Rhizobia diversity and their effect on the distribution of indigenous legumes in the Cape Floristic Region

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Declaration

I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own. The thesis is submitted for the degree of Master of Science in the Department of Botany, University of Cape Town. It has not been for any degree or examination at any other university.

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Oscar Dlodlo

Abstract

The Cape Floristic Region (CFR) includes a broad variety of bed rocks and soils are a mosaic of sandstone and shale substrates that give rise to a variety of soil types mainly sandstone, aeolian sands, shale, granite and limestone thereby creating heterogeneity in edaphic conditions. Species composition of plant communities in the CFR is predominantly associated with the parent rock, and the resultant overlying soil. The combination of edaphic and topographical variations, local climate gradients and frequent fires is undoubtedly important in promoting species diversity in the region. The family Fabaceae is the second largest family to Asteraceae in the CFR. It is currently comprised of about 760 species, in 37 genera belonging to 18 tribes. Most of these legumes are in symbiotic association with rhizobia that nodulate and fix nitrogen in the nutrient poor soils. The distribution pattern of the legumes in the CFR is such that some species form distinct populations restricted to one locality while others are widespread. It is however not understood why some CFR legumes occur in patches and there has not been studies to explore the role of symbiotic rhizobia to the unique pattern. It was, therefore, hypothesized that rhizobia isolates from indigenous legumes of the CFR will cluster phylogenically according to soil types and that the distribution of rhizobia limited that of their compatible host.

Rhizobia were isolated from a single nodule from each plant collected from 74 species in over 14 genera covering different soil types of the CFR. A purified culture from one colony was cultivated on Yeast Extract Mannitol Agar (YEMA) and phenotypic characteristics recorded. Deoxyribonucliec acid (DNA) was extracted from freshly grown isolates on Tryptone Yeast (TY) broth and sequenced using 16S rRNA. Rhizobia phylogeny reconstruction was done using Bayesian inference method. Phylogenetic characterization of rhizobial isolates revealed very high genetic diversity among symbionts associated with CFR legumes belonging to seven distinct genera in both alpha and beta classes of Proteobacteria. Betaproteobacteria class consisted of two genera namely *Burkholderia* and *Achromobacter*, while Alphaproteobacteria comprised of the five main rhizobia genera: *Azorhizobium, Agrobacterium/Rhizobium, Bradyrhizobium, Mesorhizobium* and *Sinorhizobium*. The rhizobia isolates were widely distributed on various soil types of the CFR with *Mesorhizobium* and *Burkholderia* occurring on six out of the eight soil types collected. The results also revealed that there were some degrees of legume and rhizobia specificity at the generic and even tribal level in that all rhizobial isolates of *Rafnia, Virgilia oroboides and*

Podalyria species from eight different localities of varying soil types and soil pH were associated with *Burkholderia* species exclusively. Thus, *Burkholderia* lineages seemed to be the most preferred symbiont of the Podalyrieae and in some genera of Crotalarieae including *Rafnia* and *Aspalathus*. Among the Alpha rhizobia, *Mesorhizobium* lineages were the main preferred symbionts of the Psoraleeae and most members of the Crotalarieae, and were the most common and widely distributed rhizobia in the CFR.

The other study involved inoculating a total of 19 legume species selected according to their distribution in the CFR and also to cover phylogenetic diversity of the CFR legumes with soil from five main soil types of the CFR identified as shale, sandstone, granite, limestone and coastal sand collected from the field within legume stands. The aim was to determine whether legumes species would nodulate with soil collected from areas they do not naturally grow. Plants were allowed to grow for up to 60 days in a glasshouse before assessment of nodulation. Majority of legume species were observed to nodulate with different types of soil including soils from sites they do not naturally grow. Similarly to the field study results, the members of the Podalyrieae were associated with the *Burkholderia* lineage regardless of the soil types they grew in.

Overall, the endosymbionts present in the nodules of CFR legumes revealed a large diversity of rhizobia belonging to both Alpha and Beta proteobacteria, with *Mesorhizobia* being the most predominant rhizobia. This study also showed that generally soil type did not limit the distribution of rhizobia in the CFR.

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Chapter 1

1.1 General Introduction

Ecosystem ecology together with phytogeography deal with the vegetation of the world particularly its composition, local productivity and distribution in various parts of the earth according to environmental factors. Environmental factors on the other hand can be defined as all external forces and matter affecting the growth, structure, and reproduction of a particular plant (Billings 1952). The interaction of these factors ranging from climatic, edaphic, topographic and biotic, creates unique conditions that result in plant variability from one region to another and in turn influences distribution patterns of plants (Essl et al. 2009; Reed et al. 2009; William and Pilmanis 1998; Grace 1987; Salisbury 1926; Billings 1952). Since plants are normally in contact with their environment through these two distinct directions (lithospheric and atmospheric), it therefore makes sense that the variations of the conditions of the atmosphere and the soil have direct effects on the distribution of plants.

Climatic Factors

Climate, of all the other factors affecting the biogeography of plants, was found to be the key factor determining the presence or absence of plants in a given area (Silva et al. 2012; Eyre and Woodward 1988; Polunin 1960). The classification of climate in regard to plant distribution is generally associated with four components: temperature, moisture, light and wind. The interaction of these climatic factors create unique plant environments that impose the vegetation types (biomes) of the world and their distribution on several continents (Adams 2010; Davis and Shaw 2001). And the fact that climate exerts a dominant control over the natural distribution of species is undoubtedly the central principle of biogeography (Pearson and Dawson, 2003). Climatic factors are reported in literature to either promote or adversely affect plant growth and distribution causing them to either persist or be excluded in certain areas.

a. Temperature

Temperature is said to be the most fundamental climatic factor as it is a direct function of the shape of the earth and its position with regard to the position of the sun (Good 1974). Temperature is reported to limit distribution of plants in a twofold manner, simply expressed as either maximum or minimum temperatures required for the proliferation of a particular

plant species. The two extremes will determine therefore whether a plant species can occur in a particular area or not. A large body of literature is in agreement that the correlation between climate and plant distribution could only be explained with more clarity in the case of temperature than with any other variable (Eyre and Woodward, 1988; Richardson and Bond, 1991; Prentice et al., 1992; Walther et al., 2002). As a result, the common broad classifications of plants are referred in terms of such words as tropical, temperate, hardy or tender according to their tolerance of different temperature gradients (Good 1974). On the other hand, recently increasing temperature in the context of global change has led to predictions of widespread shifts in plant distribution with some studies actually showing the shifts (Kelly and Goulden 2008; Silva et al. 2012; Parmesan 2006; Parmesan and Yohe 2003). The distribution of the then six largest grasses tribes of the world was reported to have shown that each tribe was either characteristically tropical or temperate with an exception of one which they attributed to the group probably not being natural (Good 1974).

The Cape Floristic Region (CFR) on the other hand shows that temperature changes have probably been the one most important factor to explain the current vegetation distribution in the subcontinent (Goldblatt and Manning 2000). Since the establishment of the cold Benguela Current in the Miocene, the west coast of Southern Africa is said to be characterized by cool temperatures with a drying effect (Meadows and Sugden, 1991). The work of Coertzee and Rogers (1982) reveal that in the late mid-Miocene the vegetation of the west coast around Saldanha Bay was comprised of a fairly rich subtropical flora but today is comprised of mostly treeless, succulent or sclerophyllous shrubland. Among a few studies documenting the history of the CFR, Meadows and Sugden (1991) is in agreement with the idea that temperature fluctuations as a result of climate change could be the most probable explanation of the current vegetation distribution in the CFR. Among other plant families, the Fabaceae (legumes) is documented as one of the major CFR plants that resulted from the vegetation changes at the Miocene/Pliocene boundary particularly the Podalyrieae and the Crotalarieae in response to the arising mediterranean climate (Edwards and Hawkins, 2007).

b. Moisture

Moisture as a climatic factor affecting distribution of plants comes second to temperature. It is commonly expressed in terms of humidity, dew and snow, but it is mostly referred to as precipitation or rainfall. The availability and amount of rainfall that a particular area receives, among other factors, is said to constitute a factor of outstanding importance as it regulates the

occurrence and the net primary productivity of plants (Polunin 1960). The differences in plant distribution between areas often are mainly because of their differences in levels of rainfall and temperature they experience. Some studies have reported these factors to largely determine the general latitudinal distribution of deserts, tropical forests, taiga, tundra, and ice field (Osmond et al., 1987; Polis, 1999). For example the pronounced rainfall gradient across the east-west 160 km breadth of the Namib Desert was reported to be correlated to the characteristic feature of grass community complexity (Jacobson, 1997). In another study distribution of C_4 plants has been noted to be associated with areas of low rainfall commonly known as water limited environments because of their high water-use efficiency (Osmond et al., 1987). The general simplified distribution of rainfall world over is correlated with the distribution of vegetation types of the world. Good (1974) illustrated this by pointing out that areas of maximum rainfall were generally all equatorial, areas such as lowlands of Brazil, parts of West Africa, with Malaysia being entirely equatorial. He also pointed out that South Africa falls under what he termed as nearly continuous ranges of low rainfall. The distribution of rainfall as a factor determining the distribution of plants was said to be made more apparent by the close correspondence of some of the floristic regions of the world with it, even though rainfall totals alone couldn't fully show this correlation (Good 1974). Toledo et al. (2012) studying the distribution patterns of tropical woody species found climatic to be the major factor influencing species distribution with rainfall determining the distribution of 91% of the species. The CFR experiences a Mediterranean climate with winter rainfall in most parts of the region and rainfall pattern being orographic such that precipitation increases with increasing altitude (Bond and Goldblatt, 1984). As a result, rainfall varies distinctly according to topography. In the lowlands, it ranges between 300-500 mm and over 1000 mm in the mountains because of the persistence of clouds, fog and snow that falls in winter (Linder 2003; Goldblatt and Manning 2000; Bond & Goldblatt 1984; Goldblatt 1978). It has been observed though that areas of high precipitation which is fairly evenly distributed throughout the year are characterized by forest vegetation. As precipitation becomes lower, erratic and seasonal the shrubs tend to take precedence over forest vegetation, and at precipitation levels as low as 300-250 mm per year the moisture limitation give rise to a succulent shrubland (Goldblatt and Manning 2000, Fraser 1988, Bond and Goldblatt 1984).

