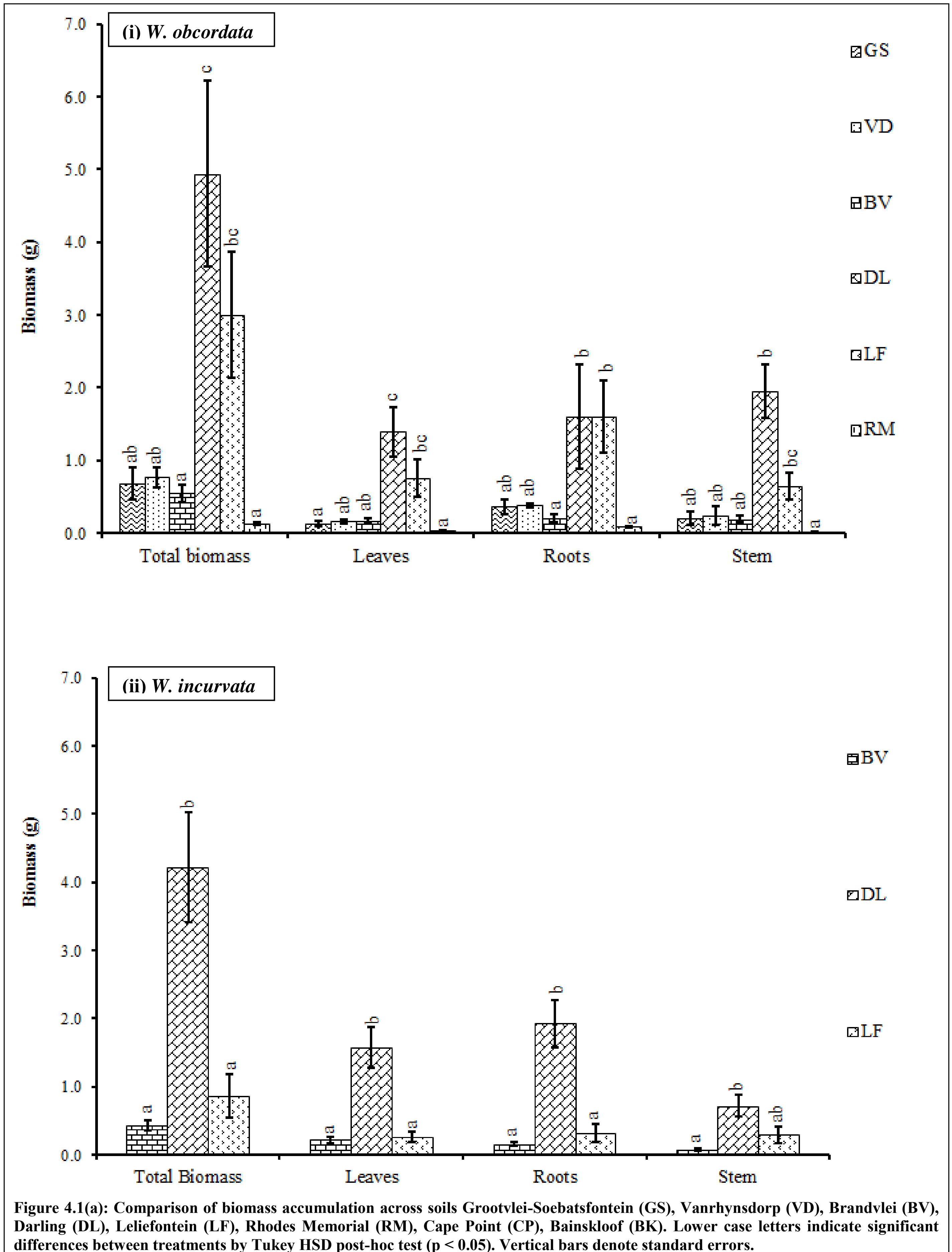
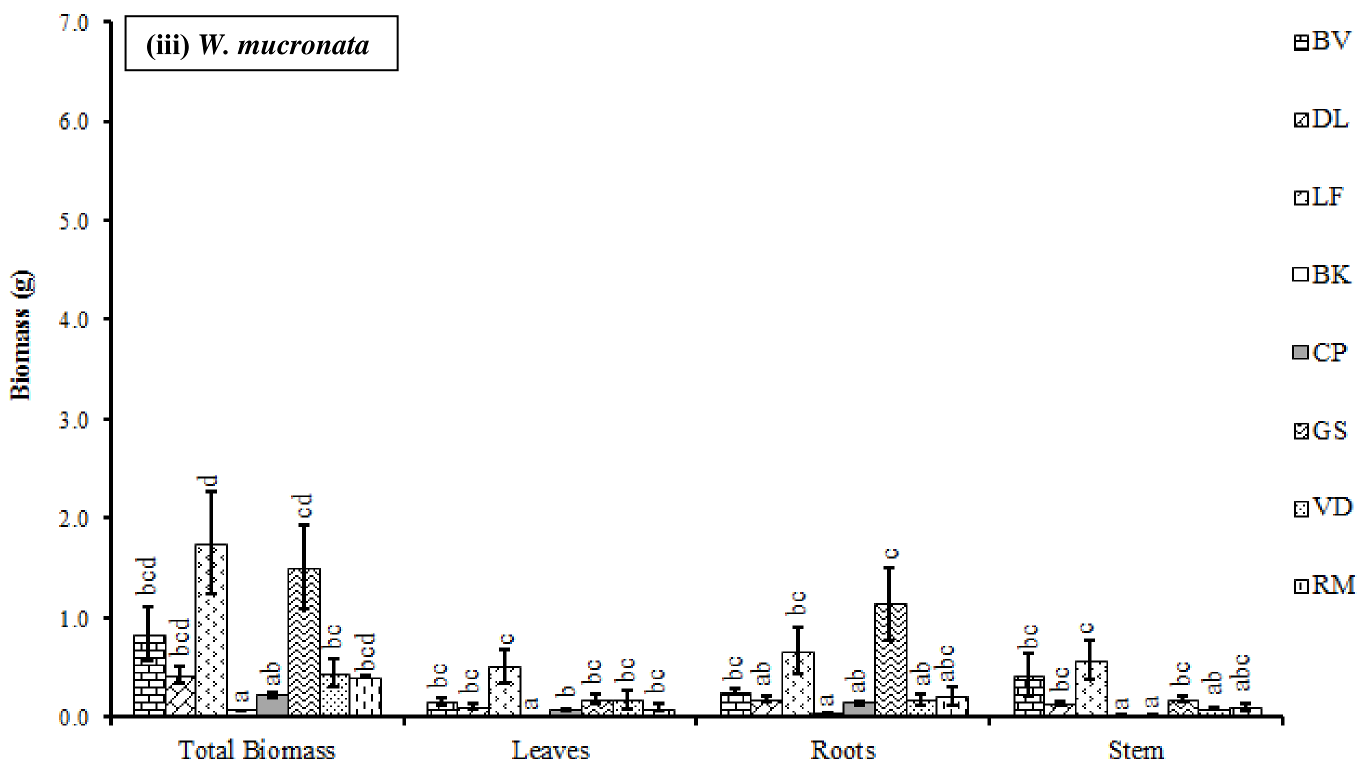


Figure 4.1(a): Comparison of biomass accumulation across soils Grootvlei-Soebatsfontein (GS), Vanrhynsdorp (VD), Brandvlei (BV), Darling (DL), Leliefontein (LF), Rhodes Memorial (RM), Cape Point (CP), Bainskloof (BK). Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.



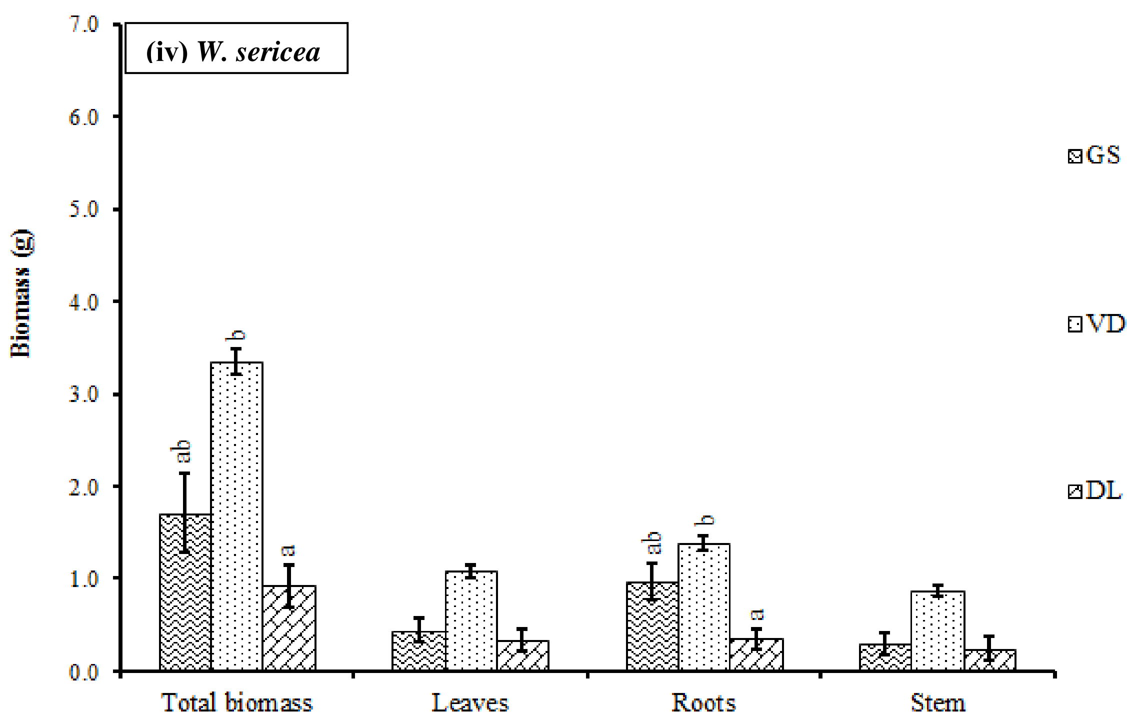


Figure 4.1(b): Comparison of biomass accumulation across soils from Grootvlei-Soebatsfontein(GS), Vanrhynsdorp (VD), Brandvlei (BV), Darling (DL), Leliefontein (LF), Rhodes Memorial (RM), Cape Point (CP), Bainskloof (BK). Lower case letters indicate significant differences between sites by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

4.3.2(b) Species biomass accumulation in soils from one location

(i) Darling

W. obcordata and *W. incurvata* both accumulated the highest total biomass (about 4 g) which was five times higher than that of both *W. sericea* and *W. mucronata* (about 0.8 g) when grown in soils from Darling. The Darling soil is habitat to both *W. mucronata* and *W. obcordata*, and non-habitat to *W. incurvata* and *W. sericea*; these differences in total biomass between habitat and non-habitat species were significant [Figure 4.2(a) i]. A similar pattern is observed for the leaf biomass, where *W. obcordata* and *W. mucronata* both accumulated about 1 g of leaf biomass, whilst *W. mucronata* and *W. sericea* accumulated about 0.2 g. Looking at root biomass, *W. incurvata* accumulated the highest root biomass of 1.6 g, followed by *W. obcordata* with a root biomass of 1.6 g, whilst *W. mucronata* and *W. sericea* accumulated the lowest and intermediate root biomass of 0.17 g and 0.34 g respectively. The stem biomass accumulation was highest in was highest in *W. obcordata* at 1.95 g, whilst lowest in both *W. sericea* and *W. mucronata* with values ranging around 0.13-0.24 g; *W. incurvata* accumulated a relatively low stem biomass of 0.7 g, however not as low as *W. sericea* and *W. mucronata*. The differences for total, leaf, stem and root biomass were all significant with *p*-values of 0.00001, 0.0001, 0.0021, and 0.0027 respectively [Figure 4.2(a) i].

(***ii) Vanrhynsdorp***

Wiborgia sericea accumulated the highest levels of total, leaf, root, and stem biomass of 3.3 g, 1.08 g, 1.3 g, and 0.87 g respectively, whilst both *W. obcordata* and *W. mucronata* accumulated the lowest levels of total, leaf, root, and stem biomass of 0.4-0.7 g, 0.15-0.17 g, 0.18-0.37 g, and 0.09-0.27 g respectively [Figure 4.2(a) ii]. These differences for total, leaf, root, and stem biomass were all significant with *p*-values of 0.001, 0.004, 0.002, and 0.008 respectively. Vanrhynsdorp is habitat to both *W. sericea* and *W. obcordata*, and non-habitat to *W. mucronata*.

(***iii) Rhodes memorial***

Plant growth for *W. mucronata* and *W. obcordata* in soils from the non-*Wiborgia* site, Rhodes Memorial, was variable (p-value 0.10), with *W. mucronata* accumulating the highest biomass whilst *W. obcordata* accumulated the lowest; these differences were significant with a *p*-value of 0.008 [Figure 4.2(b) iii]

(iv) Brandvlei, (v) Grootvlei-Soebtsfontein, and (vi) Leliefontein

Plant growth for *W. obcordata*, *W. mucronata* and *W. incurvata* did not vary in soils from Brandvlei (*p*-value 0.42) and Leliefontein (*p*-value 0.19) [Figures 4.2(b) iv and vi respectively]; where Brandvlei is habitat to *W. mucronata* and non-habitat to *W. obcordata* and *W. incurvata*, whilst Leliefontein is habitat to *W. incurvata* and non-habitat to both *W. mucronata* and *W. obcordata*. Similarly, plant

growth for *W. sericea*, *W. obcordata* and *W. mucronata* did not vary in soils from Grootvlei-Soebatsfontein (p-value 0.18) [Figure 4.2 (b) v], which is habitat to both *W. sericea* and *W. mucronata*, and non-habitat to *W. obcordata*. Biomass accumulation seemed to generally be lowest in plants from both Rhodes Memorial and Brandvlei [Figures 4.2 (b) iii and iv respectively].