c. Light and Wind

Light and Wind are reported as rather secondary climatic factors limiting distribution of plants on the earth's surface because their influence is based on their modification of the two

principal variables mentioned above (Good 1974). Light is often considered closely related to heat (temperature) by the virtue that both are a direct influence of the sun. With humidity similarly related to heat, and which in turn affects the probability of rainfall, it becomes difficult to clearly separate these climatic factors. Light, however, is regarded as probably the least important climatic variable in relation to the distribution of plants as it is sufficient almost everywhere except for areas of very high latitudes (Osmond et al. 1987, Good 1974, Polunin 1960, Salisbery 1926). On the other hand, wind can to some extent, influence both temperature and precipitation as it affects the build up of humidity and accumulation of temperature in a particular area or habitat. In the CFR there are evidence of the effect of wind as a factor influencing temperature and precipitation is shown by the reported change of the vegetation type of the region in the Miocene (the cold Benguela Current) (Goldblatt and Manning 2000).

Edaphic Factors

Edaphic factors are those that are associated with the substratum (soil) upon which the plants grow and from which they derive their mineral nutrients and most of their water supply (Rajakaruna, 2004). The effect of soil on the distribution of plants is undoubtedly overwhelming and much research on soil-plant interactions confirms this (Rendig and Taylor 1989; Eyre and Woodward 1988; Good 1974; Polunin 1960). Soil is principally made up of parent rock material which forms various complexes when it interacts with climate and living organisms. Its texture is said to be dependent on water and frost action at large and other forms of weathering, while its organic matter content (humus) are a result of input and activities of inhabiting plants and animals (Polunin 1960). The two primary functions of soil as far as plant distribution is concerned are to provide water and mineral nutrients and anchorage to the growing plant. Therefore edaphic factors are ranked second to climatic factors as the major environmental determinants of plant distribution (Eyre and Woodward, 1988). Some literature present soil on the other hand as a little world characterized by its physical structure, chemical composition, atmosphere and biota (flora and fauna) (Eyre and Woodward 1988, Good 1974, Polunin 1960). In regions where climatic conditions maybe similar, variations in vegetation type is often explained by variation in soil types making soil a very important factor in plant geography (Polunin 1960).

The physical structure of the soil as a limiting factor in plant distribution is comprised of three variables (soil depth, texture and chemistry) closely related to climatic factors. The importance of soil depth in the distribution of plants comes by the fact that it determines available moisture content of the soil. Shallow soil can only hold a limited amount of moisture and anchor mainly vegetation types predominated by grasses and small herbaceous shrubs. On the other hand, deeper soils are usually associated with vegetation types where trees predominate (Clark et al. 1998; Osmond et al. 1987). Soil texture is the physical factor (Good 1974) of the soil that controls the physical constitution of the soil in their proportionate combinations of basically sand, clay and humus. Compared to soil depth, their principal function is closely tied to the water relations and aeration of the soil. Sand is said to provide air space in the soil and clay bind the sand together, and has high water holding capacity, while humus enhanced the water retention (Good 1974). Idealistically a good soil texture would comprise of all the three in their right proportions. In a study by Toledo et al. (2012) on the distribution patterns of tropical woody species found climatic factors as the major driver of plant distribution, and soil texture was responsible for the distribution of 44 % of the species. In addition, Prentice et al. (1992) found soil depth and texture too as very important factors affecting plant growth, success, diversity and distribution in highly seasonal climates.

Soil chemistry on the other hand is regarded as the most complex of the edaphic factors limiting the distribution of plants because of the many different chemical compounds (minerals) that occur in nature (Good 1974). Some studies are reported to have documented a detailed account of plant species whose distribution correlated with definite minerals (Polunin 1960). The distribution of plants in nature showed that various minerals reacted favourably or unfavourably on the occurrence and non occurrence of plants species respectively. To put it generally, four major chemical constituents of rocks are of paramount importance in the distribution of plants on the earth's surface and these namely are quartz giving rise to sandy soils; aluminium silicate giving rise mainly to clay; calcium carbonates which results in chalk and limestone; and finally humus which is mainly organic compounds (Good 1974). It is therefore the distribution of these four in their proportional representation that form chemical distinction between soils, which in turn will determine the presence or absence of a particular plant in a given area. In a study to assess the role of the edaphic factor in plant evolution, Rajakaruna (2004) found out that edaphic islands such as limestone outcrops among others gave rise to localized patterns of plant distributions. In addition the

above mentioned four major chemical constituents in interaction with other environmental factors determine soil pH. Much literature is in agreement with pH being another aspect of soil chemistry that has been found to be largely correlated to plant distribution (Osmond et al., 1987; Eyre and Woodward, 1988). Some plants are said to be acid tolerant while others maybe be termed neutral and some are alkaline tolerant. Acid sensitive plants may be excluded in soils of high acidity (low pH) and the same will be true for alkaline sensitive plants in saline soils (high pH). Downes and Beckwith (1951) showed that pH variations as great as a single pH unit could occur over a distance of a few metres and that although it did not affect distribution of perennial plant species it affected that of several annual species.

The species composition of plant communities in the CFR is predominantly associated with the parent rock, and the resultant overlying soil that has varied edaphic factors (Mucina & Rutherford 2006). Geologically, the CFR consist of a mosaic of sandstone and shale substrates that give rise to a variety of soil types. The mountains are mostly composed of quartzitic rock which is erosion-resistant giving rise to the coarse grained sandy soils, while the valleys and the coastal plain exhibit relatively richer soils derived from shale (Fraser 1988; Bond and Goldblatt 1984). Granitic schists occur locally exposed in deep valleys, with limestones occurring near the coast, and Aeolian sandy soils on the coastal plains (Bond & Goldblatt 1984; Goldblatt and Manning 2000).The combination of edaphic and topographical variations, local climate gradients and frequent fires is undoubtedly important in promoting species diversity in the CFR (Goldblatt and Manning 2000). Previous studies have shown that soils have been very influential in the distribution of plants in the CFR (Richard, Cowling and Stock 1997). Richard et al. earlier in 1995 carried out a study to investigate the vegetation-environment relations in Soetanysberg hills and identified five plant communities associated with distinct soil types. Soil type in the CFR plays a very important role in the distribution of plants and is particularly more evident in conjunction with precipitation levels experience by various areas (Fraser, 1988; Linder, 2003). Forest vegetation in the CFR is associated with deeper soils especially in areas where precipitation is high and spread throughout the year. As the soil type become more and more sandy (the dominant substrate in the CFR), the fynbos (sclerophyllous) vegetation dominate. The characteristic renosterveld, a shrubland dominated by shrubby, microphylous Asteraceae is a feature of areas of predominantly clays soils. The mosaic of the varying soil types is said to be a contributing factor to increased diversity and their marked differences isolate vegetation type by limiting growth of another type in favour of a specific adapted type (Linder 2003; Goldblatt and Manning 2000; Dean et al. 1995;

Meadows and Sugden 1991; Fraser 1988; Bond and Goldblatt 1984). While Asteraceae is the largest plant family of the CFR reported to dominate in clays soils, on number two is the Fabaceae. The Fabaceae is not particularly restricted to any particular soil type, but has been observed to occupy habitats varying from water seeps, river valleys, and mountain slopes covering different soil types.

Biotic Factors

Biotic factors in the general view signify all living organisms, including animals and plants which range from man, herbivores, trees and the socially often lowly regarded but very important soil microorganisms. Most, if not all, biotic factors seem to be largely external in origin affecting plant distribution either directly or indirectly (Polunin 1960). Some research has revealed that herbivores effect on plant distribution is reduced plant fitness which is further compounded by competition (Maron and Crone, 2006; Huang et al., 2012). The effect of man on plant distribution on the other hand ranges from the obvious clearing of land for human habitation to the subtle indirect effects of industrialization which are making headlines in the Global Change context (Polunin 1960; Adams 2008; Kelly and Goulden 2008). Currently, fire usually controlled by man has made headlines as an important factor affecting plant distribution. Severe fires have been reported in other studies on biotic factors limiting distribution of plants to completely change dominant plant communities to ones which are adapted to them (Bond and van Wilgen 1996). Another biotic factor often overlooked by much plant scientific research was life below the ground surface (soil microbes). Soil microbes are important regulators of plant productivity, especially in nutrient poor ecosystems where plant symbionts are responsible for acquisition of limiting nutrients (van der Heijden et al., 2008). Bacteria and Fungi are common examples of soil microbes which have proved vital as factors limiting plant distribution (Mishra et al., 2012). In addition to bacteria and fungi, earthworms importance has since the time of Darwin (Good 1974) been noted for the role they play in mixing and aerating the soil and altering its physical condition (texture, depth) explained earlier in this chapter (Osmond et al., 1987; Toledo et al., 2012). In nutrient poor soils, plants depend mostly on symbiotic relationships with rhizobia and mycorrhiza for their N and P nutrition. Therefore in such cases the absence of these symbionts could be very vital in determining the distribution of their host. It is therefore important also to understand the factors that limit the distribution of soil microbes such as bacteria and fungi.