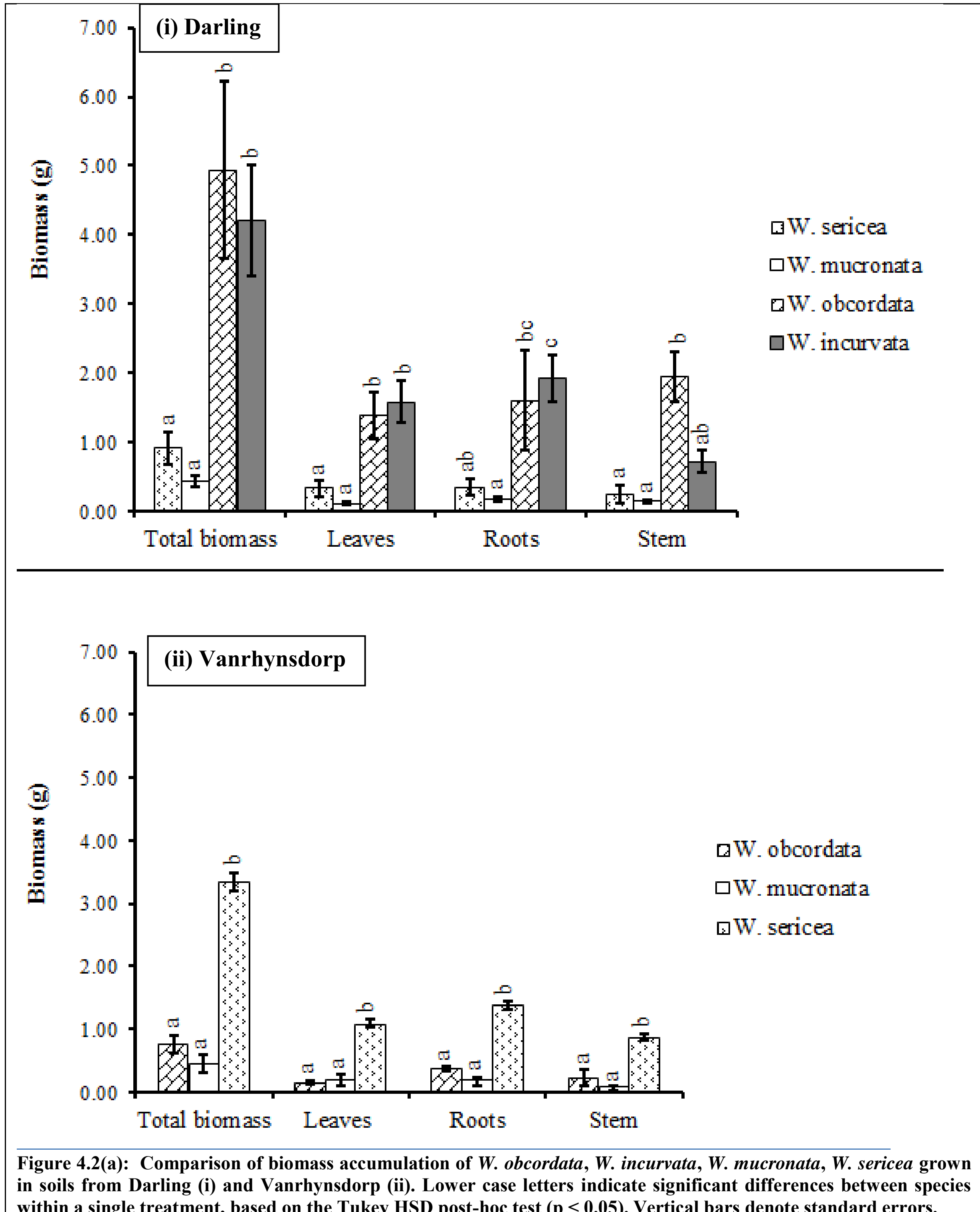


Figure 4.2(a): Comparison of biomass accumulation of *W. obcordata*, *W. incurvata*, *W. mucronata*, *W. sericea* grown in soils from Darling (i) and Vanrhynsdorp (ii). Lower case letters indicate significant differences between species within a single treatment, based on the Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

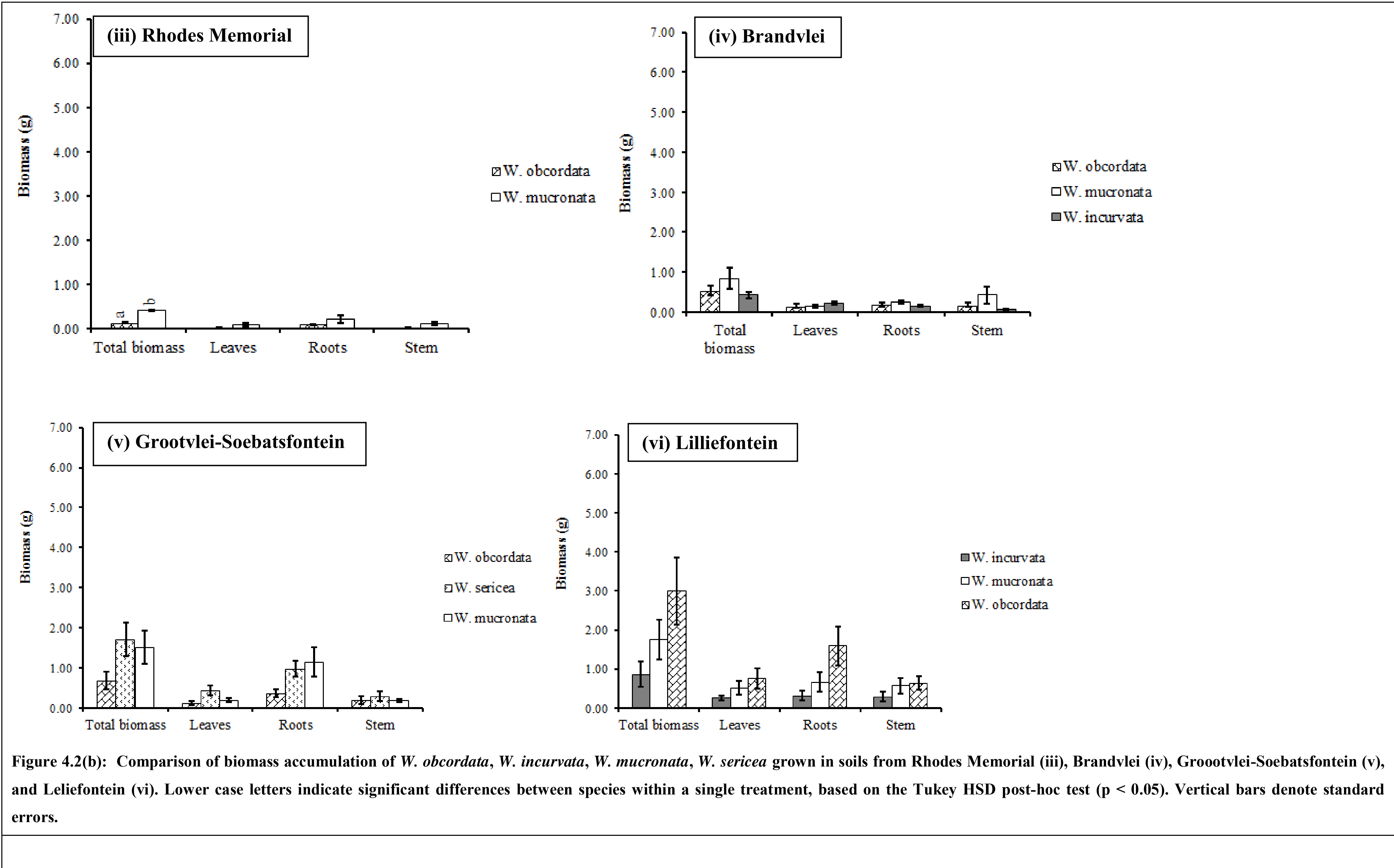


Figure 4.2(b): Comparison of biomass accumulation of *W. obcordata*, *W. incurvata*, *W. mucronata*, *W. sericea* grown in soils from Rhodes Memorial (iii), Brandvlei (iv), Groootvlei-Soebatsfontein (v), and Leliefontein (vi). Lower case letters indicate significant differences between species within a single treatment, based on the Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

4.3.3 Plant tissue nutrient analysis

Nutrient concentration was not assessed from plants from some locations where biomass accumulation was too little for the analyses. For instance, the nutrients measured for *W. mucronata* were from the plants harvested from Brandvlei, Grootvlei-Soebatsfontein, Leliefontein and Vanrhynsdorp treatments, whilst plants from Bainskloof, Cape Point, Darling, and Rhodes Memorial all yielded low biomass quantities which were not adequate to allow for inclusion in the analyses. Whilst for *W. obcordata*, the nutrients measured were from plants harvested from Brandvlei, Darling, Grootvlei-Soebatsfontein, Leliefontein, and Vanrhynsdorp treatments, whilst plants from Rhodes Memorial yielded low biomass quantities which were not adequate to allow for inclusion in the analyses. Then for *Wiborgia sericea*, the nutrients measured were from plants harvested from Grootvlei-Soebatsfontein and Vanrhynsdorp, whilst plants from Darling yielded low biomass quantities which were not adequate to allow for inclusion in the analyses. For *W. incurvata* only plants harvested from Darling could be analysed for the different nutrients whilst plants from Leliefontein and Brandvlei all yielded low biomass quantities which were not adequate to allow for inclusion in the analyses, thus for comparison purposes the results for *W. incurvata* will not be presented due to there being data from only a single treatment.

4.3.3 (a) Species nutrient concentration in different soil treatments

(i) Wiborgia obcordata

Similar to the biomass accumulation results, K and Mg concentrations were highest in plants from Darling soil (habitat soils) and Leliefontein (non-habitat soils) (0.9% and 0.12-0.14% for K and Mg respectively) which had the highest biomass, and lowest in plants harvested from Vanrhynsdorp (habitat soils), Grootvlei-Soebatsfontein(non-habitat soils) and Brandvlei (non-habitat soils (0.32-0.38% and 0.05-0.07% for K and Mg respectively) which had the lowest biomass[Figure 4.3(a) i]. Calcium and P concentrations were highest in plants from Darling (0.48% and 0.059% for Ca and P respectively), intermediate in plants from Leliefontein (0.27% and 0.027 for Ca and P respectively), and lowest in plants from Brandvlei, Vanrhynsdorp and Grootvlei-Soebatsfontein (0.09-0.17% and 0.01-0.02% for Ca and P respectively) [Figure 4.3(a) i]. All the differences for K, Ca, Mg, and P are significant with *p*-values of 0.001, 0.003, 0.01, and 0.002 respectively.

Manganese concentration was highest in plants harvested from Darling (habitat soil with highest biomass) (826 mg/kg), intermediate in plants from Brandvlei (non-habitat soil with low biomass), Grootvlei-Soebatsfontein (non-habitat soil with low biomass) and Leliefontein (non-habitat soil with low biomass) (271-296 mg/kg), and lowest in plants from Vanrhynsdorp (habitat soil with low biomass) (99 mg/kg) [Figure 4.3(a) i]. Iron concentration was highest in plants from Leliefontein

(non-habitat soil with high biomass accumulation) and Brandvlei (332-600 mg/kg), intermediate in plants from Grootvlei-Soebatsfontein and Darling (234-276 mg/kg), and lowest in plants from Vanrhynsdorp (107 mg/kg) [Figure 4.3(a) i]. On the other hand, Zn concentration levels are highest in plants harvested from Darling (190 mg/kg), and lowest in plants form Brandvlei, Grootvlei-Soebatsfontein, Leliefontein and Vanrhynsdorp (24-72 mg/kg) [Figure 4.3(a) i]. All the differences for Mn, Fe, and Zn were significant with *p*-values of 0.001, 0.014, and 0.0006 respectively. Copper concentration was highest in plants from Darling (9.25 mg/kg), and lowest in plants from Brandvlei, Grootvlei-Soebatsfontein, Leliefontein and Vanrhynsdorp (2-4.5 mg/kg) [Figure 4.3(a) i]. Boron concentration was highest in plants from Leliefontein (16 mg/kg), intermediate in plants from Brandvlei and Darling (6-12 mg/kg, and lowest in plants from Grootvlei-Soebatsfontein and Vanrhynsdorp (5.3-5.6 mg/kg) [Figure 4.3(a) i]. These differences for Cu and B were all significant with *p*-values of 0.0005 and 0.009. Concentration for Na was not significantly different between all different soil treatments, with a *p*-value of 0.15 [Figure 4.3(a) i].

(ii) Wiborgia mucronata

For all plants grown in soils from Grootvlei-Soebatsfontein (habitat soil with highest biomass) and Leliefontein (non-habitat soil with high biomass), concentrations of nutrients such as K (*p*-value 0.21), Ca (*p*-value 0.50), Mg (*p*-value 0.91), P (*p*-value 0.84), Na (*p*-value 0.36), Mn (*p*-value 0.57), Fe (*p*-value 0.45), Zn (*p*-value 0.40), Cu (*p*-value 0.63), and B (*p*-value 0.43) were all not significantly different [Figure 4.3(b) ii].

(iii) Wiborgia sericea

Plants grown in soils from Vanrhynsdorp (habitat soil with highest biomass) had the highest concentrations for both Na and Mn (385 mg/kg and 516 mg/kg respectively), whilst those from Grootvlei-Soebatsfontein (habitat soil with intermediate biomass) had the lowest concentrations for both Na and Mn (188 mg/kg and 200 mg/kg respectively). These differences for Na and Mn were significant with *p*-values of 0.033 and 0.0035 respectively [Figure 4.3(c) iii]. The rest of the concentrations for nutrients such as K (*p*-value 0.36), Ca (*p*-value 0.32), Mg (*p*-value 0.13), P (*p*-value 0.91), Fe (*p*-value 0.26), Zn (*p*-value 0.15), Cu (*p*-value 0.90), and B (*p*-value 0.47) were not significantly different between plants grown in Vanrhynsdorp and Grootvlei-Soebatsfontein soils [Figure 4.3(c) iii].

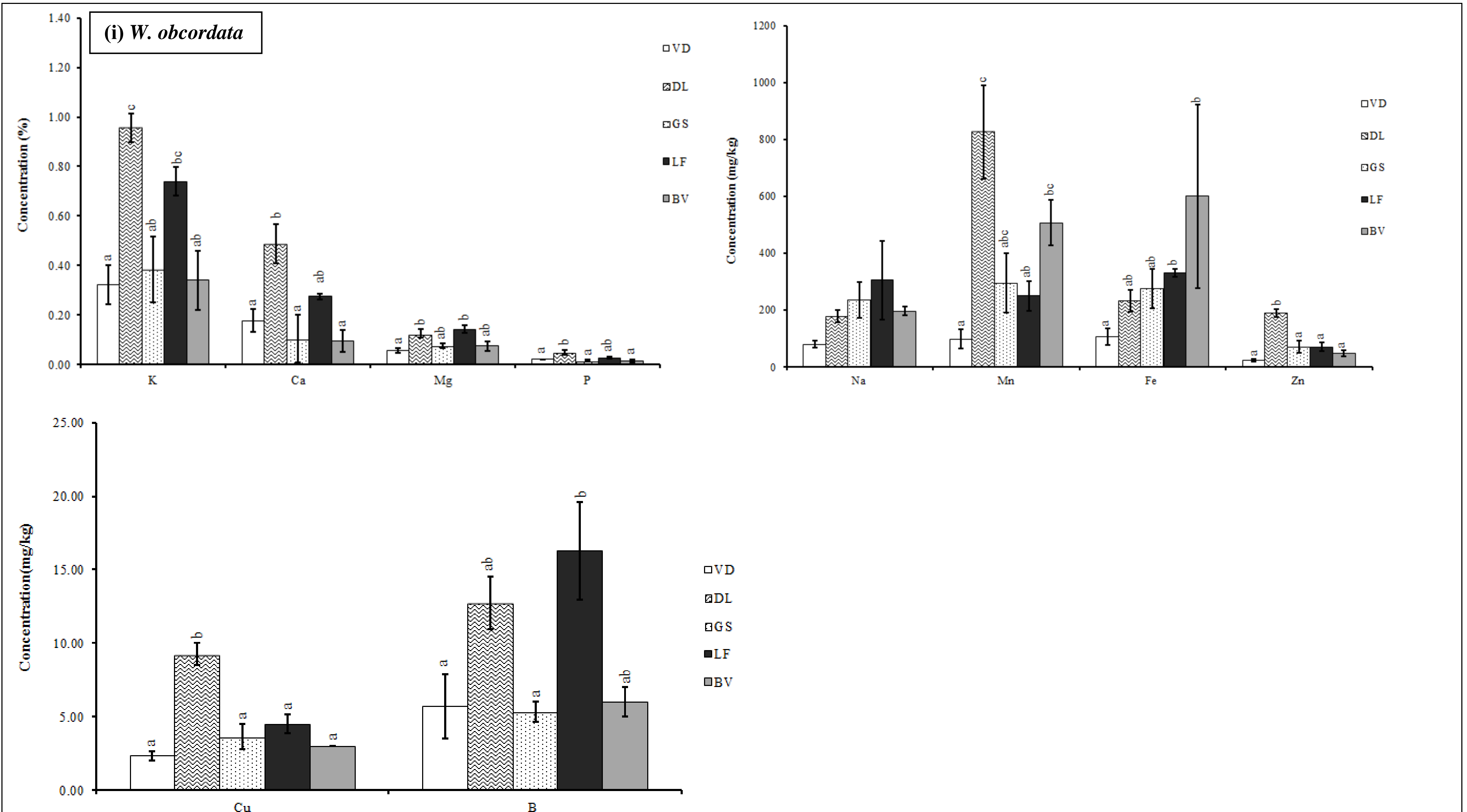


Figure 4.3(a): Comparison nutrient concentrations (Na, Mn, Fe and Zn) in the shoot tissue *W. obcordata* across soils from Brandvlei (BV), Darling (DL), Grootvlei-Soebatsfontein (GS), Leliefontein (LF), and Vanrhynsdorp (VD), Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