As early as 1934, the Dutch microbiologist L. M. G. Baas-Becking was reported to be the first to address the issue of bacteria biogeography (Staley, 1999). He hypothesized that bacteria were everywhere and supported it by pointing out that bacteria were readily dispersed from one area on earth to another by both abiotic and biotic means. With the advent of molecular phylogenetic methods, in the late 1990s,, certain cyanobacteria species have been reported to be cosmopolitan based on 16S rDNA sequence analysis (Staley, 1999). Some soil microbes such as rhizobia have been suggested to be cosmopolitan since they are adapted to various climatic and soil conditions. In the absence of their host plants they are said to be free living in the rhizosphere as saprophytes and their reproductive organs can remain viable in dormancy for long periods of time if environmental conditions are not conducive (Sprent 2007; Silva et al. 2005; Somasegaran and Hoben 1985). Other studies, however, agree that rhizobia are free living but they are not fully convinced that rhizobia are everywhere (Makatiani and Odee 2007; Bala et al. 2003a; Staley 1999; Woomer et al. 1988). Some studies have shown that rhizobia diversity is highest in the hosts' centre of diversity suggesting co-evolution of legume host and their symbiotic bacteria (Bala et al., 2003b). As a result, factors that affect the distribution of plants (host) including soil type as discussed in this chapter may therefore also be responsible for the distribution of their rhizobia symbionts. Most literature on the role of rhizobia-legume symbiosis is in agreement that the success of legumes in productivity and occurrence worldwide has been attributed to their association with these often underestimated soil microbes. Much research has been done concerning the systematics of rhizobia but studies on the biogeography of rhizobia is still lacking in other regions (Sprent 2012; Essl et al. 2009; Küper et al. 2006; Santos et al. 2001).

Fabaceae is reported in most literature as one of the most species rich and widely distributed plant families of the world (Young and Haukka, 1996; Moulin et al., 2001; Willems, 2006; Raychaudhuri et al., 2007; Sprent, 2012). Commonly known as legumes, the Fabaceae is divided into three subfamilies: Caesalpinoideae, Mimosoideae, and Papilionoideae (Sprent 2009; Sprent 2007; Gepts et al. 2005; Somasegaran and Hoben 1985). The legumes claim to fame and earner of its overwhelming attention among plant scientific research was probably their ability to fix atmospheric nitrogen in symbiosis with rhizobia. In addition, because they can fix nitrogen legumes have an added advantage to overcome the limitations nitrogen in many nutrient poor soils, hence their wide distribution. It should also be noted however that nodulation ability is most common in subfamily Papilionoideae followed by Mimosoideae and remains uncommon in Caesalpinioideae, a phenomenon that corresponds with the species

diversity and distribution of these sub families (Sprent 2007; Somasegaran and Hoben 1985). The Papilionoideae remains the most species diverse and widely distributed. In recent studies (Sprent 2012), researchers in the legume field have began to show that distribution and evolution of different taxa are on-going processes and are most of the times closely related to biogeographical features such as climate, soil and isolation. In addition, she mentioned that it is also becoming clearer that nodulation success could also be related to the biogeographic features. More studies are required to investigate whether the biogeography of legumes is affected by rhizobia and to clarify on the possible role played by these rhizobia in the distribution patterns currently observed. The distribution of CFR legumes which are mostly papilionoids (Elliott et al., 2007; Mishra et al., 2012) are well documented (Goldblatt and Manning 2000; Bond and Goldblatt 1984) with some species being restricted to certain areas while others are widespread all over the region. However there has not been a biogeographic study to investigate the likely effect of rhizobia symbionts occurring on various soil types of the CFR on the distribution of their legume host.

Rhizobia Diversity

Rhizobia is a term collectively used to infer bacteria that cause nodulation and fix nitrogen in symbiosis with legumes (De Meyer et al. 2011; Sprent 2009; Raychaudhuri et al. 2007; Willems 2006). The knowledge of these rhizobia has traditionally been known to be restricted to a limited number of genera in the family Rhizobiales in the Alpha-Proteobacteria (Graham 2008; Sahgal and Johri 2003; Young and Haukka 1996). Classified according to their growth rate in growth media, the Alpha-rhizobia as they are alternatively known are divided into six genera: the fast to moderately fast growing being the *Rhizobium, Allorhizobium, Ensifer (Sinorhizobium)* and *Mesorhizobium*, and the slow growing falling into genera *Bradyrhizobium* and the *Azorhizobium*, the later commonly associated with stem nodules (De Meyer et al. 2011; Raychaudhuri et al. 2007). In addition phylogenetic analysis based primarily on the 16S rRNA gene marker has allocated the above mentioned Alpha-bacteria into four distinct branches. These groups comprise of the following combinations: *Mesorhizobium-Sinorhizobium-Rhizobium/Agrobacterium/Allorhizobium* clade *Bradyrhizobium* clade, *Azorhizobium* branch and the *Methylobacterium* clade being the most recently described of the four (Raychaudhuri et al. 2007; Sahgal and Johri 2003; Moulin et al. 2001).

In the recent past in what has been referred to as surprises in rhizobia taxonomy (Sahgal and Johri, 2003) was the report on the identification of proteobacteria from the β-subclass that nodulate legumes in a study by Moulin et al. (2001). More recent studies after Moulin (2001) have conclusively established legume nodulation by Beta-rhizobia to be associated with the legume genus *Mimosa* (Elliott et al., 2009) and also other mimosoids and some papilonoids (dos Reis et al., 2010), such as *Cyclopia* as reported by Elliott et al. (2007) and *Rhynchosia* (Garau et al., 2009). The Beta-bacteria are known to include *Burkholderia spp.* and *Cupriavidus taiwanensi* previously named *Ralstonia taiwanensis* (Elliott et al., 2009; dos Reis et al., 2010). Other bacteria phylogenetically outside the Alpha and Beta rhizobia groupings now considered as rhizobia include *Methylobacterium, Devosia, Ochrobacterium* and *Phyllobacterium* (Raychaudhuri et al., 2007). The history of rhizobia taxonomy reveals that rhizobia studies have been on the rise since the end of the 19th century resulting in the increase in number of rhizobia species known (Raychaudhuri, 2007). The reasons to this has mainly been attributed to the contemporary advancement in the molecular techniques in the study of rhizobia and the increase in the number of legume taxa sampled (Willems, 2006). The current list of valid published names of rhizobia species numbers 63 spread over 11 genera according to Raychaudhuri et al. (2007) as compared to 23 species from 5 genera at the end of the 19th century (Willems, 2006).

Studies in the CFR on the diversity of legume root nodule rhizobia have revealed that they include *Bradyrhizobium, Rhizobium, Mesorhizobium, Sinorhizobium and Burkholdeira* and *Herbasprillum* but limited to a few genera such as *Aspalathus, Cyclopia, Lotononis, Lebeckia* or *Lessertia* (Le Roux, 2003; Kock, 2004; Elliott et al., 2007; Phalane et al., 2008; Hassen et al., 2011; Gerding et al., 2012). Consequently considering the area covered by the CFR and the distribution of many wild legumes in the region, further studies including more genera and covering the whole area of the CFR was necessary for a better understanding of rhizobial diversity in this region.

Aims and Rationale

The CFR classified as the smallest among the other five floral kingdoms of the world, but unique with its richness in diversity of species (Goldblatt and Manning 2000; Bond and Goldblatt 1984). The region has therefore been a hub for much plant scientific research as many researchers were fascinated by the plants. In an effort to understand such species

diversity one of the unique observations was the presence of wild legumes with N_2 –fixing rhizobia that occur in distinct populations in various soil types. However it is not known whether the rhizobia associated with these legumes also form distinct populations in the varying habitats and soil types. The objectives of this study were to assess the distribution and diversity of rhizobia isolates from nodules of indigenous legumes growing in different soil types of the CFR and to determine their effect on the distribution of the host species. It was hypothesized that:

1. The rhizobia isolates from indigenous legumes of the CFR would cluster phylogenetically according to soil types
2. The legume plant distribution in the CFR is constrained by the presence of their compatible rhizobia

Thesis Outline

This study investigated the diversity of rhizobia associated with CFR legumes and the possible effects of their interactions thereof in an effort to understand the legume host species diversity and distribution as affected by their compatible rhizobia. The two hypotheses mentioned above formed the basis of the two data chapters.

The research chapters constituting this thesis were each written as stand alone chapters and therefore there is a degree of repetition in some instances.

Chapter 2

Characterization of rhizobia from different host legumes and soil types of the CFR

1.1. INTRODUCTION AND LITERATURE REVIEW

The Cape Floristic Region (CFR) is located at the southwestern tip of the African continent. The region. is bordered by the Indian and Atlantic oceans coastline, between latitudes 31^{o} and 34^{o}30' S, and comprises of approximately 90 000 km^{2} land area of mountain chains and lowlands (Mucina et al,. 2006; Goldblatt and Manning 2000). The region has a Mediterranean climate and is mostly covered with fynbos vegetation, a sclerophyllous shrubland, occurring mostly on acid sands of poor nutrients derived from Table Mountain Sandstones (Goldblatt and Manning 2000). The CFR is the smallest among the other five floral kingdoms of the world, but unique with its richness in diversity of species. The region has therefore been a hub for much plant scientific research as many researchers continue to be fascinated by the rich diversity.

Out of the about 20 500 plant species in Southern Africa, the CFR is home to about 9000 species with 69% of these endemic to the region and such diversity is comparable with many of the richest tropical forests, and exceptional among temperate and African floras (Mucina and Rutherford 2006; Goldblatt and Manning 2000). The fynbos is dominated by sclerophyllous to microphyllous shrubs of many families with Proteaceae, Ericaceae and Rutaceae being common in woody vegetation types while ground cover consists mainly of Restionaceae and Cyperaceae (Bond and Goldblatt 1984). In the CFR context, the family Fabaceae is currently comprised of about 760 species, in 37 genera (seven genera are endemic) belonging to 18 tribes (Goldblatt and Manning 2000). Most of these legumes are in symbiotic association with rhizobia that nodulate and fix nitrogen in nutrient poor soils. The legume species in the CFR occur in distinct populations in varying habitats ranging from water seeps, river valleys, and mountain slopes covering different soil types. However it is not known whether the rhizobia associated with the legumes also form distinct populations in the varying habitats. Among the many documented reasons for the complexity of the flora and vegetation of the CFR, Barraclough (2006) summarised the causes of the complexity and put the factors under six headings, including topographical factors, edaphic factors, pollinator specialization, fire and short dispersal distances. However there was no mention of plant microbial associations contributing to the plant distribution pattern.

Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria that are capable to nodulate members of the leguminoseae and fix nitrogen (Bontemps et al. 2010; Masson-Boivin et al. 2009; Sprent 2009). Earlier on, two groups of rhizobia were identified

as fast and slow growers, and subsequently called *Rhizobium* and *Bradyrhizobium* respectively (Jordan 1982). The term rhizobia in its strictest sense is said to refer to members of the genus *Rhizobium* and was initially restricted to the family Rhizobiaceae within the α – subclass (Young and Haukka 1996). Over the years the concept of rhizobia has been expanded to include all bacteria that are capable of nodulation and nitrogen fixation in association with legumes (Willems, 2006; Raychaudhuri et al., 2007; Garcı, 2010). Currently, rhizobia taxonomy includes five genera belonging to four families: Rhizobiaceae, Phyllobacteriaceae, Nitrobacteraceae and Hyphomicrobiaceae (Garcı, 2010). All bacteria that fix nitrogen are restricted to the gram-negative Proteobacteria which have five subdivisions (α, β, γ, δ, and ε) with only the first two groups having members proven to nodulate legumes (Raychaudhuri et al. 2007; Sprent 2007; Willems 2006; Moulin et al. 2001).

The history of rhizobia is said to date back to the end of the 19th century when it was realized that atmospheric N was being assimilated through root nodules of legume plants (Beijerinck 1888). Beijerinck was reported to be the first microbiologist to obtain pure bacteria culture from nodule suspension and established that the bacteria were actually responsible for the N-fixation process and named the bacteria *Bascillus radicola*. Later *Bascillus radicola* was changed to *Rhizobium leguminosarum* (Beijerinck 1888 and Frank 1889 cited by (Garcı 2010). In recent years, other N-fixing bacteria in *Bradyrhizobium, Mesorhizobium, Sinorhizobium, and Azorhizobium* belonging to Alphaproteobacteria and members of the Betaproteobateria such as *Cupriavidus* and the recently described *Burkholderia lineages*, and other non-Rhizobiaceae α-Proteobacteria rhizobia genera such as *Methylobacterium, Devosia, Ochrobacteria* and *Phyllobacterium* have been identified (Garcı 2010; Graham 2008; Elliott et al. 2007; S. Raychaudhuri et al. 2007; Willems 2006; Sahgal and Johri 2003; L Moulin et al. 2001). Most of the Alpha rhizobia have been reported to be widespread in nature with specific genera predominating in particular environments. Rhizobia biogeographic studies have reported the general global predominance of *Bradyrhizobium* in nutrient poor soils (Rodríguez-Echeverría et al., 2011; Stępkowski et al., 2012), *Rhizobium* in acidic soils, *Mesorhizobium* at intermediate pH, *Sinorhizobium* under alkaline conditions and *Azorhizobium* in water logged soils (Bala and Giller 2006). On the other hand, among the Beta rhizobia, *Burkholderia* although often associated with acidic soils (Bontemps et al., 2010), it has been reported to be widespread in nature with its centres of diversity being South Africa and South America (Chen et al., 2003; Mishra et al., 2012). The genus

Culpriavidus has been reported to predominate in Taiwan and some parts of China and South America (Liu et al., 2012; Mishra et al., 2012).

Superior and new developments in the methods to study cell DNA and RNA has revealed that within each rhizobia genus there are hundreds of known strains, differing in characteristics and performance with different hosts (Bontemps et al. 2010; Sprent 2007; Willems 2006; Sahgal and Johri 2003). For example, characterization of 111 rhizobial strains isolated from wild legumes in Xinjiang, China defined nine genomic species belonging to four genera of *Rhizobium, Mesorhizobium, Ensifer and Bradyrhizobium* (Han et al., 2008). Previous studies have reported rhizobia isolates from nodules of CFR legume plants to include *Bradyrhizobium, Herbasprillum, Rhizobium, Mesorhizobium, and Burkholdeira,* but were limited to a few genera such as those from *Aspalathus, Cyclopia, Lotonis* and *Lebeckia* (Hassen et al. 2011; Phalane 2008; Elliott et al. 2007; Kock 2004; Spriggs 2004; Le Roux 2003). In this study, the entire legume family was targeted to assess rhizobia diversity and to investigate whether there are links between the distribution of rhizobia and host legumes on various soil types of the CFR. It was hypothesized that the rhizobia isolates from indigenous legumes of the CFR would cluster phylogenetically according to soil types.

2.0. MATERIALS AND METHODS

2.1. Nodule and soil Collections

Nodules were collected from the roots of about 65 legume species from 15 genera (Table 1)growing as re-seeders and re-sprouters in about 20 localities of varying soils types in the 5 phytogeographic regions of the Cape Floristic Region (Figure 1; Goldblatt and Manning 2000; Bond and Goldblatt 1984). The global positioning system (GPS) was used to record the coordinates of the localities of each sample. On all sites, legume species that had nodules were collected. Only viable nodules attached to vegetatively growing plants were collected together with soil in plastic bags and transported to the laboratory for strain isolation. In addition, host plant samples were collected for the purpose of herbarium specimens and each was allocated a voucher number.

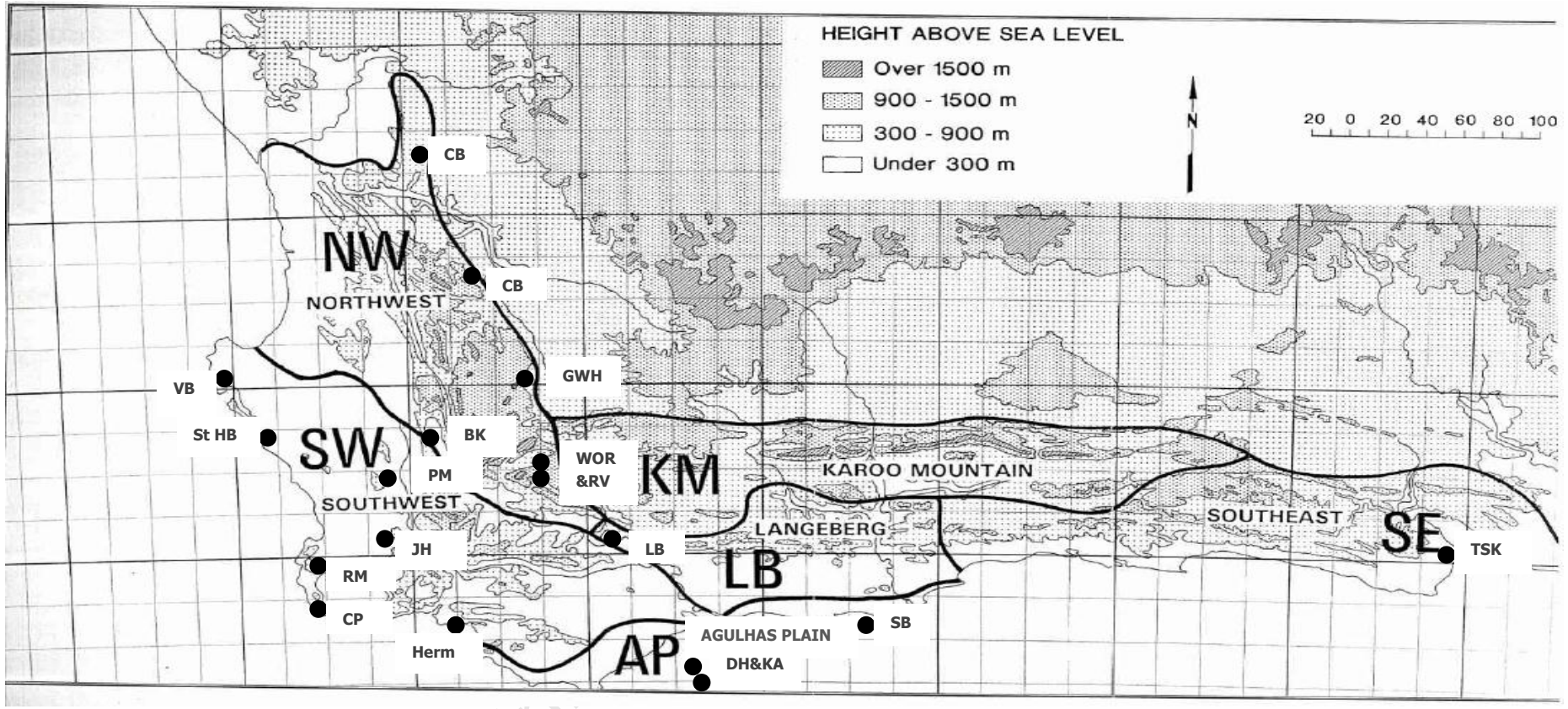

Figure 1. The phytogeographic divisions of the Cape Floristic Region (Goldblatt and Manning 2000) showing study sites.CB (Cederberg), CP (Cape Point), RM (Rhodes Memorial), St HB (St Helena's Bay),VB (Vredenburg), Herm (Hermanus), JH (Jonkershoek), PM (Paarl Mountain), BK (Bainskloof), LB (Langeberg), WOR (Worcester), RV (Rawsonville), GWH (Grootwinterhook), DH (De Hoop), KA (Koppie Alleen), SB (Stilbaai) and TSK (Tsitsikamma).