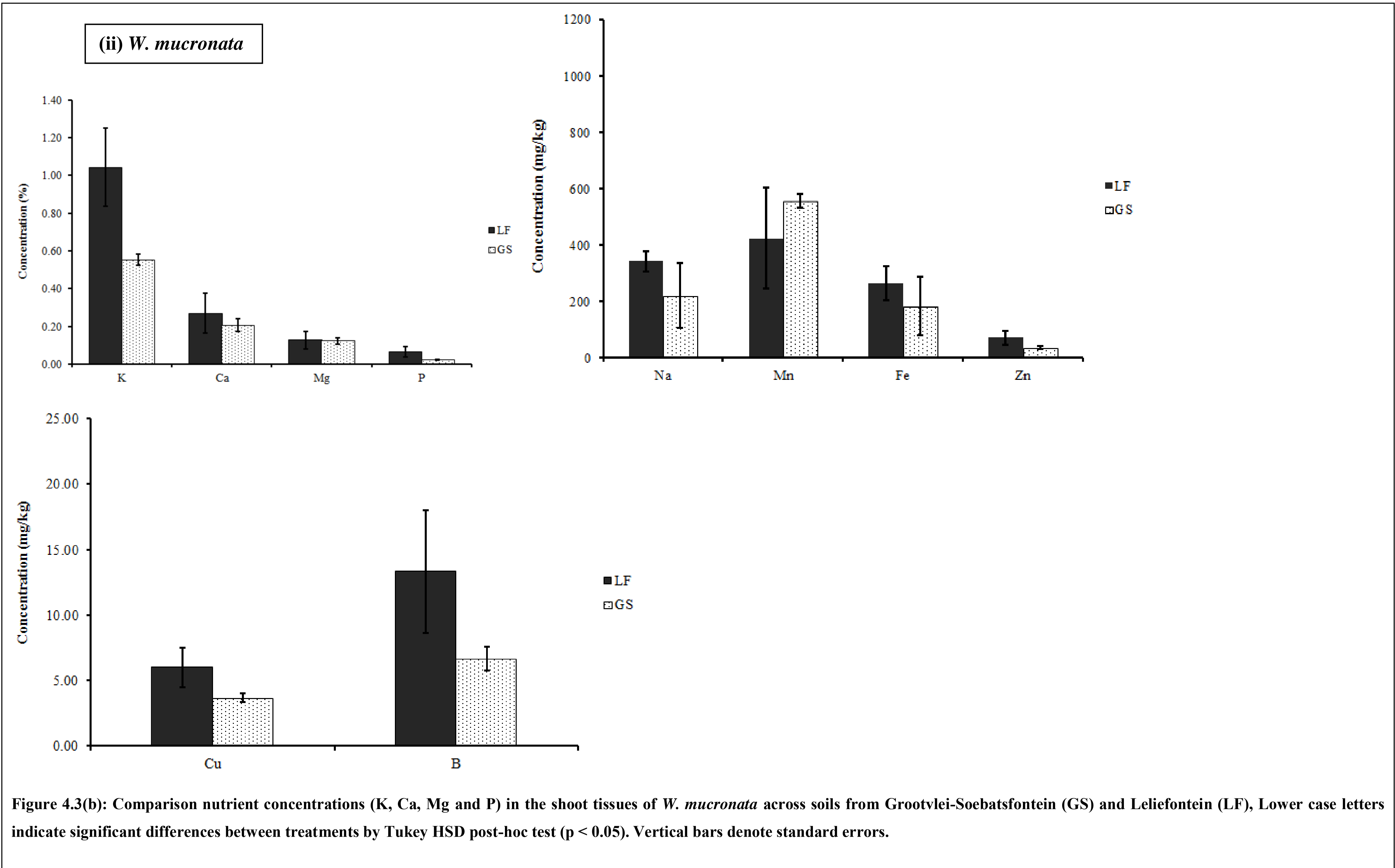
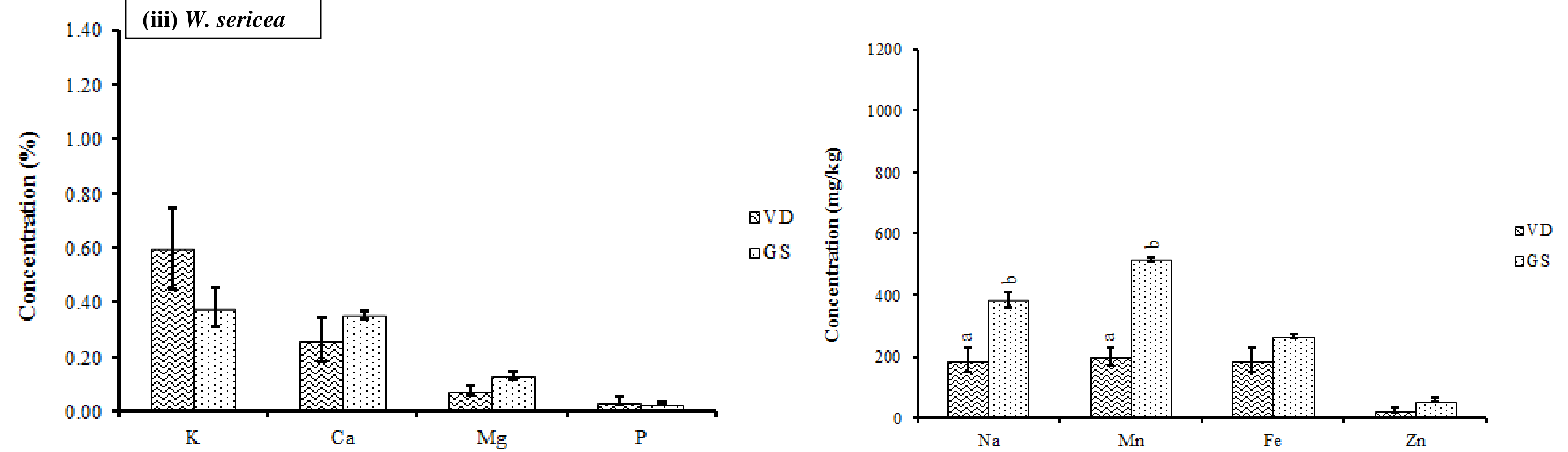
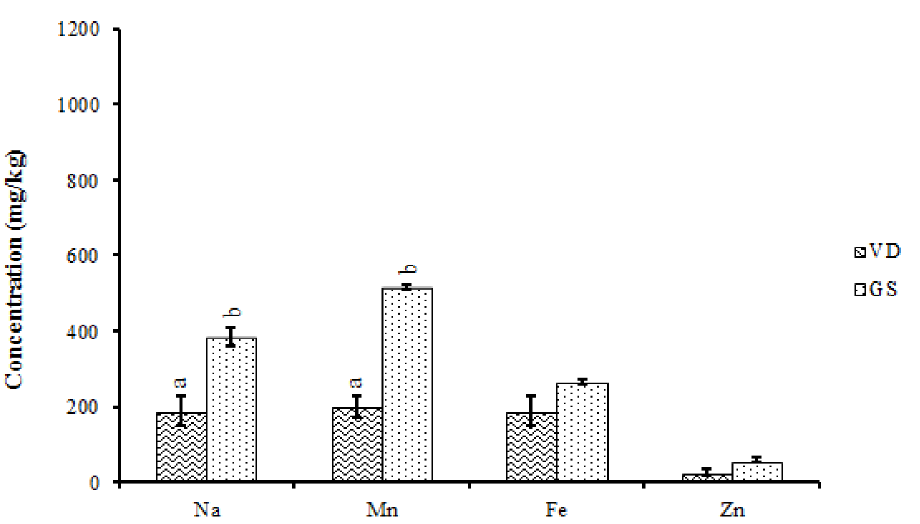


Figure 4.3(b): Comparison nutrient concentrations (K, Ca, Mg and P) in the shoot tissues of *W. mucronata* across soils from Grootvlei-Soebatsfontein (GS) and Leliefontein (LF), Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.





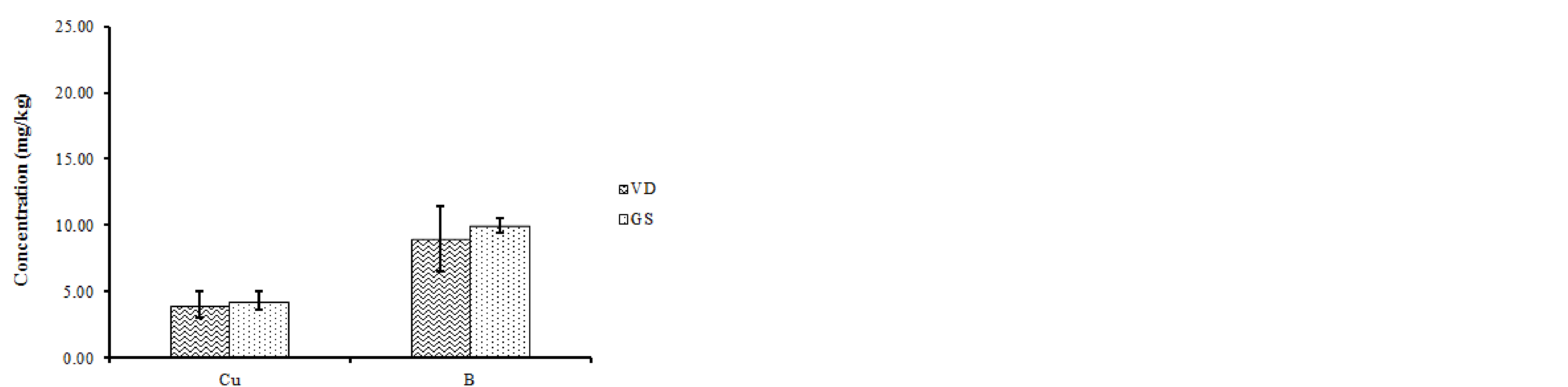


Figure 4.3(c): Comparison nutrient concentrations (Cu and B) in the shoot tissues of *W. sericea* across soils from Grootvlei-Soebatsfontein (GS) and Vanrhynsdorp (VD) (both soils are habitat). Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

4.3.4 Species nutrient concentration in the same soil treatment

(i) Darling

Manganese concentrations were highest in *W .obcordata* grown in Darling soil (species in habitat soil with highest biomass) (826 mg/kg), with a concentration four times greater than *W. incurvata* (species in non-habitat soil) which had a concentration of 200 mg/kg. Similarly Zn variation patterns were highest in *W. obcordata* (190 mg/kg) with a concentration close to seven times than in *W. incurvata* which had a concentration of and 27 mg/kg. Moreover Cu concentration was highest in *W. obcordata* (9.2 mg/kg) and lowest in *W. incurvata* (4 mg/kg). The differences in concentrations for Mn and Zn were significant both with *p*-values of 0.002, whilst that for Cu was 0.02 [Figure 4.4(a) i]. The rest of the concentrations for nutrients such as K (*p*-value 0.26), Ca (*p*-value 0.23), Mg (*p*-value 0.13), P (*p*-value 0.57), Na (*p*-value 0.38), Fe (*p*-value 0.078), and B (*p*-value 0.39) were not significantly different between *W. obcordata* and *W. incurvata* in Darling soil [Figure 4.4(a) i].

(ii)Grootvlei-Soebatsfontein

Calcium concentration levels were highest in *W. sericea* (species in habitat soil) (0.35%), followed by *W. mucronata* (also habitat species) (0.20%), and lowest in *W. obcordata* (non-habitat species) (0.10%). These differences were significant both with a *p*-value of 0.0009. Boron concentration levels on the other hand were more variable; with *W. sericea* having the highest concentration of 10 mg/kg, followed by *W. mucronata* with a concentration of 6.6 mg/kg, whilst *W. obcordata* had the lowest B concentration of 5.3 mg/kg. These differences in B concentration were significant with a *p*-value of 0.021 [Figure 4.4(b) ii]. The rest of the concentrations for nutrients such as K (*p*-value 0.15), Mg (*p*-value 0.08), and P (*p*-value 0.16) were not significantly different between *W. mucronata*, *W. obcordata*, and *W. sericea* [Figure 4.4(b) ii].

(iii) Leliefontein

For all species grown in Leliefontein soils (*W. mucronata* and *W. obcordata* both which are non-habitat species), nutrient concentrations were not significantly different: K (*p*-value 0.41), Ca (*p*-value 0.60), Mg (*p*-value 0.79), P (*p*-value 0.69), Na (*p*-value 0.11), Mn (*p*-value 0.22), Fe (*p*-value 0.43), Zn (*p*-value 0.47), Cu (*p*-value 0.74), and B (*p*-value 0.81) [Figure 4.4(c) iii].

(iv) Vanrhynsdorp

Sodium concentration was highest in *W. sericea* (habitat species with highest biomass) (215 mg/kg) and lowest in *W. obcordata* (habitat species with low biomass) (80 mg/kg). And these differences were significant with a p-value of 0.03 [Figure 4.4(d) iv]. The rest of the concentrations for nutrients such as K (*p*-value 0.07), Ca (*p*-value 0.20), Mg (*p*-value 0.18), and P (*p*-value 0.22), Mn (*p*-value 0.48), Fe (*p*-value 0.50), Zn (*p*-value 0.63), Cu (*p*-value 0.21), and B (*p*-value 0.21) were not significantly different between *W. sericea* and *W. obcordata* [Figure 4.4(d) iv].

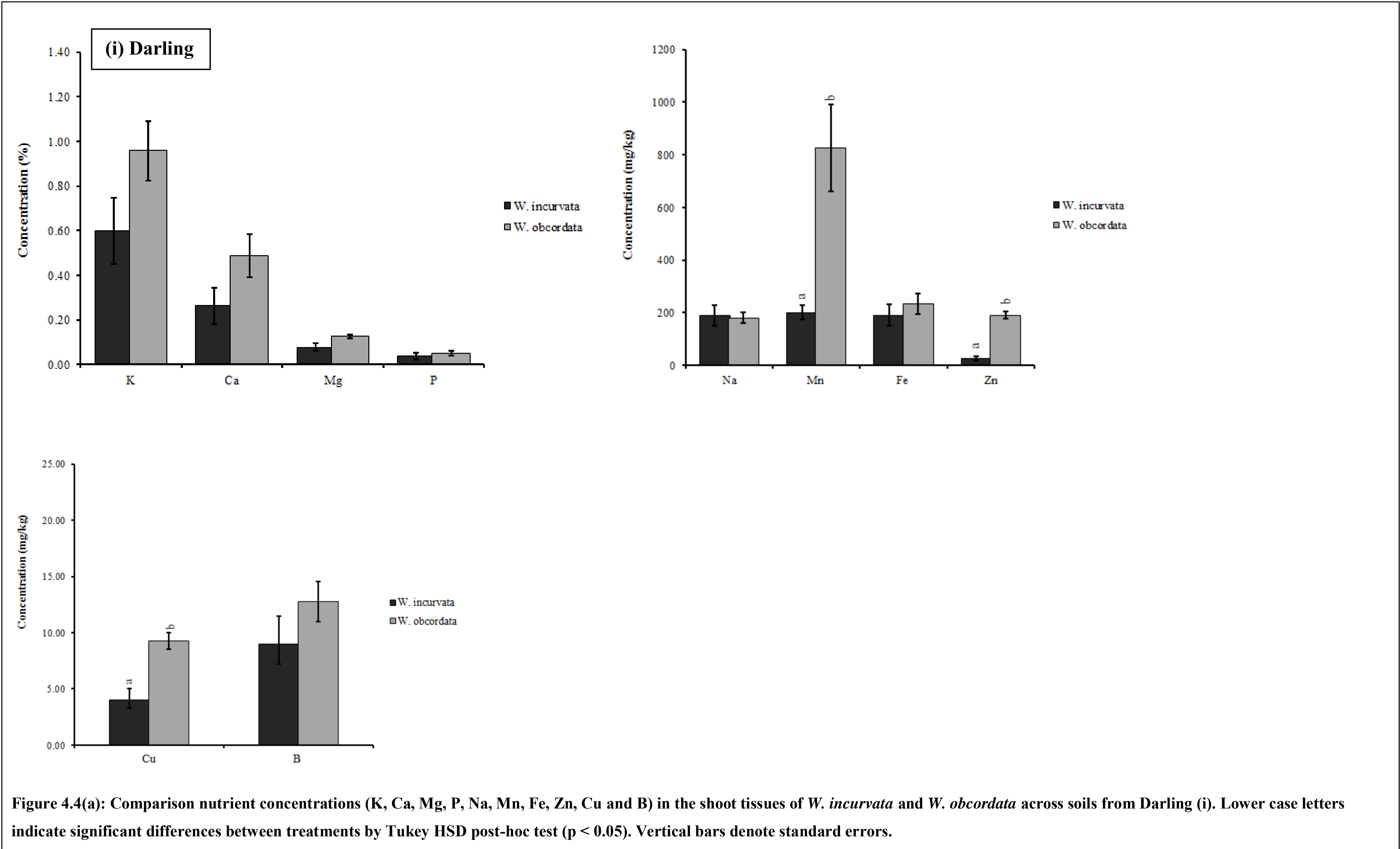


Figure 4.4(a): Comparison nutrient concentrations (K, Ca, Mg, P, Na, Mn, Fe, Zn, Cu and B) in the shoot tissues of *W. incurvata* and *W. obcordata* across soils from Darling (i). Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