Three replicate soil samples were collected within the legume stands per site using an auger or a trowel to a depth of 15 cm for the determination of soil pH. The pH was determined by adding 50 ml 1 M KCl to 20 g soil, stirred for 10 minutes and was left to stand for another 30 minutes. After 30 minutes, the mixture was stirred again before passing through a filter. The pH was measured on the filtrate using a Waterproof pHTester 10-pH meter (Fierer and Jackson, 2006).

2.2. Rhizobia Strain Isolation from Root Nodules

One viable nodule was selected per legume species to isolate rhizoba following the procedure by Vincent (1970). Sterile distilled water was used to wash the nodules and dipped in 95% ethanol after which they were inundated in 0.1% acidified $HgCl_2$ for 3 minutes. Nodules were rinsed in at least six changes of sterile distilled water to remove the acid solution. Isolation of bacteria was achieved by crushing a single nodule aseptically in a plate. Using a sterile wire loop, the nodule squash were streaked on yeast extract mannitol (YEM) agar plate in a manner that will progressively dilute the suspension to obtain single colonies (Vincent, 1970). The plates were incubated for up to 10 days at 28 ^{o}C. Plates were daily checked for morphological traits such as growth rate, colony growth form, colony type and colour (Hassen et al. 2011; Bala et al. 2003a; Somasegaran and Hoben 1985). These were noted for morphological characterization of rhizobia isolates. The rhizobia isolates were purified from a single colony of each nodule, and stored in the 0˚C room for subsequent PCR analysis. Every three months the purified strains were re-cultivated to maintain their viability and purity. Three replicates of each selected strain were stored with glycerol for long term storage at -80 ˚C (Hassen et al. 2011; Estrella et al. 2009; Odee et al. 1997).

2.3. Genomic DNA Extraction

DNA was extracted from freshly grown isolates on Tryptone Yeast (TY) broth using a standard protocol obtained from the lab of Dr Emma Steenkamp, University of Pretoria. Bacterial isolates were grown for four days on a shaker at 28 ºC. Bacteria cells were harvested by centrifuging a total of 3mml at 6000 rpm for 3 minutes at room temperature and the resultant cell pellet was re-suspended in 250 µl TES buffer and 30 µl of Proteinase K (10 µg/µl). Isolates were frozen at -70 ºC for 15–20 minutes after which they were incubated at 62–64 ºC for 60 minutes. A 3/10 volume of NaCl and 1/10 volume of CTAB were added to 280 µl and incubated for 10 minutes at 65 ºC. Isolates were washed by adding 1 volume (392 µl) of phenol:chloroform:iso-amyl alcohol solution and after incubation on ice bath for 30

minutes, the isolates were centrifuged at room temperature for 30 minutes at 14 000 rpm. The upper liquid phase was transferred to a new 1.5 µl tube and incubated with 0.6 volume cold isopropanol and incubated at -20 ºC for at least 48hrs for the precipitation of DNA. The isolates were then centrifuged at 4 ºC for 30 minutes @ 14 000 rpm to obtain the DNA pellet. The pellet was rinsed with 250 µl 70% ethanol and centrifuged for 15 minutes at 4 ºC at 14 000 rpm. The supernatant was carefully removed and the pellets were dried using a heating block set to 37 ºC. Finally the pellet was re-suspended in 50 µl sterile distilled H_2O. Estimation of DNA concentration was done by visualizing under UV light a stained agarose gel after electrophoresis.

Table 1. Voucher numbers of the host legume of rhizobia isolates and their biogeography.

Tribe	Collector	Voucher No.	Genus	Species	Locality	Elev-ation (m)	Soil type	pH
Acacieae	Oscar	122	*Acacia*	*mearnsii* De Wild.	Tsitsikama	421	River sand	6.4
Crotalarieae	Oscar	13	*Aspalathus*	*cordata* (L.) Dahlg.	Jonkershoek	296.3	Alluvium	4.0
Crotalarieae	Oscar	18	*Aspalathus*	*astroites* L.	Jonkershoek	296.3	Alluvium	4.0
Crotalarieae	Oscar	26	*Aspalathus*	*uniflora* L. *subsp. Uniflora*	Jonkershoek	272	Alluvium	3.6
Crotalarieae	Oscar	31	*Aspalathus*	*ericifolia* L. *subsp. Ericifolia*	Paarl Mtn	581	Granite	5.2
Crotalarieae	Oscar	48	*Aspalathus*	*sp.*	De Hoop	11.4	Limestone	6.4
Crotalarieae	Oscar	49	*Aspalathus*	*sp.*	De Hoop	11.4	Limestone	6.7
Crotalarieae	Oscar	53	*Aspalathus*	*sp.*	Stilbaai	107.1	Limestone	6.5
Crotalarieae	Oscar	108	*Aspalathus*	*Ciliaris*	Cederberg	522.8	Sandstone	4.8
Crotalarieae	Oscar	5352	*Aspalathus*	*ericifolia* L.*subsp. ericifolia*	Jonkershoek	272	Alluvium	4.9
Crotalarieae	Oscar	5361	*Aspalathus*	*ciliaris* L.	Jonkershoek	296.3	Alluvium	4.3
Crotalarieae	Oscar	5372	*Aspalathus*	*laricifolia* Berg. *subsp. laricifolia*	Jonkershoek	330.3	Alluvium	3.6
Crotalarieae	Oscar	5398	*Aspalathus*	*spicata* Thunb.	Rawsonville Farm	368	Alluvium	4.1
Crotalarieae	Oscar	5440	*Aspalathus*	*spicata* Thunb.	Worcester-Dam	229	Acid sand	3.9
Crotalarieae	Oscar	5477	*Aspalathus*	*callosa* L.	Cape Point	10	Acid sand	4.6
Crotalarieae	Oscar	5496	*Aspalathus*	*ericifolia* L. subsp. Ericifolia	Cape Point	57	Acid sand	3.7
Crotalarieae	Oscar	5618	*Aspalathus*	*ericifolia* L. subsp. Ericifolia	Paarl Mtn	582	Granite	5.2
Crotalarieae	Oscar	5734	*Aspalathus*	*uniflora* L. subsp. Uniflora	Cederberg	348	Sandstone	3.5
Crotalarieae	Oscar	5757	*Aspalathus*	*perfoliata* (Lam.) Dahlg. subsp. perfoliata	Cederberg	667	Sandstone	3.6
Crotalarieae	Stirton	13166	*Aspalathus*	*Ciliaris*	Groot Hogelkraal	28	Acid sand	4.1
Crotalarieae	Oscar	120	*Crotalaria*	*sp.*	Tsitsikama	421	Sandstone	4.6
Crotalarieae	Oscar	22	*Rafnia*	*acuminata* (E. May.) G.J. Campbell & B.-E. Van Wyk	Jonkershoek	272	Alluvium	3.6
Crotalarieae	Oscar	28	*Rafnia*	*sp.*	Jonkershoek	272	Alluvium	3.6
Crotalarieae	Oscar	55	*Rafnia*	*triflora* (L.) Thunb.	Stilbaai	107.1	Limestone	6.5
Galegeae	Oscar	46	*Lessertia*	*sp.*	Koppie Alleen	4.7	Coastal sand	7.3
Genisteae	Oscar	14	*Argyrolobium*	*Cordata*	Jonkershoek	296.3	Alluvium	3.2
Genisteae	Oscar	47	*Argyrolobium*	*sp.*	De Hoop	11.4	Limestone	6.1
Indigofereae	Muthama	5746	*Indigofera*	*sp.*	Cederberg	611	Sandstone	4.1
Indigofereae	Oscar	45	*Indigofera*	*sp.*	De Hoop	7.1	Limestone	6.1
Indigofereae	Oscar	5378	*Indigofera*	*sp.*	St Helena's Bay	68	Granite	4.9
Indigofereae	Oscar	5392	*Indigofera*	*frutescens* L.f.	Rawsonville Farm	427	Alluvium	4.2
Indigofereae	Oscar	5397	*Indigofera*	*sp.*	Rawsonville Farm	368	Alluvium	4.1
Indigofereae	Oscar	5419	*Indigofera*	*superba* C.H. Stirt.	Hermanus	352	Sandstone	3.6
Indigofereae	Oscar	5621	*Indigofera*	*ericifolia* L. subsp. Ericifolia	Paarl Mtn	584	Granite	5.2
Indigofereae	Muthama	5878	*Indigofera*	*sp.*	Groot Hogelkraal	40	Acid sand	4.1

Millettieae	Oscar	5405	*Tephrosia*	*capensis* (Jacq.) Pers.	Hermanus	45	Sandstone	3.8
Phaseoleae	Oscar	29	*Bolusafra*	*bituminosa* (L.) Kuntze	Jonkershoek	272	Alluvium	3.2
Podalyrieae	Oscar	5482	*Amphithalia*	*ericifolia* L. subsp. Ericifolia	Cape Point	20	Acid sand	4.6
Podalyrieae	Oscar	25	*Podalyria*	*calyptrata* (Retz) Willd.	Jonkershoek	272	Alluvium	3.6
Podalyrieae	Oscar	5337	*Podalyria*	*calyptrata* (Retz) Willd.	Bain'skloof	420	Shale	4.6
Podalyrieae	Oscar	5384	*Podalyria*	*sericea* (Andrews) R. Br.	Langeban	91	Granite	5.3
Podalyrieae	Muthama	5875	*Podalyria*	*Spicata*	Groot Hogelkraal	28	Acid sand	4.1
Podalyrieae	Oscar	123	*Virgilia*	*divaricata* Adamson	Tsitsikama	421	River sand	5.3
Podalyrieae	Oscar	5366	*Virgilia*	*oroboides* (Berg.) Salter	Jonkershoek	296	Alluvium	4.2
Psoraleeae	Oscar	32	*Otholobium*	*hirtum* (L.) C.H. Stirt.	Paarl Mtn	581	Granite	5.2
Psoraleeae	Oscar	42	*Otholobium*	*bracteolatum* (Eckl. & Zeyh.) C.H.Stirt. subsp. limnophilum	De Hoop	11.4	Limestone	6.7
Psoraleeae	Oscar	5333	*Otholobium*	*virgatum* (Burm.f.) C.H. Stirt.	Rhodes Memorial	203	Shale	5.3
Psoraleeae	Oscar	5334	*Otholobium*	*hirtum* (L.) C.H. Stirt.	Rhodes Memorial	203	Shale	5.3
Psoraleeae	Oscar	5357	*Otholobium*	*virgatum* (Burm.f.) C.H. Stirt.	Jonkershoek	296.3	Alluvium	4.3
Psoraleeae	Oscar	5369	*Otholobium*	*sp.*	Jonkershoek	296	Alluvium	4.2
Psoraleeae	Oscar	5370	*Otholobium*	*parviflorum* (E.Mey.)C.H.Stirt.	Jonkershoek	330.3	Alluvium	4.2
Psoraleeae	Oscar	5376	*Otholobium*	*hirtum* (L.) C.H. Stirt.	St Helena's Bay	68	Granite	4.9
Psoraleeae	Oscar	5382	*Otholobium*	*hirtum* (L.) C.H. Stirt.	Vredenburg	38	Granite	4.6
Psoraleeae	Muthama	5675	*Otholobium*	*zeyheri* (Harv.) C.H. Stirt.	Houw Hoek Mts	610	Sandstone	3.6
Psoraleeae	Oscar	15	*Psoralea*	*asarina* (Berg.) Salter	Jonkershoek	296.3	Alluvium	3.2
Psoraleeae	Oscar	24	*Psoralea*	*gigantea* M.N. Dludlu, A.M. Muasya & C.H. Stirt.	Jonkershoek	272	Alluvium	4.0
Psoraleeae	Oscar	52	*Psoralea*	*sp.*	Stilbaai	107.1	Limestone	6.5
Psoraleeae	Oscar	5336	*Psoralea*	*pinnata* L.	Rhodes Memorial	141	Shale	5.3
Psoraleeae	Oscar	5343	*Psoralea*	*rigidula* C.H. Stirt.	Bainskloof	753.2	Sandstone	3.3
Psoraleeae	Oscar	5360	*Psoralea*	*asarina* (Berg.) Salter	Jonkershoek	296.3	Alluvium	4.3
Psoraleeae	Oscar	5364	*Psoralea*	*usitata* C.H. Stirt.	Jonkershoek	296	Alluvium	4.2
Psoraleeae	Oscar	5413	*Psoralea*	*pullata* C.H. Stirt.	Hermanus	352	Sandstone	2.3
Psoraleeae	Oscar	118	*Psorelea*	*Oligophylla*	Tsitsikama	421	Shale	4.6
Psoraleeae	Oscar	119	*Psorelea*	*laxa* Salter	Tsitsikama	421	Shale	4.6
Sesbanieae	Muthama	5717	*Sesbania*	*punicea* (cav.) Benth.	Olifant Algeria	113	Riversand	6.6

2.4. PCR Amplification

PCR amplifications were done in a GeneAmp® PCR system 2700 (Applied Biosystems, Foster City, Califonia USA) with the universal primers: forward primer (16f27 5´-AGAGTTTGATCCTGGCTCAG –3´) and the reverse primer (16r1485 5´-TACCTTGTTACGACTTCACCCCA-3´) amplifying nearly 1200 base pairs of the 16S rRNA gene (Estrella et al. 2009; Lane, 1991). The primers were produced by the Molecular and Cell Biology department of UCT. The PCR reactions were prepared to a final volume of 30 µl containing PCR buffer (3 µl), $MgCl_2$ (3 µl), dNTPs (1.2 µl), forward and reverse primers (1 µl) each, Kapa-Taq (0.2 µl) and water (18.6 µl). An initial denaturation of 94 ˚C for 2 minutes followed by 30 cycles of denaturation at 94 ˚C for 1 minute, annealing at 55 ˚C for 1 minute and extension at 72 ˚C for 1 minute was conducted. Finally an elongation step of 72 ˚C for 7 minutes was followed by a final holding temperature of 20 ˚C. The PCR product was viewed on agarose gel electrophoresis (Hassen et al., 2011). The unpurified PCR product was sent to Stellenbosch University Central DNA Sequencing Facility for post PCR clean up and sequencing reaction. Sequencing primers for 16S rRNA were the same as for the initial PCR.

2.5. Reconstruction of Rhizobial Phylogeny

Sequences obtained in this study were edited using Bio Edit version 7.0.9.1. These sequences were aligned, together with the sequences downloaded from GenBank are listed in (Table 2), using MUSCLE v. 3.8.31 (Edgar 2004) and edited manually. Reconstruction of the rhizobia phylogeny was done using Bayesian inference and the trees were rooted with the species from the main bacterial lineages of Proteobacteria (Stackebrandt et al., 1988).

Model testing was done using the MRAIC v. 1.4.4 PERL script (Nylander 2004), implemented in PHYML v. 3.0 (Guindon and Gascuel 2003). The model that best fit the data was HKY+I+Γ, according to both AIC and BIC, and this model was used in the Bayesian tree reconstruction. The phylogeny was reconstructed in MRBAYES v. 3.1.2 (Ronquist and Huelsenbeck 2003). The analysis was run with four simultaneous Metropolis-coupled chains for 20 million generations, sampling a tree every 1000 generations, with one cold and three heated chains at a temperature setting of 0.1. TRACER v. 1.5 (Drummond and Rambaut 2007) was used to evaluate the effective sample size of each parameter: These were all above 800, indicating that the MCMC algorithm had been run long enough. Burn-in was assessed by inspecting the trace for each run; the first 10 % of the samples were discarded as burn-in. A 50 % majority-rule consensus tree was created from the post-burn-in parameter estimates in MRBAYES, with posterior probabilities (*PP*) of nodes indicating clade support values.

Table 2. 16S rRNA sequences downloaded from the GenBank used as reference strains and as outgroups (in bold) in this study.

Class	GenBank accession no.	Genus	Species
Acidobacteria	**NR043386**	***Acidobacterium***	***capsulatum***
Alphaproteobacteria	NR041396	*Agrobacterium*	*tumefaciens*
Alphaproteobacteria	EF522124	*Agrobacterium*	*rhizogenes*
Alphaproteobacteria	NR041839	*Azorhizobium*	*doebereinerae*
Alphaproteobacteria	HQ706108	*Azorhizobium*	*caulinodans*
Alphaproteobacteria	JN392462	*Bradyrhizobium*	*elkanii*
Alphaproteobacteria	HQ844501	*Ensifer*	*adhaerens*
Alphaproteobacteria	DQ100068	*Mesorhizobium*	*plurifarium*
Alphaproteobacteria	FJ491264	*Mesorhizobium*	*huakuii*
Alphaproteobacteria	HQ424937	*Mesorhizobium*	*loti*
Alphaproteobacteria	HQ877490	*Mesorhizobium*	*amorphae*
Alphaproteobacteria	JF496403	*Rhizobium*	*alkalisoli*
Alphaproteobacteria	JN208895	*Rhizobium*	*etli*
Alphaproteobacteria	JN208903	*Rhizobium*	*leguminosarum*
Alphaproteobacteria	JN208906	*Rhizobium*	*tropoci*
Alphaproteobacteria	HQ406753	*Rhizobium*	*galicum*
Alphaproteobacteria	JF496403	*Sinorhizobium*	*meliloti*
Alphaproteobacteria	JN105985	*Sinorhizobium*	*medicae*
Betaproteobacteria	HE578794	*Achromobacter*	*insolitus*
Betaproteobacteria	NR042021	*Achromobacter*	*denitrificans*
Betaproteobacteria	FN908407	*Burkholderia*	*tuberum*
Betaproteobacteria	FN908408	*Burkholderia*	*mimosarum*
Betaproteobacteria	FN908409	*Burkholderia*	*phymatum*
Betaproteobacteria	AY860233	*Capriavidus*	*gilardii*
Betaproteobacteria	AY860244	*Capriavidus*	*resiraculi*
Betaproteobacteria	EU024156	*Capriavidus*	*oxalaticus*
Betaproteobacteria	JN545038	*Pseudomonas*	*aeruginosa*
Deltaproteobacteria	**NR044916**	***Desulfovibrio***	***indonesiensis***
Epsilonproteobacteria	**AF348748**	***Helicobacter***	***cinaedi***
Gammaproteobacteria	**JN545038**	***Pseudomonas***	***aeruginosa***
Zetaproteobacteria	**EF493244**	***Mariprofundus***	***ferrooxydans***

2.6.Plant growth in Leonard jars for authentication of rhizobia

Out of the 65 legume species used in this study for the sequencing of rhizobia only 17 were selected for the authentication process (Table 7) because of the availability of seed at the time of nodule collection and also from local seed companies. A total of 17 test species were used in the authentication process (Table 7). All the rhizobia isolates were tested on host species except *A. crenata* and *L. frutescens*. Seeds of the test species were germinated as explained in Section 3.2. (Chapter 3) with the exception of that the growth medium was sterilised and germination took place in a controlled environment to prevent bacterial contamination.