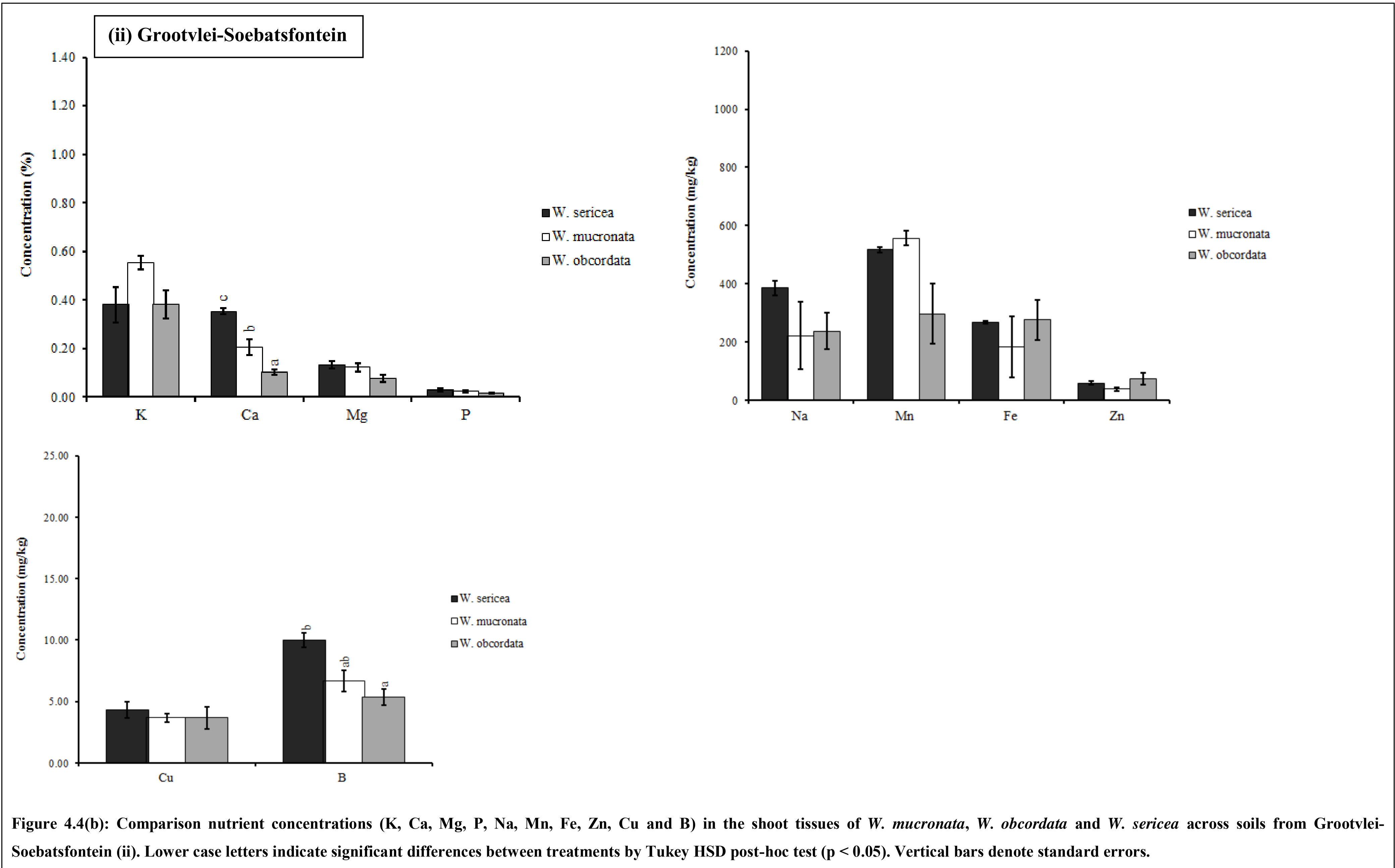


Figure 4.4(b): Comparison nutrient concentrations (K, Ca, Mg, P, Na, Mn, Fe, Zn, Cu and B) in the shoot tissues of *W. mucronata*, *W. obcordata* and *W. sericea* across soils from Grootvlei-Soebatsfontein (ii). Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

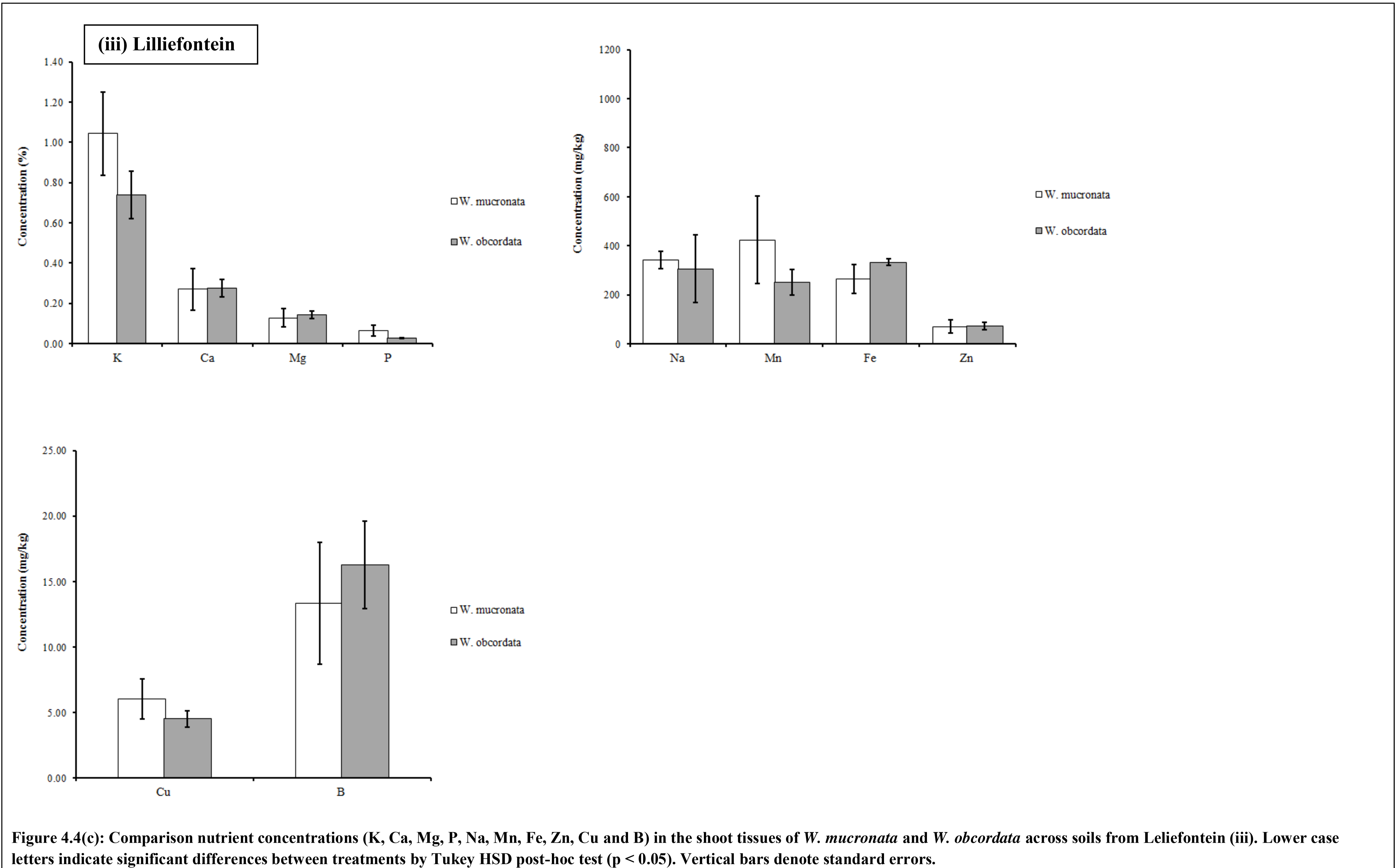


Figure 4.4(c): Comparison nutrient concentrations (K, Ca, Mg, P, Na, Mn, Fe, Zn, Cu and B) in the shoot tissues of *W. mucronata* and *W. obcordata* across soils from Leliefontein (iii). Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

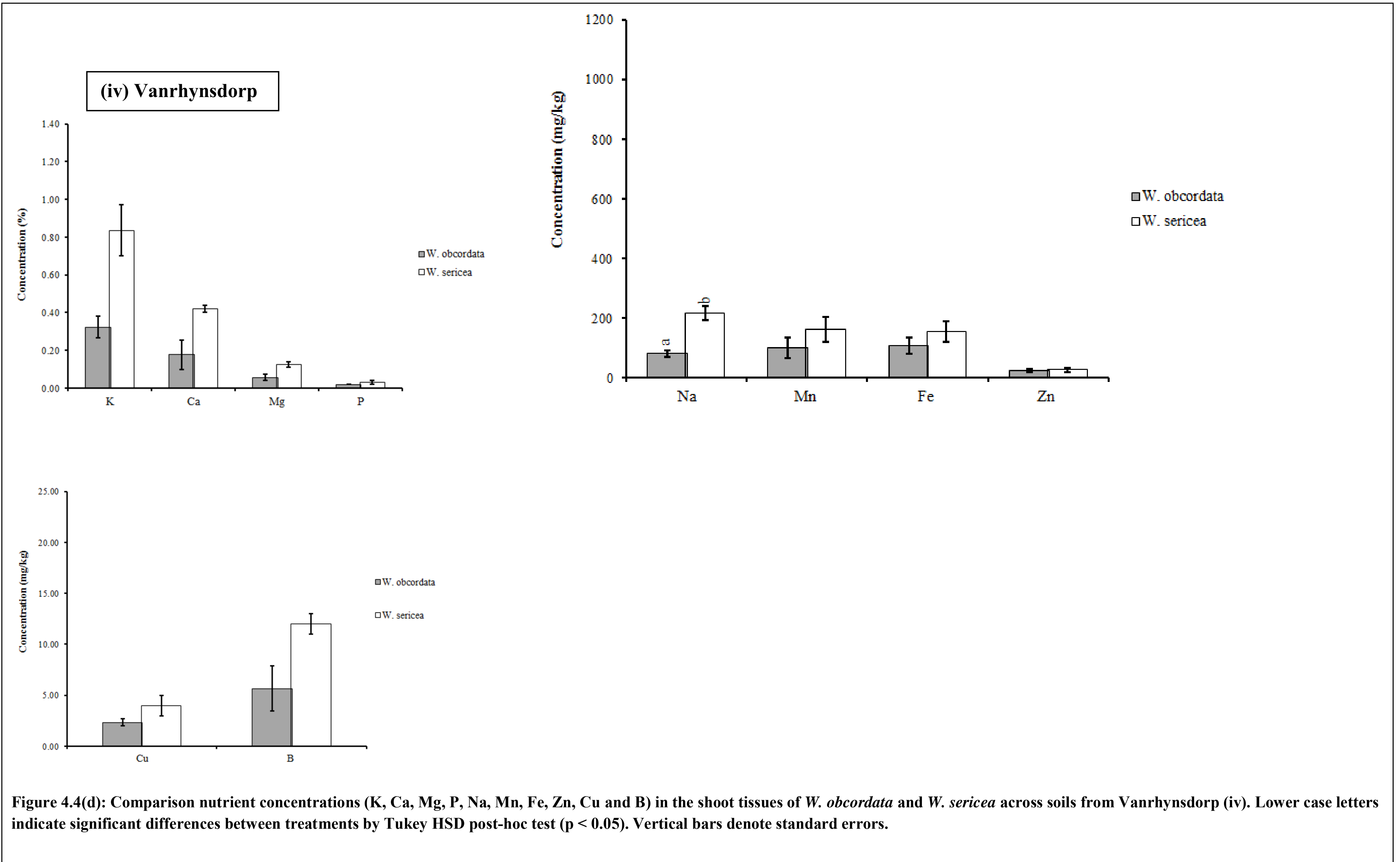


Figure 4.4(d): Comparison nutrient concentrations (K, Ca, Mg, P, Na, Mn, Fe, Zn, Cu and B) in the shoot tissues of *W. obcordata* and *W. sericea* across soils from Vanrhynsdorp (iv). Lower case letters indicate significant differences between treatments by Tukey HSD post-hoc test ($p < 0.05$). Vertical bars denote standard errors.

4.3.5 Rhizobia

From the plant growth experiment, the nodulation data obtained was not enough to allow for statistical analyses whereby majority of the plants for a single treatment only nodulated in 2 replicates, and also not all of the species within a single treatment were able to nodulate, therefore no comparison of nodule biomass could be made. Therefore only results of the species which were able to nodulate in specific treatments will be presented. *Wiborgia mucronata* nodulated in soils from Cape Point (0.146 and 0.0134 g for replicates 1 and 2), Brandvlei (0.062 g), Leliefontein (0.532 g), Vanrhynsdorp (0.185 and 0.068 g for replicates 1 and 2), and Darling (0.081 g). *Wiborgia obcordata* nodulated in soils from Brandvlei (0.019 g), Vanrhynsdorp (0.006 g), and Darling (1.346 and 0.159 g for replicates 1 and 2). *Wiborgia sericea* nodulated in soils from Grootvlei-Soebatsfontein (0.354 and 0.094 g for replicates 1 and 2), Vanrhynsdorp (0.003 g), and Darling (0.293 g). *Wiborgia incurvata* nodulated in soils from Leliefontein (0.123 g), and Darling (0.212 g). For the rest of the treatments not reported here, the species did not nodulate.

A total of 39 accessions of nodules were collected from eight species of *Wiborgia* (all species except *W. monoptera*) across the distribution range. These 39 accessions were from *W. mucronata* (16 accessions), *W. sericea* (7 accessions), *W. obcordata* (6 accessions), *W. incurvata* (4 accessions), *W. fusca* (3 accessions), *W. leptoptera* (2 accessions), *W. tetraptera* (2 accessions) and *W. tenuifolia* (1 accession) (Table 4.3). From the abovementioned nodule accessions, 30 isolates were successfully obtained from the eight host species of *Wiborgia* (shown in bold in Table 4.3). The 30 isolates were initially classified into rhizobial genera through the assessment of the *16S* rRNA, *recA, nodC, nodA*, and *nifH* sequences by comparison using the BLASTN search tool on the Genbank database (Altschul *et al*., 1990).

16S rRNA

The *16S* rRNA BLAST results placed the 30 bacterial strains into different genera of both rhizobial and non-rhizobial lineages. For the rhizobial lineages, eight of the 30 strains were placed in the different genera of both Alpha- (*Rhizobium, Mesorhizobium*) and Beta- (*Burkholderia*) classes of the Proteobacteria with a high sequence similarity (99-100%) of the *16S* rRNA sequences with those already published known rhizobial lineages in Genbank. The blast results (>95% similarily) confirm presence of *Mesorhizobium* in the nodules of *W. tenuifolia*_38 (habitat soil, Brandvlei dam), *W. sericea*_41 (habitat, Ceres) and *W. obcordata*_6931-6928 (glasshouse, Leliefontein). Similarly, *Burkholderia* was endophyte in the nodules of *W. obcordata*_6931-3-1a (glasshouse, Darling), *mucronata*_6930-6953 (glasshouse, Cape Point), and *Rhizobium* was endophyte in the nodules of *W. mucronata*_6930-6931 (glasshouse, Vanrhynsdorp). The rest of the isolates (22 strains) were placed in genera of non-rhizobial lineages such as *Pseudomonas*, *Bacillus*, *Pantoea*, *Enterobacter, Rahnella, Paenibacillus*.

recA

The BLAST results for both *recA* loci (*recA* F-R, *recA* 63F-504R) revealed six strains belonging to the rhizobia mainly in the genera *Burkholderia* (Betaproteobacteria), *Rhizobium* and *Agrobacterium* (Alphaproteobacteria). The two sets of primers used for amplifying the *recA* gene were specific for Betaproteobacteria [labelled *recA* (F-R) on Table 4.3 below] and Alphaproteobacteria [labelled *recA* (63F-504R) on Table 4.3 below]; and looking at the Betaproteobacteria, the strain of *W. mucronata*_6930-18 (which could only be sequenced for *recA* and *nifH*) isolated from plants grown in soils from Brandvlei dam slope in a glasshouse experiment was closely associated with *Burkholderia*. The strain of *W. obcordata*_6931-3-1a isolated from plants grown in soils from Darling in a glasshouse experiment was closely associated with *Burkholderia rynchosiae* (98%). Lastly, the strain of *W. obcordata* isolated from plants grown in its habitat soil from Vanrhynsdorp in a glasshouse experiment was closely associated with *Burkholderia fangorum* (99%). Looking at the Alphaproteobacteria, strains of *W. mucronata*_6930-6922 isolated from plants grown in one of the species' range soils from Grootvlei-Soebatsfontein was closely associated with *Rhizobium tropici* (94%) and this strain could only be amplified for *recA* and *nodA*. The strain of *W. sericea*_6923-6931 isolated from plants grown in soils from Vanrhynsdorp was closely associated with *Agrobacterium tumefaciens* (96%). And the strain from *W. incurvata* isolated from plants grown in the species' habitat soils from Leliefontein was closely associated with *Rhizobium tropici* (98%). Meanwhile the rest of the strains could not be amplified or sequenced for the *recA* gene as shown on Table 4.3.

nodC, *nodA*, and *nifH*

BLAST results for the nodulation gene *nodC* revealed five isolates which were associated with three genera within the Alphaproteobacteria i.e. *Mesorhizobium*, *Rhizobium* and *Phyllobacterium*, however the level of similarity between the five strains in comparison with the reference strains in Genbank was not very strong (80-83%) (Table 4.3). In addition to the traditional rhizobia revealed by the BLAST results, one strain isolated from nodules of *W. mucronata*_32 was associated with the non-rhizobial strain *Rahnella aquatilis* (88% similarity level). Another nodulation gene, *nodA*, was amplified and sequenced for only one accession, and was also assessed using the BLAST tool. From this assessment, the strain isolated from *W. sericea*_41 was closely associated with *Mesorhizobium* with a sequence similarity level of 97% (Table 4.3). For both *nodC* and *nodA* results, strains from five *Wiborgia* species were associated with traditional rhizobial lineages: *W. tenuifolia*, *W. sericea*, and *W. obcordata* were all associated with *Mesorhizobium* with similarity levels of 82%, 80%, 82% respectively; whilst two accessions of *W. mucronata* from Grootvlei and Rhodes Memorial were associated with *Phyllobacterium* (83%) and *Rhizobium* (81%) respectively. Looking at the nitrogen fixation gene, *nifH*, only ten out of the 30 isolates could be amplified and sequenced. The BLAST results for the ten isolates for the *nifH* gene revealed four strains closely associated with the genus *Mesorhizobium* with a high level of similarity (96-99%); these were isolated from *W. tenuifolia*_38,

*W. sericea*_41, *W. obcordata*_6931-6928, and *W. mucronata*_6930-7-2 collected from plants growing in the soils from Brandvlei dam slope, Ceres, Leliefontein, and Rhodes Memorial respectively. Only one isolate was associated with the species *Burkholderia xenovorans* with a high similarity level of 100%, and this was the strain isolated from *W. mucronata*_6930-18 collected from plants growing in soils from Brandvlei dam slope. The other five isolates were associated with *Enterobacter, Rahnella*, and *Paenibacillus* all with a high similarity level of 94-99%.

Table 4.3: BLAST results for strains isolated in this study in comparison with reference strains from GenBank. - denotes to strains that could not be amplified; $^{\times}$ denotes *nodC* sequence and *recA* (F-R) primer pair; * denotes the *nodA* sequence and the *recA* (63F-304R) primer pair. Strains closely associated with rhizobia are shown in bold

Host plant	Accession	Locality	Gene region/Blast results Closest species (identity %)			
			16S	***recA*(F-R)$^{\times}$/*recA*(63F-304R)***	**nodC$^{\times}$ /nodA***	**nifH**
W. fusca	23	Piketberg-ElandsbaaiB		-	-	*Rahnella aquatilis*(94%)
W. incurvata	6928_3_1	Da ling	*Pseudomonas koreensis*(100%)	-	-	-
W. incurvata	**6928_69 8**	**Leliefontein**	***Rhizobium miluonense*(100%)**	***Rhizobium tropici*(98%)***	**-**	**-**
W. leptoptera	31	Darling	*Pseudomonas* (99%)	*Pseudomonas* (96%) $^{\times}$	-	-
W. leptoptera	29	PiketbergB	*Pseudomonas*(10 %)	-	-	-
W. mucronata	**6930_18**	**Brandvlei slope**	**-**	***Burkholderia fangorum*(99%) $^{\times}$**	**-**	***Burkholderia xenovorans*(100%)**
W. mucro ata	36	Brandvlei banks	*Bacillus subtilis*(100%)	-	-	-
W. mucronata	**6930_6953**	**Cape Point**	***Burkholderia*(99%)**		**-**	**-**
W. mucronata	30	Darling	*Pantoea*(99%)	*Pseudomonas*(96%) $^{\times}$	-	-
W. mucronata	6930_3_1	Darling	*Enterobacter*(100%)	-	-	-
W. mucronata	**6930_6922**	**Gro tvlei-Soebatsfontein**	**-**	***Rhizobium tropici*(94%)***	***Phyllobacterium* (83%) $^{\times}$**	**-**
W. mucronata	32	Klipbok	*Enterobacter*(98%)	-	*Rahnella aquatilis*(88%) $^{\times}$	*Rahnella aquatilis*(99%)
W. mucronata	6930_6928	Leliefontein	-			-
W. mucronata	21	Piketberg-ElandsbaaiB	*Pseudomonas*(95%)	-	-	-
W. mucronata	26	PiketbergA	*Pseudomonas*(99%)	-	-	-

W. mucronata	**6930_7_2**	**Rhodes Memorial**	**-**	**-**	***Rhizobium jaguaris*(81%)** ×	***Mesorhizobium*(99%)**
W. mucronata	6930_6930	Studer's Pass	*Paenibacillus*(99%)	-		*Paenibacillus* (99%)
W. mucronata	**693 _6931**	**Vanrhynsdorp**	***Rhizobium*(99%)**	**-**	**-**	**-**
W. obcordata	6931_18	Brandvlei slope	*Enterobacter cloacae*(95%)	*Pseudomonas*(94%) ×	-	-
W. obcordata	**6931_3_1a**	**Darling**	***Burkholderia cenocepacia*(100%)**	***Burkholderia rynchosiae*(98%)** ×	**-**	**-**
W. obcordata	**6931_6928**	**Lillie ontein**	***Mesorhizobium* (93%)**	**-**	***Mesorhizobium*(82%)** ×	***Mesorhizobium*(97%)**
W. obcordata	27	PiketbergA	*Rahnella aquatilis*(99%)	-	-	*Rahnella aquatilis*(99%)
W. sericea	**41**	**Ceres**	***Mesorhizobium*(99%)**	**-**	***Mesorhizobium*(80%)***	***Mesorhizobium*(96%)**
W. sericea	6923_3	Darlin	*Enterobacter ludwigi*(99%)	*Pseudomonas*(95%)×	-	-
W. sericea	6923_6922	Grootvlei-Soebatsfontein	*Pantoea* (99%)	-	-	-
W. seric a	6942_6912	Khoisan's Kitchen	*Bacillus aryabhattai* (100%)	-	-	-
W. sericea	**6923_69 1**	**Vanrhynsdorp**	***Rhizhobium*(99%)**	***Agrobacterium tumefaciens* (96%)***	**-**	**-**
W. tenuifolia	**38**	**Brandvlei slope**	***Mesorhizobium*(99%)**	**-**	***Mesorhizobium mediterraneum* (82%)** ×	***Mesorhizobium*(97%)**
W. tetraptera	24	Piketberg-ElandsbaaiB	*Enterobacter ludwigi*(99%)	-	-	-

Rhizobia phylogenetic relationships

16S rRNA

The Bayesian analysis of the 1*6S* rRNA matrix produced a phylogenetic tree with eight well supported (PP=1) groups representing genera of both rhizobial as well as non-rhizobial lineages. Groups made up of rhizobial lineages consisted of strains from both the Alpha- (*Rhizobium* and *Mesorhizobium*) and Betaproteobacteria (*Burkholderia*) subclasses. The *Burkholderia* clade [group 1 Figure 4.5(a)], was made up of symbionts of *W. mucronata*_6930-6953 and *W. obcordata*_6931-3-1a (isolated from plants which nodulated in soils from Cape Point and Darling respectively), as well as those symbionts previously isolated from Cape Crotalarieae (*Aspalathus callosa*, *Rafnia triflora* and *Rafnia acuminata),* Phaseoleae (*Bolusafra bituminosa)* and Podalyrieae (*Podalyria calyptrata* and *Virgilia oroboides*). The clade also included the reference strains *Burkholderia ambifaria*, *B. cenocepacia*, *B. glumae*, *B. seminalis B. mallei, B. pseudomallei, B. thailandensis* and *B. oklahomensis B. tuberum, B. xenovorans*, *B.phymatum*, and *B. phytofirmans* [Figure 4.5(a)]. The *Mesorhizobium* clade [group 7 Figure 4.5(a)], was made up of symbionts of *W. tenuifolia*_38, *W. sericea*_41 and *W. obcordata*_6931_6928 (isolated from plants which nodulated in soils from Brandvlei dam slope, Ceres, and Leliefontein respectively), as well as symbionts previously isolated from Cape legumes from the Psoraleeae (*Otholobium hirtum*, *O. virgatum, Psoralea pinnata*, and *P. asarina)* and Crotalarieae (*Aspalathus ciliaris*, and *A. spicata*); and the reference strains *Mesorhizobium australicum*, *M. loti, M. huakuii*, *M. caraganae* and *M. gobiense*. The *Rhizobium* clade [group 6 Figure 4.5(a)], sister to the *Mesorhizobium* clade, (PP=1), was made up of symbionts of *W. incurvata*_6928-6928 and W. sericea_6923-6931 (isolated from plants which nodulated in soils from Leliefontein and Vanrhynsdorp respectively), as well as the symbiont previously isolated from the Podalyrieae (*Virgilia divaricata*); and the reference strains *Rhizobium hainanense*, *R. tropici*, *R. phaseoli*, *R. indigoferae,* and *R. galegae* [Figure 4.5(a)]. The *Bradyrhizobium* clade [group 8 Figure 4.5(a)] which was sister to both the *Rhizobium* and *Mesorhizobium* clades (PP=1) did not contain any strains isolated from *Wiborgia* hosts [Figure 4.5(a)].

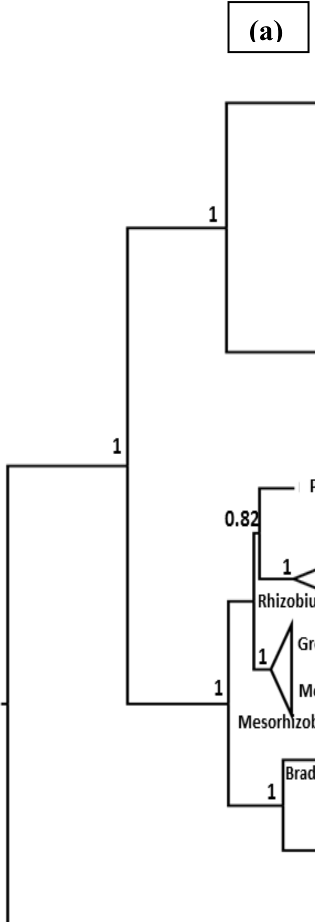
Groups made up of non-rhizobial lineages were made up of strains belonging to six different genera (*Bacillus*, *Paenicillus*, *Rahnella*, *Pantoea*, *Enterobacter*, and *Pseudomonas*) which formed three well supported clades (PP=1). The *Bacillus* clade [group 4 Figure 4.5(a)], was made up of isolates from *W. sericea*_6942-6912 and *W. mucronata*_36 (obtained from plants grown in soils from Khoisan's kitchen and Brandvlei dam banks respectively), and the reference strains *Bacillus aryabhattai* and *B. subtilis*. The *Paenibacillus* clade [group 5 Figure 4.5(a)], sister to the *Bacillus* clade, (PP=1) was made up of symbionts of *W. mucronata*_6930-6930 (obtained from plants grown in soil from Studer's Pass), and the reference strains *Paenibacillus cineris* and *P. rhizosphaerae*. The *Rahnella*, *Pantoea*, and *Enterobacter* alliance [group 2 Figure 4.5 (a)] which consists of three subclades (not shown in

detail) corresponding to the three genera. The *Rahnella* subclade was made up of the symbiont of *W. mucronata*_32 (isolated from plants growing in Klipbok reserve), and the reference strain *Rahnella aquatilis*. The *Pantoea* subclade was made up of symbionts of *W. sericea*_6923-6922 and *W. mucronata*_30 (isolated from plants in soils from Grootvlei and Darling respectively), and the reference strains *Pantoea agglomerans* and *P. brenneri*. The *Enterobacter* subclade was made up of symbionts of *W. mucronata*_6930-3-1, *W. tetraptera*_24, and *W. sericea*_6923-3 (isolated from plants which nodulated in soils from Darling, Lambertsbaai, and Darling respectively), and the reference strain *Enterobacter ludwigi*. The *Pseudomonas* clade [group 3 Figure 4.5 (a)], sister to the entire *Rahnella*, *Pantoea* and *Enterobacter* alliance (PP=1), was made up of the symbionts of *W. incurvata*_6928-3-1, *W. leptoptera*_29, and *W. mucronata*_26 (isolated from plants which nodulated in soils Darling, PiketbergB, and PiketbergA respectively), and the reference strains *Pseudomonas poae*, *P. rhodesiae*, *P. fluorescens*, and *P. koorensis* [Figure 4.5(a)].

recA

The Bayesian analysis of the *recA* gene region produced a phylogenetic tree with five well supported (PP=1) clades [groups 1-5 Figure 4.5(b)], representing genera of both rhizobial as well as non-rhizobial lineages. Groups made up of rhizobial lineages consisted of strains from both the Alpha- (*Bradyrhizobium, Rhizobium* and *Mesorhizobium*) and Betaproteobacteria (*Burkholderia*) subclasses. The *Burkholderia* clade [group 1 Figure 4.5(b)], was made up of symbionts of *W. mucronata*_6930-18, *W. obcordata*_6931-6931, *W. obcordata*_6931-3-1a (isolated from plants grown in soils from Brandvlei, Vanrhynsdorp, and Darling respectively), as well as those symbionts previously isolated from Cape Crotalarieae (*Aspalathus callosa*), Podalyrieae (*Podalyria calyptrata* and *Virgilia oroboides*), and Phaseoleae (*Bolusafra bituminosa)*, and the reference strains, *Burkholderia tuberum, B. phymatum*, *B. rhynchosiae*, *B. fangorum*, *B. cenocepacia*, *B. pseudomallei*, *B. nodosa*, and *B. mimosarum* [Figure 4.5(b)]. The *Bradyrhizbium* clade [group 3 Figure 4.5(b)], sister the *Rhizobium-Mesorhizobium* alliance, (PP=1) was made up of only the reference strains, *Bradyrhizobium betae*, *B. japonicum*, *B. elkanii*, *B. liaoningense*, and *B. yuanmingense*, but none of the *Wiborgia* isolates from our study belonged to this clade. The *Rhizobium* clade [group 4 Figure 4.5(b)] was made up of the symbionts of *W. incurvata*_6928-6928, *W. mucronata*_6930-6922, *W. sericea*_6923-6931 (isolated from plants grown in soils from Leliefontein, Grootvlei, and Vanrhynsdorp respectively), and the reference strains *Rhizobium vignae*, *R. indigoferae*, and *R. phaseoli* [Figure 4.5(b)]. The *Mesorhizobium* clade [group 5 Figure 4.5(b)], was made up of symbionts previously isolated from Cape Crotalarieae (*Aspalathus callosa* and *A. callosa*), Psoraleeae (*Otholobium hirtum*, *O. virgatum, Psoralea pinnata*, and *P. asarina*), and reference strains, *Mesorhizobium australicum*, *M. mediterraneum*, *M. mediterraneum*, *M. loti*, *M. metallidurans*. None of the isolates from our study (i.e. isolated from *Wiborgia* species) belonged to this clade [Figure 4.5(b)].