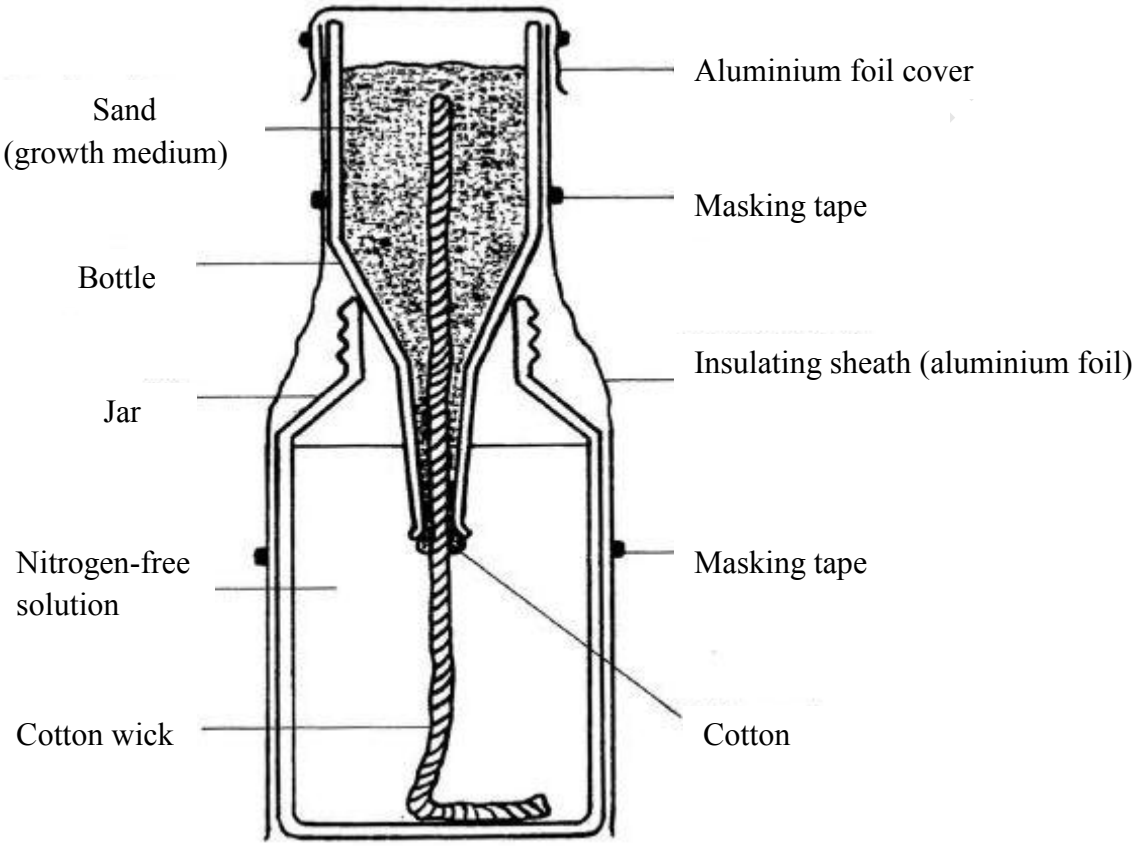

Figure 2. Modified Leonard jar assembly (Somasegran and Hoben 1985).

The assembling of the Leonard jars (Figure 2) was as described by Somasegaran and Hoben (1985). A cotton wick obtained from strands of a mop was passed through the mouth of a 750 ml quart bottle which had its base cut off to a smooth finish. A small amount of cotton wool was used to secure the position of the wick into the neck of the bottle and also to prevent the growth medium settling in the reservoir.

The bottle was inverted into a 1000 ml glass reservoir previously filled to ¾ of its volume with modified N-free solution (section 3.2, Chapter 3) originally formulated at the University of Western Australia (UWA). Holding the cotton wick centrally, the bottle was filled to 5 cm below its surface with acid washed sand (medium) previously passed through 2 mm sieve to minimise air spaces. The growth medium was moistened to saturation by adding ¼ modified N-free solution of the reservoir's volume. The complete assembly was wrapped in aluminium foil secured by masking tape at critical points along the jar and autoclaved for 2 hours at 120 ºC.

The assembly was conveniently allowed to cool in the autoclave over night. Pre-germinated seedlings were transplanted into the jars in a laminar-flow hood using sterile forceps. A single vigorously growing seedling was planted into each Leonard jar and seedlings in three Leonard jars were inoculated directly onto their roots with 1 mL of inoculant (rhizobia broth culture; Section 2.3). A 2 cm layer of sterilised vermiculite was placed over the sand to prevent moisture loss and as a barrier to bacteria contamination. Seedlings in two Leonard jars were inoculated with yeast extract broth without rhizobia as controls.

3.0. RESULTS

3.1. The host legume sampled and their ecology

A total of 65 isolates were obtained from 65 species (Table 1) collected from five phytogeographic regions of the CFR (North-west, South-west, South-east, Langeban and Agulhas plain). The genera *Aspalathus, Podalyria, Psoralea, Otholobium*, and *Indigofera* are among the largest legume genera in the CFR (Table 3), and their species were the most common in most localities covering a wide range of pH (2.3 to 7.3; Table 1). The soil types were grouped into eight distinct groups including sandstone, granite, shale, limestone, alluvium, coastal sand acid sand, and river sand. Sandstone and acid sand were in the range of pH 2.3 to 4, alluvium, granite and shale were in the pH range 4 to 5, while river sand and limestone were within the pH range of 6 to 7 and as expected coastal sand had the highest pH of 7.3.

Table 3. Sampling effort among the tribes of Fabaceae in the CFR. Shown are number of sampled taxa against the total in brackets (Goldblatt and Manning 2000)**.**

Tribe	# genera sampled	# Species Sampled	% Species sampled
Sesbanieae	1(1)	1(1)	100
Millettieae	1(1)	1(3)	33
Psolareeae	2(3)	22(94)	23
Acacieae	1(1)	1(8)	12.5
Indigofereae	1(1)	9(80)	11
Crotalarieae	3(6)	28(297)	9
Podalyrieae	2(8)	8(116)	7
Genisteae	1(3)	2(34)	6
Galegea	1(1)	1(19)	5.2
Phaseoleae	1(8)	1(22)	4.5

The CFR host species belonged to 15 genera from 10 tribes as indicated in the Table 3. The proportion of the species sampled to their total in CFR was about 9% (Table 3) which was similar to that of dos Reis et al. (2010) and Bontemps et al. (2010) who sampled a total of 70 species out of over 500 *Mimosa* species in Cerrado and Caatinga biomes of Brazil. Three of the tribes had more than one genus sampled, the *Crotalarieae (Aspalathus Crotalaria, Rafnia), Podalyrieae (Podalyria, Virgilia)* and the *Psoraleae (Psoralea, Otholobium)* (Table 3). On the basis of the number of species sampled, Crotalarieae predominates with over 28

species, Psolareeae had 22, Indigofereae had 9 species and Podalyrieae had 8 species (Table 3).

3.2. Morphological characterization of CFR rhizobia isolates

The CFR isolates obtained from this study were categorised into four colony types: white opaque, creamy opaque, creamy translucent, and watery (Table 4). On all the four colony types, shapes were either raised or semi-raised (Table 4). Among the isolates collected, 38% showed fast growth rate (< 3 days), 44% were intermediate (4-6 days) while 18% recorded slow growth rate (>7 days; Table 4). The soil types including alluvium, acid sand, river sand and sandstone contained rhizobia isolates belonging to all stages of growth rates (Table 5). However, shale soil lacked isolates with slow growth rate whereas granite soil lacked isolates with fast growth rate. Rhizobia isolates from limestone and coastal sand showed intermediate growth rates (Table 5).

Table 4. Growth rates, shape and colony types of rhizobial isolates grown on YEM agar.

Growth rate	Shape	Colony type	Propotion of Isolates (%)
Fast (<3days)	raised, semi-raised	white opaque, creamy opaque, creamy translucent, watery	38
Intermediate (4 - 6days)	raised, semi-raised	white opaque, creamy opaque, creamy translucent, watery	44
Slow (>7days)	Semi-raised, raised	Creamy translucent	18

Table 5. Distribution of growth rates of the isolates on different soil types

Soil types	No. of isolates: fast	intermediate	Slow
Alluvium	11	5	6
Acid sand	3	2	1
Sandstone	5	6	2
River sand	1	1	1
Shale	5	1	0
Coastal sand	0	1	0
Limestone	0	8	0
Granite	0	6	2
Total of growth rate types	26	30	12
Percentage of total isolates	38%	44%	18%

3.3. Phylogenetic characterization of the CFR rhizobial isolates

The phylogenetic groupings of the CFR isolates was achieved by sequencing of the 16S rRNA gene marker and results revealed that these were associated with two main divisions of the gram negative Proteobacteria (Figure 3) and these were the Alpha-proteobacteria and Beta-proteobacteria (Brenan et al. 2012; Sahgal and Johri 2003). The beta rhizobia were represented by three genera on the phylogentic tree (Figure 4): *Burkholderia, Achromobacter and Cupriavidus* (Brenan et al. 2012; Benata et al. 2008; Elliott et al. 2006). The CFR isolates formed membership with the *Burkholderia and Achromobacter lineages* with a very strong posterior probability support (pp) = 100% and none were associated with the *Cupriavidus.*