Only one non-rhizobial lineage was included in the *recA* analysis, and this was the genus *Pseudomonas*. The *Pseudomonas* clade [group 2 Figure 4.5(b)], sister to the *Burkholderia* clade, was made up of the (PP=1), consisting of symbionts of *W. mucronata*_30, *W. leptoptera*_31, *W. obcordata*_6931-18, W. sericea_6923-3 (isolated from plants grown in soils from Darling, Brandvlei, and Darling respectively), and the reference strains, *Pseudomonas fluorescens*, *P. koreensis*, *P. lini*, *P. marginalis, P. huida, P. poae* and *P. rhodesiae* [Figure 4.5(b)].



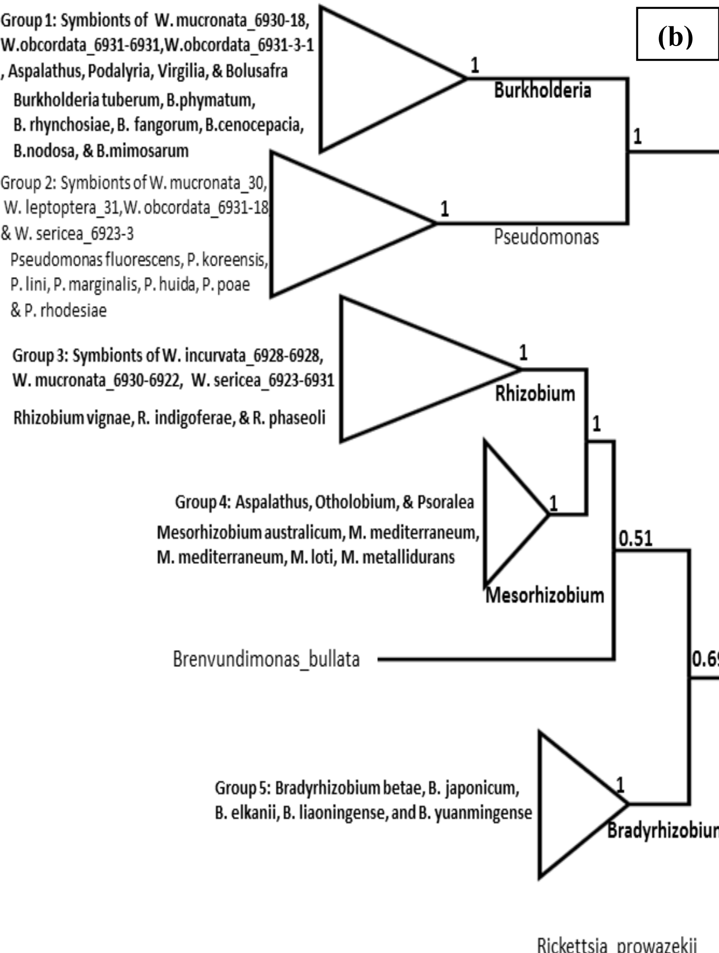


Figure 4.5: Phylogenetic trees based on partial (a) *16S* rRNA and (b) *recA* sequences of isolates. Support (posterior probability) values for the Bayesian analysis are given at the nodes above branches; Generic names of the bacterial groups studies are given below branches. Clades have been collapsed to highlight bacterial generic Groups 1-8 for *16S* rRNA (a) and 1-5 for *recA* (b) Strains closely associated with rhizobial lineages are highlighted in bold.

4.4 Discussion

4.4.1 Plant biomass and nutrient concentrations

In this study, four *Wiborgia* species were grown in different soils which were either habitat or non-habitat soils. The total biomass accumulation for *W. obcordata* was relatively similar between the non-*Wiborgia* site (Rhodes Memorial) and most *Wiborgia* sites, except Leliefontein (non-habitat soil) and Darling (habitat soil) where the species accumulated very high total biomass levels [Figure 4.1(a) i] and nodulated (Table 4.2) in soils from those two localities in the ECR and CCR. Similarly *W. mucronata* accumulated similar total biomass levels [Figure 4.1(b) iii] and nodulated (Table 4.2) in treatments from Rhodes Memorial, Darling and Brandvlei dam, of which Rhodes Memorial is the non-habitat site whilst the latter two sites are both habitat sites. This similar accumulation of total biomass thus suggests that the species could be able to grow in Rhodes Memorial. Interestingly *Wiborgia incurvata* grew well in soils from Darling which is non-habitat site to this species, thus actually showing that the species would potentially grow in Darling soils and persist. In addition, the species also accumulated similar biomass levels in soils from both Leliefontein (habitat soil) and Brandvlei dam, where Brandvlei dam slope is not habitat to *W. incurvata*, but rather habitat to *W. tenuifolia* which is another narrowly distributed species. Based on these biomass results, it could then be said that these two species (*W. incurvata* and *W. tenuifolia)* would be able to co-occur in the same soils of Brandvlei. These results then to a large extent mean that *Wiborgia* species are actually not constrained by soil nutrients or lack of compatible rhizobia because species were able to grow and nodulate in their non-habitat soil. Our results are in contrast with those of Mustart & Cowling (1993), where they did transplant experiments in the Agulhas Plain and found that growth and survival of particular *Protea* and *Leucadendron* species was greater when grown on their native soil types as compared to neighbouring soil types, thus suggesting specific nutrient requirements by these species (Mustart & Cowling 1993; Richards *et al*., 1997b). Another example is that of Richards *et al*. (1997a) suggesting that contrasting nutritional demands of fynbos species could closely be linked with soil nutrient availability which influences the distribution of these species, thus suggesting that the association of different fynbos species with a variety of soils differing in nutrient concentrations could mainly be driven by varying nutritional requirements of these fynbos species (Richards *et al*., 1997b).

For *W. obcordata*, biomass accumulation seemed to be directly related with the nutrient concentrations in the tissues which were generally highest in plants which accumulated more biomass and low in plants that accumulated the least biomass. This observation may be interpreted that nutrient availability limited plant growth in the plants with low biomass and nutrient concentration. Several authors have suggested that plant growth can generally be expected to increase with increasing levels of soil nutrients (Lynch & Brown, 2001). The positive correlation between plant

biomass and nutrient concentration was however not consistent for other species such as *W. mucronata* and *W. sericea*; for example biomass accumulation of plants grown in Rhodes Memorial [non-*Wiborgia* site with high levels of most nutrients (Chapter 3)] and Brandvlei dam [*Wiborgia* site with intermediate levels of most nutrients (Chapter 3)] was low when compared to biomass accumulation of plants grown Darling (habitat to *W. mucronata*) and Leliefontein (habitat to *W. mucronata* and *W. sericea*) which are *Wiborgia* sites shown to have intermediate to low nutrient levels compared to Rhodes Memorial. This therefore implies that although the species were grown in soils with low and high nutrient concentrations, the biomass accumulation did not seem to be directly proportional to soil nutrient thus suggesting that perhaps nutrient availability did not play a role in biomass accumulation. Other examples of non-*Wiborgia* sites however, cannot be used in drawing the above patterns because for example; the low biomass accumulation of *W. mucronata* in Cape Point and Bainskloof cannot be definitively linked to limitations in soil nutrition because the nutrient concentrations in the tissues for all *W. mucronata* plants across all sites could not be analysed (due to reasons pointed out above), and those that were analysed did not show any significant differences, highlighting the fact that although plants showed variation in biomass accumulation across treatments they had similar nutrient concentrations in the tissues. These results therefore highlight the point that higher nutrients in the soil will not necessarily result in high biomass accumulation, similarly low nutrients in the soil will not necessarily result in low biomass accumulation due to species differences in response to nutrient availability. This point is further supported by the suggestion of Maistry *et al*. (2013 & 2015) where they indicate that a balanced allocation of resources to processes which are important at that specific time of the plant's life enables the plants to grow relatively well even during conditions of limited nutrient availability.

4.4.2 Rhizobia

Our results revealed a diversity of rhizobial strains which seemed to be closely associated with four genera from the Alphaproteobacteria (*Mesorhizobium, Rhizobium, Agrobacterium*, and *Phyllocaterium*) and one genus from the Betaproteobacteria (*Burkholderia)*. These rhizobia genera have also been reported for other Cape legume species from the tribes Crotalarieae, Podalyrieae, Psoraleeae, and Phaseoleae (Kock 2004; Elliott *et al.*, 2007a; Garau *et al.*, 2009; Gyaneshwar *et al.*, 2011; De Meyer *et al*., 2011; Kanu and Dakora 2012; Hassen *et al.*, 2012; Gerding *et al.*, 2012 Beukes *et al.*, 2013; De Meyer *et al*., 2013; Howieson *et al.*, 2013; Sprent *et al.*, 2013; De Meyer *et al*., 2014; Lemaire *et al*., 2015a & b). However, to my knowledge, this is the first reports of rhizobial diversity in *Wiborgia* species.

One of the hypotheses for this Chapter was that the *Wiborgia* species are unable to diversify into other niches outside their range due to lack of compatible rhizobia. With the results at hand, there is evidence that three of the widespread species *(W. mucronata, W. obcordata, W. sericea)* are nodulated

in non-habitat soil by rhizobia that are widespread in the Fynbos biome (Beukes *et al*., 2013; Lemaire *et al*., 2015a & b). Thus the absence of such taxa in in some areas of the CCR may not be limited by lack of suitable rhizobia. On the other hand, species which are regarded as narrowly distributed are observed to have formed associations (when grown in their habitat soils) with strains from the Alphaproteobacteria only (*Rhizobium* and *Mesorhizobium*). And from our study we identified that *W. incurvata* (a narrowly distributed species) in its habitat soils (Leliefontein in the ECR) is nodulated by strains closely associated with *Rhizobium* species; this the only report of *Wiborgia* species being nodulated by strains closely associated with *Rhizobium* when growing in soils of the ECR. These observations based on single occurrence need to be verified with larger sampling before rhizobia specificity can be invoked as the main driver of the distribution of these two species. Previous studies mainly focussed on the CCR [prior to that of Lemaire *et al* (2015a)] have identified *Burkholderia* to be the most common root-nodulating genera in the CCR (Kock 2004; Elliott *et al.*, 2007; Garau *et al.*, 2009; Gyaneshwar *et al.*, 2011; Beukes *et al.*, 2013; Howieson *et al.*, 2013; Sprent *et al.*, 2013), and particularly within the Podalyrieae. However a recent study, (Lemaire *et al*., 2015a) reported both *Burkholderia* and *Mesorhizobium* to be common within the Crotalarieae, and our study confirms *Wiborgia*, being one of the Cape endemic Crotalarieae genera, to also be nodulated by both *Burkholderia* (*W. mucronata* and *W. obcordata*) and *Mesorhizobium* (*W. obcordata*, *W. sericea*, and *W. tenuifolia*).

The widespread species (*W. mucronata, W. obcordata*, and *W. sericea*) which are distributed across different soil types along both the ECR and CCR were nodulated by strains associated with both Alphaproteobacteria and Betaproteobacteria lineages. For example, *W. mucronata* was nodulated by strains associated with *Burkholderia* when grown in soils from Brandvlei dam [Figure 4.5(b)] as well as the non-*Wiborgia* site, Cape Point [Figure 4.5(a)], whereas the same species was nodulated by strains associated with *Rhizobium* species when grown in soils from Vanrhynsdorp and Rhodes Memorial (another non-*Wiborgia* site) [Figures 4.5(a) and (b)]. This ability by *W. mucronata* to form symbiosis with strains such distant rhizobial lineages suggests that this widespread species could be highly promiscuous. These results are similar to those of Kanu & Dakora (2012), who demonstrated that eight *Psoralea* species have a degree of promiscuity, by isolating strains of *Mesorhizobium*, *Rhizobium* and *Burkholderia* from their nodules. Previously, *Burkholderia* has been reported to nodulate legumes growing in Cape Point (*Aspalathus callosa*) and Rhodes Memorial (*Bolusafra bituminosa*, *Podalyria calyptrata*) (Lemaire *et al*., 2015a & b). Similarly, *W. obcordata* which when grown on its habitat soils (Vanrhynsdorp and Darling) was nodulated by strains associated with *Burkholderia* species, but when grown in non-habitat soil (Leliefontein) was nodulated by strains closely associated with *Mesorhizobium*, thus indicating a level of promiscuity similar to *W. mucronata*. These two species (*W. mucronata* and *W. obcordata*) are the most widespread in the genus, and this could be attributed to their ability to form symbiosis with strains from both Alpha- and

Betaproteobacteria. *Wiborgia sericea*, also a relatively widespread species, showed a certain level of promiscuity where it was nodulated (in soils from Vanrhynsdorp and Ceres which are both habitat soils of this species) by Alphaproteobacteria strains closely associated with *Rhizobium* and *Mesorhizobium*.

Legumes have been reported to house a variety of endophytes within their nodules (Muresu *et al*., 2008; Ibanez *et al*., 2009; Muresu *et al*., 2010; De Meyer *et al*., 2015). From this study, a large diversity of non-rhizobial endophytes (NREs) belonging to the genera *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Pantoea, Rahnella*, and *Enterobacter* were identified. These NREs have previously been isolated from other legume lineages by several authors (Sturz *et al*., 1997; Mantelin *et al*., 2006; Zakhia *et al*., 2006; Putnam & Miller, 2007; Zurdo-Pineiro *et al*., 2007; Palaniappan *et al*., 2010; De Meyer *et al*., 2015). The function of NREs in nodules are still unknown, but some authors have suggested that through co-inoculation with rhizobia, that they may act as helper bacteria where they increase plant health and/or yield (Valverde *et al*., 2005; Ardley *et al*., 2012). Other studies have also reported that certain NRE bacteria are thought to be more beneficial to host plants, for example by promoting plant growth (Vessey, 2003; Kuklinsky-Sobral *et al*., 2004; Ibanez *et al*., 2009; El-Tarabily *et al*., 2010; Tariq *et al*., 2014), nitrogen fixation as well as increased plant stress tolerance (Andrews *et al*., 2010), defensive nature to the host against pathogens through biological control (El-Tarabily *et al*., 2010), and also siderophore mediated interactions (Rajendran *et al*., 2008; Andrews *et al*., 2010; De Meyer *et al*., 2015). On the other hand, the NREs may also act as opportunistic endophytes targeting to thrive in the nitrogen rich environment within the nodules (Dudeja *et al*., 2012). Most studies which have reported on the presence of NREs have mostly focussed on legumes of economic importance, and very few studies have actually investigated the functions of these NREs in native legumes (De Meyer *et al*., 2015); and for the GCFR there is currently (to my knowledge) no study investigating the presence and functions of these NREs in the native indigenous legumes of the GCFR. De Meyer *et al*. (2015) found a correlation of rhizobia species linked with NREs where *Mesorhizobium* species were closely linked with *Rahnella* and *Pantoea*, an oabservation similar to this study where species identified to have the ability to be nodulated by *Mesorhizobium* species were also closely linked with *Pantoea* and *Rahnella* (Table 4.3). In addition, De Meyer *et al*. (2015) also reported that *Rhizobium* species were closely linked with *Enterobacter, Pantoea*, *Bacillus* and *Pseudomonas*; a finding that is also consistent with the results of this study where species identified to be nodulated by *Rhizobium* species were also linked with *Enterobacter*, *Pantoea*, *Bacillus* and *Pseudomonas* (Table 4.3). The results of this study together with those highlighted above, provide evidence that the large number of NREs observed was not a coincidence and that there is a high incidence of specific NREs to associate with particular rhizobia species from different genera. This thus stresses the need for more detailed studies looking at the NRE association with rhizobia and also the function of these NREs in the legume nodules.

4.5 Conclusion

It was hypothesised that *Wiborgia* species would be unable to grow in soils outside their distribution range. However the results of this study leads to the rejection of that hypothesis and conclude that *Wiborgia* species have the ability to grow and nodulate in fynbos soils outside their distribution range. Based on the ability of the four species of *Wiborgia* which were able to grow relatively well in soils from different sites, including most importantly the non-*Wiborgia* sites (in the case of *W. mucronata* and *W. obcordata*), it can be concluded that these four species have the ability to establish and grow in the CCR without being restricted by soil type or availability of nutrients. The narrow geographic distribution currently observed for *W. incurvata* and *W. tenuifolia* may not necessarily be mainly driven by soil nutrient characteristics of the areas where these species do not occur. The phylogenetic relatedness of rhizobia strains isolated in this project seems not to be closely linked to or dependent on geography, soil fertility status, and soil type but rather the isolates seemed to be randomly spread across the distribution range of *Wiborgia* species, as well as non-habit sites. Based on these results, it seems that the availability of nutrients and compatible rhizobia do not limit the distribution of *Wiborgia* species in GCFR. The role of NREs in the distribution of *Wiborgia* species and how they affect nodule functioning is still unknown at this point, and their importance still needs to be identified.

CHAPTER 5

5.1 GENERAL DISCUSSION AND SUMMARY

Wiborgia is one of the species poor lineages in the 'Cape clade' of the Crotalarieae, with only nine species, endemic to the GCFR. The species in the genus are mainly distributed in lowlands north of the Cape Peninsula extending all the way to the northern parts of Namaqualand (Chapter 3; Fig. 3.1), and occurring on a variety of habitats characterized by a variety of soils derived from the Malmesbury and Cape Systems (Dahlgren, 1975). The genus, ecologically different to most sclerophyllous fynbos groups due to the characteristic mesomorphous deciduous leaves, occurs in arid types of the fynbos and mainly in a vegetation of thorny sclerophyllous scrub occurring mostly on clayey shale derived soils (renosterveld) (Dahlgren, 1975; Lewis *et al*., 2005). The main objectives of the study were based on the fact that there is currently no well resolved phylogeny showing species relationships within *Wiborgia* [apart from the study by Boatwright *et al*. (2008) which explored generic relationships within the Crotalarieae], and also the effects of compatible rhizobia as well as compatible rhizobia specificity has not been studied in *Wiborgia*; also the effects of varying soil nutritional characteristics within the different habitats of the GCFR has not previously been explored within the genus. Based on all of the above, the main objectives of this study were to (1) Test the monophyly of *Wiborgia* and infer phylogenetic relationships within the genus using multiple markers; (2) Determine whether *Wiborgia* species occupies habitats with similar nutrient concentrations, and compare whether there is a difference between the nutritional characteristics of soils occupied by *Wiborgia* species in comparison to the sites where *Wiborgia* have not been recorded to occur; and lastly (3) To determine if availability of compatible rhizobia, diversity and the phylogenetic relatedness of the rhizobia nodulating *Wiborgia* plays a role in influencing the distribution of the species within the genus. The results of this study showed that *Wiborgia* is monophyletic (Chapter 2), its distribution is not limited by soil nutritional characteristics (Chapter 3 and 4) or availability of compatible rhizobial endophytes (Chapter 4).

This study revealed that, although in overall *Wiborgia* occupies soils with a very similar nutritional profile (Chapter 3); the soils they occupy do however show a variation in their individual concentrations of the nutrients available to plant growth and functioning. For example, *W. mucronata* showed quite a wide tolerance of nutrition in a sense that the species occupied soils which had concentrations on both extremes as well as intermediate nutrient levels. Similarly *W. incurvata* showed the ability to grow better by accumulating very high levels of biomass when grown in its non-habitat soil (Darling) as compared to its habitat soil (Leliefontein) (Chapter 4). Our results not only showed that widespread species are closely related with narrowly distributed species (Chapter 2), but they also profoundly showed that both widespread and narrowly distributed species are able to

tolerate soils a wide range of nutrient concentrations (Chapter 3). And also broadly showing that the distantly related narrowly distributed species are actually occupying soils of similar nutrient to an extent (with the example of *W. incurvata* and *W. tenuifolia*), where growth and biomass accumulation of *W. incurvata* grown in Brandvlei soils showed that the species would be able to grow and persist) (Chapter 4). Thus our results showed that the distribution of these two narrowly distributed (which are distantly related taxa) is not mainly influenced by nutrient availability. The findings in these three Chapters (2, 3 and 4) regarding phylogenetics and biogeography, all point to the fact that the distribution of the species in the genus *Wiborgia* (whether closely related or narrowly distributed) in the GCFR are not necessarily limited by soil nutrition.

Rhizobial symbiont data (Chapter 4) shows that the species within the genus exhibited the ability to form symbioses with strains associated with both Alpha- and Betaproteobacteria lineages. The ability of the species to form symbioses with a variety of rhizobia shows that *Wiborgia* species are not limited by the availability of compatible rhizobia (Chapter 4). Interestingly, both the widely as well as the narrowly distributed species seem to show the ability to be nodulated by strains the Alpha- and Betaproteobacteria (Chapter 4), and there seems to be no phylogenetic conservatism with regards to rhizobial association because species from both major subclades of *Wiborgia* (Chapter 2) seem not to be showing characteristics of rhizobial specialization. Also, this study identified a large amount of *Mesorhizobium* strains as compared to *Burkholderia*, an unexpected result which has been highlighted by the recent study of Lemaire *et al*. (2015a) which identified *Mesorhizobium* as the most common genus associated with the Crotalarieae. This finding is contrary to the expectation because edaphic factors in the GCFR favour *Burkholderia* as the most predominant rhizobia in the region (Mishra *et al*., 2012). Although the ability of both the narrowly distributed as well as the widely distributed species of *Wiborgia* to form symbioses with rhizobia species from both the Alpha- and Betaproteobacterial lineages is a novel finding; the abundance of *Mesorhizobium* in the GCFR is not surprising as it has also been identified in a recent study by Lemaire *et al* (2015a). In conjunction with the results of Lemaire *et al*. (2015a), it would thus seem appropriate to point out that both *Mesorhizobium* and *Burkholderia* are both common symbionts of Crotalarieae species in the GCFR, and also that availability of rhizobial symbionts do not in any way limit the distribution of *Wiborgia* species.Interestingly, sister species seemed to show some degree of promiscuity in the choice of symbiont association, and no conservatism with regards to specializing in rhizobia species was observed. For example, the sister pair in subclade 2 (*W. mucronata* and *W. tenuifolia*) (Chapter 2) showed association with strains that are quite similar to those which nodulated other species in subclade 1 (Chapter 4). *Wiborgia mucronata* was the one species which showed a higher degree of promiscuity, by exhibiting the ability to form symbioses with species from both *Burkholderia* and *Rhizobium*, and also the ability to grow and nodulate in soils from outside its distribution range (Chapter 4). These results confirm that soils nutrition (Chapter 3) and compatible rhizobia (Chapter

4), do not limit the distribution of both the widespread as well as the narrowly distributed species in the GCFR.

Given that soil characteristics such as nutrient and availability of compatible rhizobia do not limit the diversification of *Wiborgia* species into some habitats, there is need to identify the key factors determining distribution and diversification of *Wiborgia* in various habitats of the GCFR. Fire and summer aridity, prevalent in the Fynbos biome (Cowling *et al*., 1997; Manning & Goldblatt, 2012), could be some of the key factors which limit the distribution and diversification of *Wiborgia* in the CCR. *Wiborgia* co-ocurs with *Dodonea viscosa* Jacq., a taxon prevalent in fossil record (Dupont *et al*., 2011) as part of the tropical woodland vegetation that dominated the GCFR prior to the expansion of the Fynbos and Succulent Karoo biomes in late Miocene, and now restricted to the ecotone between these two emergent biomes. *Wiborgia* has remained on the margins of the fynbos vegetation where fire is uncommon, persisting as long-lived deciduous shrubs bearing anti-herbivory traits such as spines. In contrast, its sister genus *Aspalathus* has conquered most kinds of habitats in the Fynbos biome by evolving traits to withstand fire prone habitats (sclerophyllous, resprouting and reseeding), unlike *Wiborgia* which does not possess those traits and could thus be limited by that inability.

Similar to other lineages in the GCFR, speciation in some *Wiborgia* species may be driven by ecological shifts (such as shifts in floral features as suggested by van der Niet & Johnson, 2009) among closely related species (sister species), e.g. *W. mucronata* and *W. tenuifolia* exhibit shifts in floral colour, thus indicating that there exists a pollinator specialization between these sister species. In contrast, *W. incurvata* and *W. monoptera* (which are sister species, Chapter 2), seem to have not differentiated in soil nutrition niches or pollinator specialization/floral colour shifts as both species have grossly yellow coloured flowers and occupy the same habitats. Therefore the contrast in these results suggests that there exist more intricate and subtle processes within the distribution of each of the species, and that no single account would fully explain the jagged distribution of both the narrowly/widely distributed species within the genus.

In conclusion, the phylogenetic study provided results similar to those of Boatwright *et al* (2008) which showed that *Wiborgia* is strongly supported as monophyletic; and further also showed the species relationships within the genus, some of which have already been reported by Boatwright *et al* (2008) (however without strong bootstrap support for the sister relationships) as well novel relationships. In terms of the soil nutrition study (Chapter 3), our results showed that *Wiborgia* habitat soils are in overall comparable with each other as well as with some non-*Wiborgia* soils. The results showed quite a large tolerance of *Wiborgia* species to different nutrient levels. At this point it can be speculated that the interaction of these could possibly be responsible for driving the distribution of the species.

5.2 Future directions

There is still need to identify the factors influencing the the distribution of *Wiborgia* species in the GCFR and it is recommended that other aspects such as climate, topography, land-use, fire frequency, summer aridity effects, dispersal mechanisms strategies and efficiency, pollination/pollinator aspects, and also including the point raised by Dahlgren (1975) during his taxonomic revision where he identified *Wiborgia* to have a mesomorphous deciduous leaves untypical of the sclerophyllous leaves of the fynbos genera, and this perhaps being an indicative characteristic which may possibly highlight the reasons for the absence of *Wiborgia* in true fynbos. Also studies evaluating the effectiveness of the nodulation, and addition of more sites where *Wiborgia* does not occur still need to be included in the study to further identify how different/similar these non-*Wiborgia* soils are in comparison to the *Wiborgia* soils.

Future directions regarding the phylogenetic study would be to firstly incorporate morphology onto our phylogeny in order to produce a more robust phylogeny not only based on molecular data, but also including morphological data (Wiens, 2004; Wortley & Scotland, 2006) in order to identify the congruency of morphological and molecular datasets. And secondly to do a population level study for most of the widespread species which from our study seemed to show that some processes of genetic differentiation within a single species are in progress (e.g. *W. mucronata*, *W. obcordata*, *W. fusca*, and *W. sericea*) (Chapter 2). Evolution of characters such as the presence of thorns, pubescence, habit, fruit wings within the genus also need to be reconstructed on the phylogeny.

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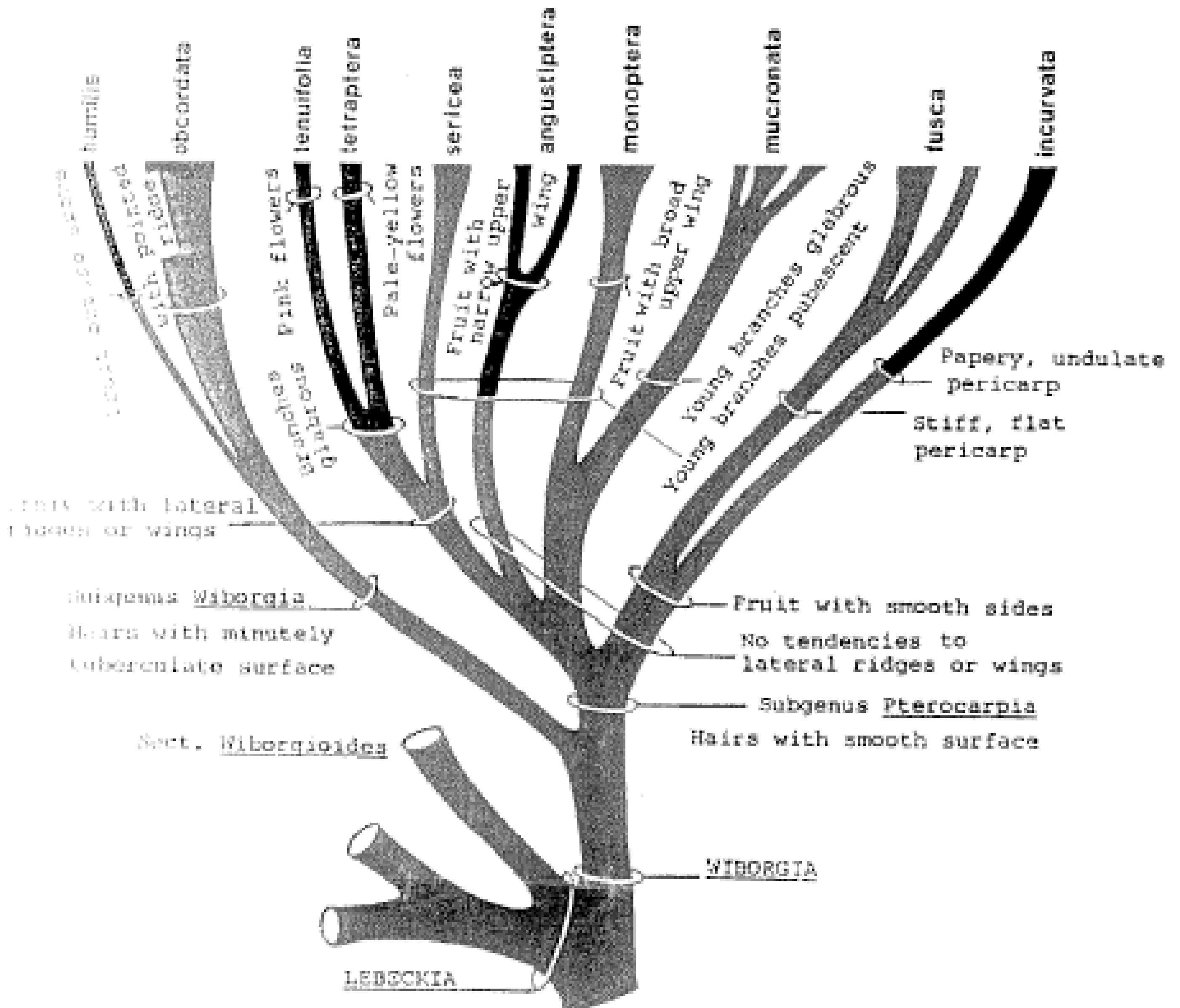
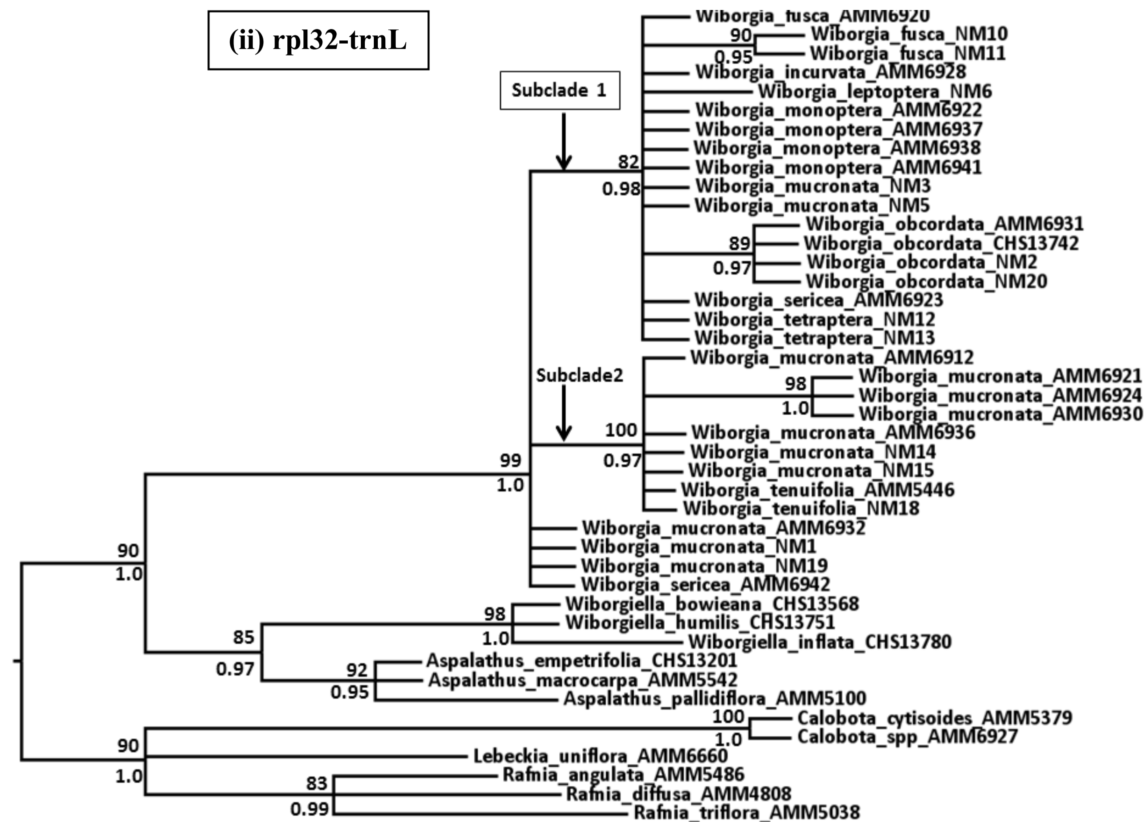
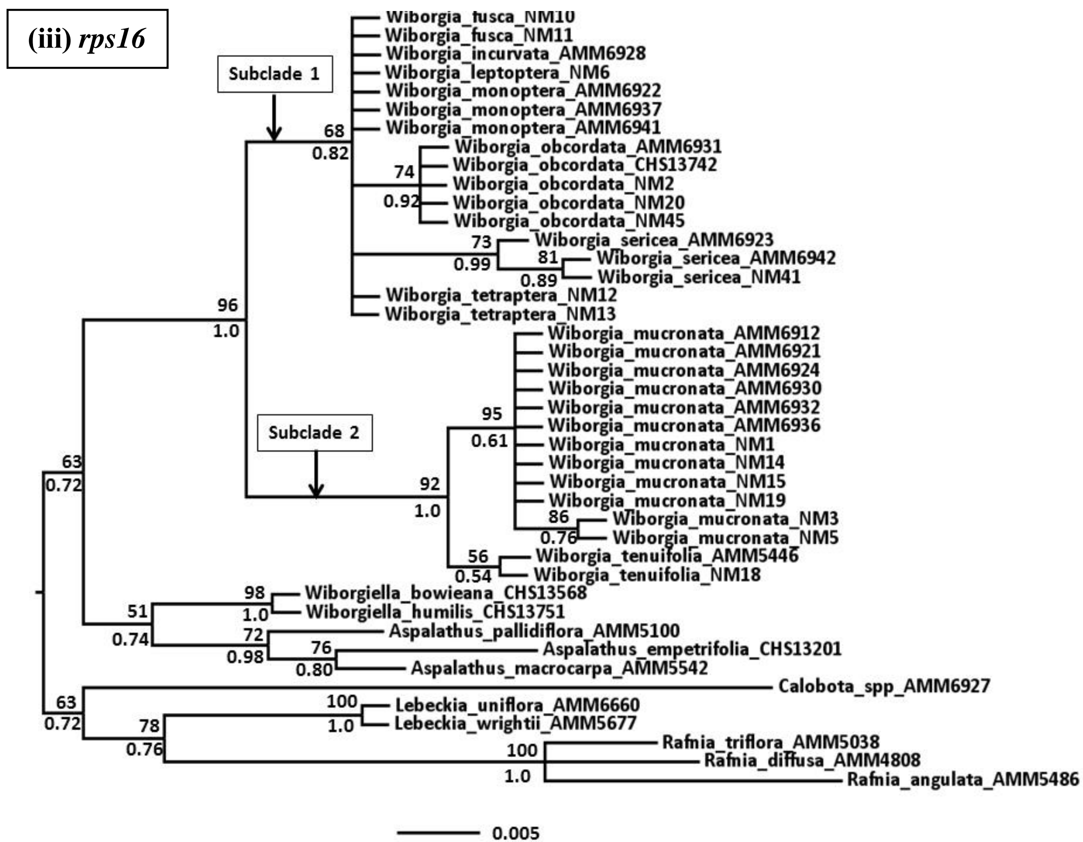
APPENDICES



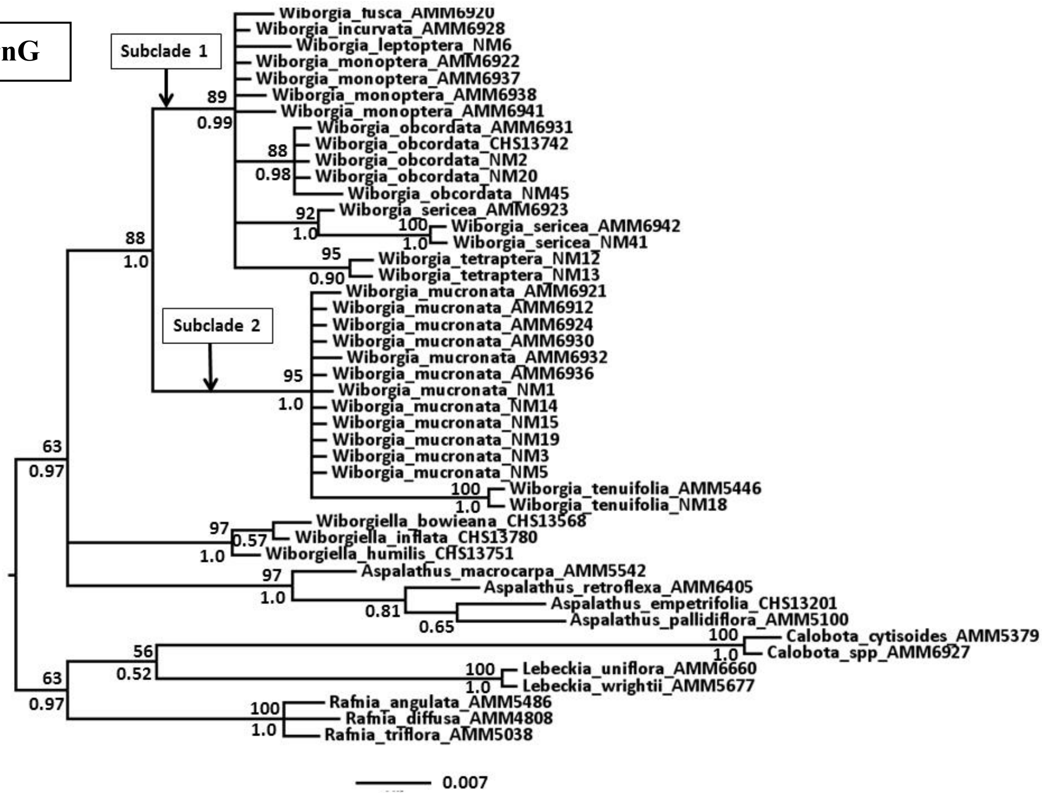
Appendix 1.1: Dahlgren's evolutionary diagram for *Wiborgia* and related *Lebeckia* sections. Figure obtained from Dahlgren (1975).



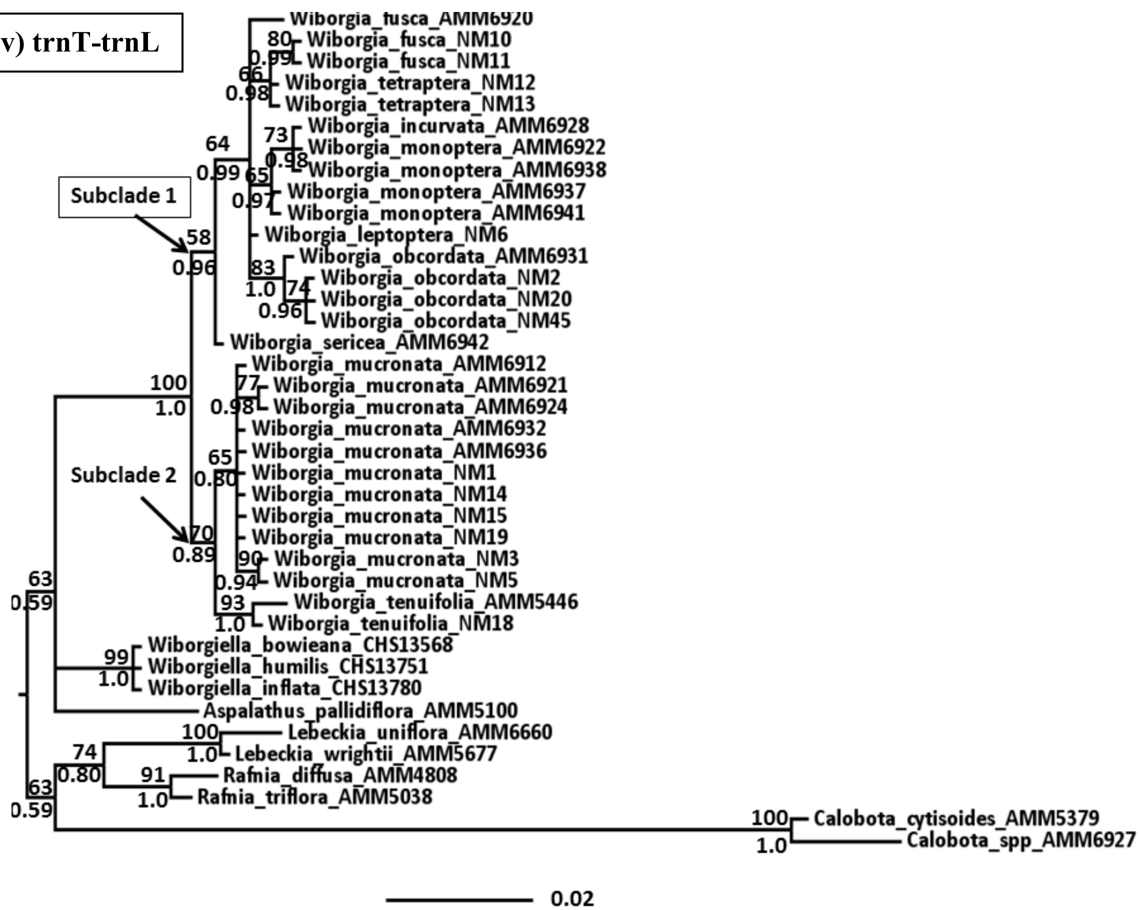


Appendix 1.2 (a): 50% majority rule consensus tree from the analysis of the (ii) rpl32-trnL and (iii) *rps16* data sets for the members of the "Cape clade" of the Crotalarieae. Numbers above branches are bootstrap percentages from maximum likelihood analysis and those below are posterior probabilities from the Bayesian analysis (only values above 50% and 0.50 are shown for the bootstrap and posterior probability respectively.

(iv) trnS-trnG



(v) trnT-trnL



Appendix 1.2 (b): 50% majority rule consensus tree from the analysis of the (iv) trnS-trnG and (v) trnT-trnL data sets for the members of the "Cape clade" of the Crotalarieae. Numbers above branches are bootstrap percentages from maximum likelihood analysis and those below are posterior probabilities from the Bayesian analysis (only values above 50% and 0.50 are shown for the bootstrap and posterior probability respectively.

Appendix 2: Nested anova reslts of the four elements which showed insignificant contribution to the cluster separation by the multivariate analyses N, K, NH_4^+, Na. Means and standard errors followed by different letters in the rows are significantly different at *p < 0.05, **p < 0.01 and ***p < 0.001

	N (%)	K (mg/kg)	NH4+ (%)	Na (mg/kg)
Cluster1	**N=24**	**N=24**	**N=24**	**N=24**
Khoisan's kitchen	0.043±0.008abcde	48.66±12.7bcdef	0.023±0.008abcd	21.33±2.7bcde
Studer's PassB	0.03±0.001abc	44.66±8.8bcdef	0.026±0.008abcd	26.33±5.1cde
Wellington	0.07±0.005bcdef	61.0±14.7bcdef	0.060±0.011abcd	22.66±3.2bcde
Darling	0.073±0.01cdef	64.0±4.1def	0.043±0.003abcd	29.0±1.5de
Brandvlei dam slope	0.08±0.005def	72.33±5.2def	0.066±0.003abcd	15.33±1.6abcd
Brandvlei dam banks	0.056±0.003abcde	55.0±16.5bcdef	0.040±0.01abcd	13.33±2.02abcd
PiketbergB	0.046±0.008abcde	39.33±6.3bcde	0.076±0.031bcd	12.33±1.7abc
Klipbok reserve	0.086±0.008ef	62.33±10.9cdef	0.076±0.008bcd	16.66±0.3abcd
Ceres	0.056±0.003abcde	56.66±0.8bcdef	0.043±0.003abcd	8.333±0.8a
Cluster2	**N=15**	**N=15**	**N=15**	**N=15**
Calvinia Rd	0.036±0.008abcd	29.33±0.3bcd	0.016±0.003ab	11.0±0.5abc
Cape Point	0.036±0.008abcd	5.333±0.3a	0.030±0.001abcd	14.33±0.3abcd
Piketberg-ElandsbaaiA	0.023±0.003ab	21.66±2.3b	0.016±0.003ab	7.66±0.3a
Rawsonville	0.053±0.01abcde	27.00±2.5bcd	0.023±0.008abcd	13.0±3.7abc
Piketberg-Citrusdal	0.023±0.003ab	34.33±6.5bcde	0.016±0.003ab	10.66±1.8ab
Cluster3	**N=18**	**N=18**	**N=18**	**N=18**
Garies-Kamieskroon	0.036±0.008abcd	43.00±4.9bcdef	0.020±0.005abc	18.66±5.4abcd
Grootvlei-Soebatsfontein	0.030±0.005abc	42.00±4.5bcdef	0.010±0.001a	11.0±0.5abc
Studer's PassA	0.013±0.003a	35.00±1.7bcde	0.010±0.001a	9.33±0.3ab
Elandsbaai	0.023±0.003ab	29.66±1.3bcd	0.010±0.001a	9.66±0.3ab
Botterkloof passA	0.040±0.005abcde	47.00±3.0bcdef	0.023±0.006abcd	10.66±1.7ab
PiketbergA	0.030±0.005abc	38.33±7.2bcde	0.013±0.003ab	14.0±4.0abcd
Cluster4	**N=9**	**N=9**	**N=9**	**N=9**
Leliefontein	0.040±0.001abcde	53.33±3.2bcdef	0.020±0.01abc	26.33±4.9cde
Barrydale	0.110±0.01f	55.00±14.7bcdef	0.086±0.003de	50.66±13.4ef
Darling-Mamre	0.073±0.008cdef	91.66±8.8ef	0.063±0.003abcd	21.66±2.3bcde
Cluster5	**N=3**	**N=3**	**N=3**	**N=3**
Botterkloof passB	0.050±0.01abcde	95.33±54.8def	0.053±0.008abcd	15.0±0.5abcd
Cluster6	**N=3**	**N=3**	**N=3**	**N=3**
Rhodes Memorial	0.376±0.02h	263.3±32.2g	0.293±0.044f	68.0±1.7f
Cluster7	**N=3**	**N=3**	**N=3**	**N=3**
Bainskloof Pass	0.180±0.01g	33.00±2.5bcde	0.150±0.005e	20.0±0.5bcd
Cluster8	**N=6**	**N=6**	**N=6**	**N=6**
Vanrhynsdorp	0.030±0.001abc	66.33±2.4def	0.016±0.003ab	12.0±1.5abc
Worcester	0.070±0.02bcdef	115.3±6.4fg	0.083±0.029cde	15.33±1.8abcd
Cluster9	**N=3**	**N=3**	**N=3**	**N=3**
Lambertsbaai	0.023±0.003ab	23.0±4.04bc	0.016±0.003ab	13.66±1.8abcd
Cluster10	**N=3**	**N=3**	**N=3**	**N=3**
Piketberg-ElandsbaaiB	0.033±0.003abcd	66.33±11.02def	0.033±0.003abcd	10.33±2.4ab
F statistic	**4.61********	**4.11********	**2.98******	**4.35********