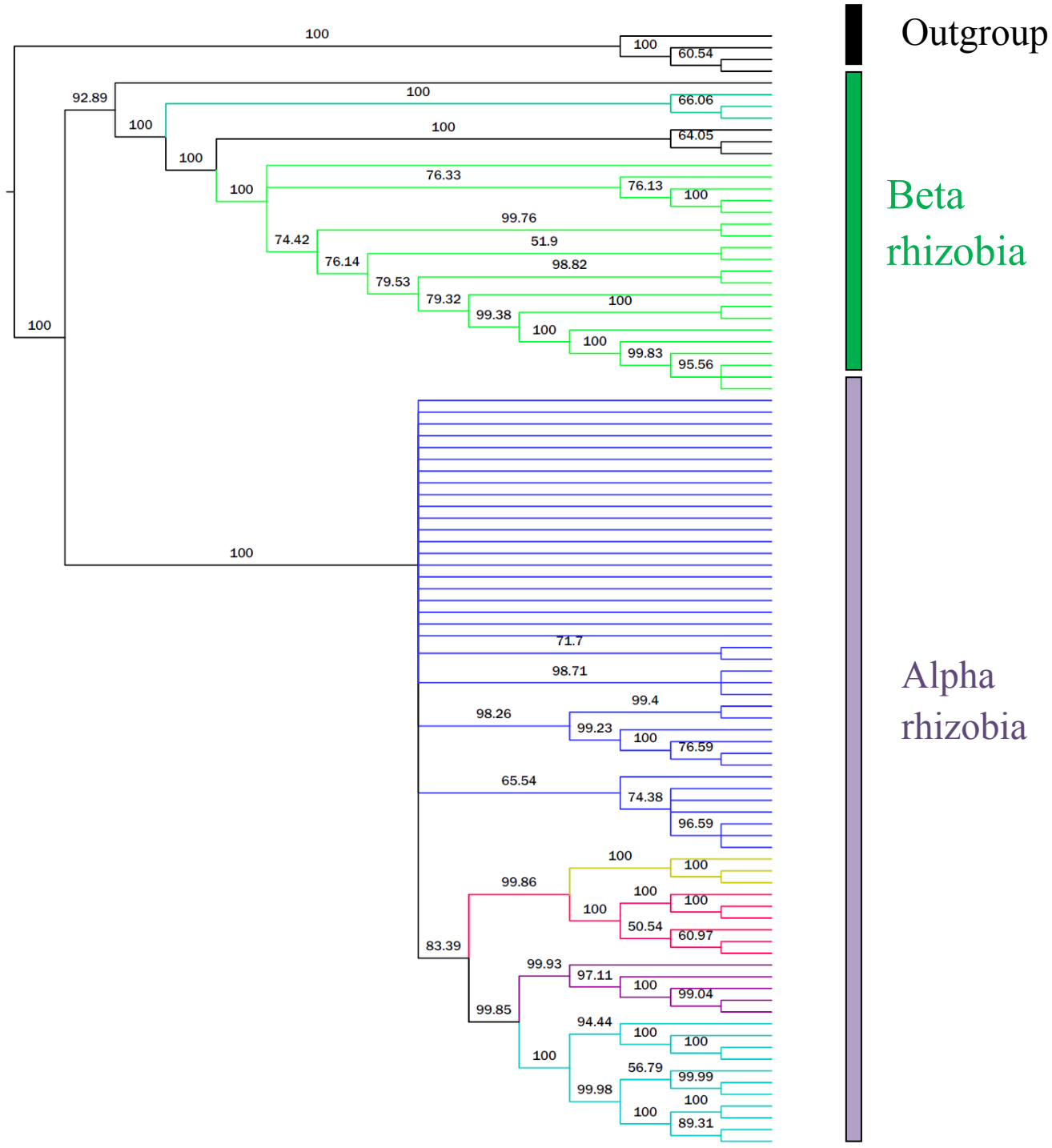

Figure 3. Bayesian 50% majority rule consensus tree indicating three main rhizobial clades based on CFR isolates and reference sequences from the GenBank.

Most of the isolates in the β-rhizobia clade were closely associated with *Burkholderia.* All rhizobial isolates of *Rafnia, Virgilia oroboides and Podalyria species* from eight different localities of varying soil types were associated with *Burkholderia* species exclusively. For

example, isolates 5337 and 25 (Figure 4) represented species growing in shale and alluvium respectively, but were shown to be nodulated by the same or similar rhizobia (pp=99.76%). However, in some cases, different host species such as *Podalyria spicata* and *Indigofera* sp. occurring in the same geographic locality were nodulated by similar rhizobia (e.g isolates 5875 and 5878 (pp=100%) from Gansbaai; isolates 5477, 5482 and 5496 (pp=95.56%) from Cape Point; Figure 4). Isolate 5419 from *Indigofera superba* (Figure 4) which distinctively occupied a patch of the eastward slope of the Vogelgat Nature Reserve in the Hermanus area was associated with *Achromobacter* (pp=100%), a bacterial lineage not commonly known to cause nodules and fix nitrogen in symbiosis with legume species.

The alpha group comprised of seven rhizobia genera, including *Azorhizobium, Agrobacterium, Bradyrhizobium, Ensifer, Mesorhizobium, Rhizobium/Agrobacterium* and *Sinorhizobium* (Figure 4), all well known to cause nodulation and N-fixation in symbiosis with legumes (Ormen et al. 2010; Menna et al. 2006; Bala et al. 2004; Bala et al. 2003a). Most of the clades containing the rhizobia isolates had strong posterior probability support of up to 100%. Out of the 50 isolates that grouped as α-rhizobia, 35 isolates came from *Crotalarieae* and *Psoraleeae* species associated with *Mesorhizobium* (Figure 5). On the other hand, *Azorhizobium* (pp=100) was associated with *Sesbania punicea* growing on the Olifant River sands in Algeria area of the Cederberg mountains. The reference strains of *Bradyrhizobia* species formed a clade (pp=100%) that contained isolates from two *Indigofera* species, and one species each from *Tephrosia* and *Acacia* (Figure 5). The *Sinorhizobia* clade (pp=97.11%) was associated with isolates from two different host species (*Tephrosia* and *Indigofera*) occurring in the same geographic locality (Figure 5).

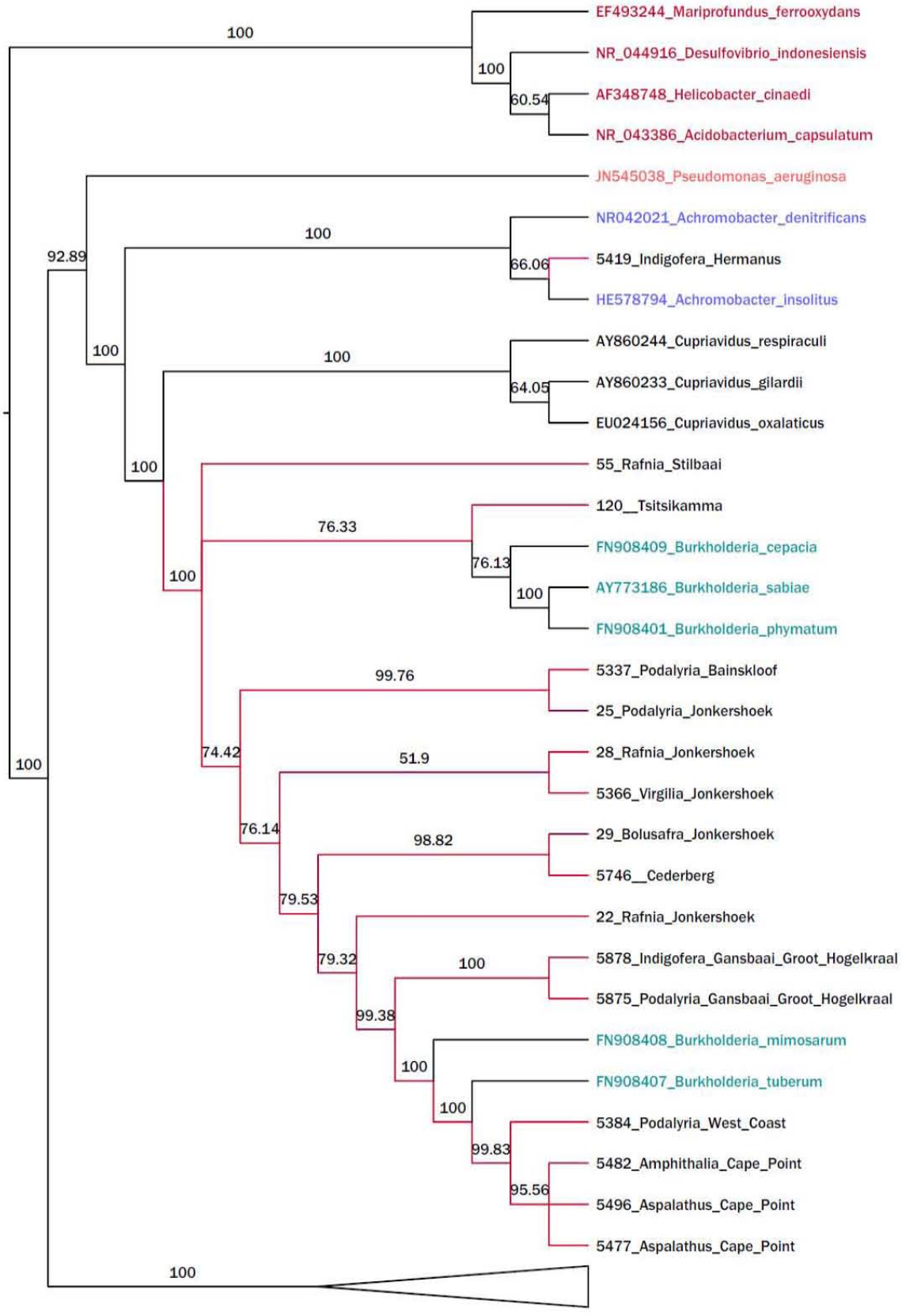

Figure 4. Bayesian 50% majority rule consensus tree showing the four main clades of the members of the betaproteobacteria based on CFR isolates sequences and reference strains from the GenBank.

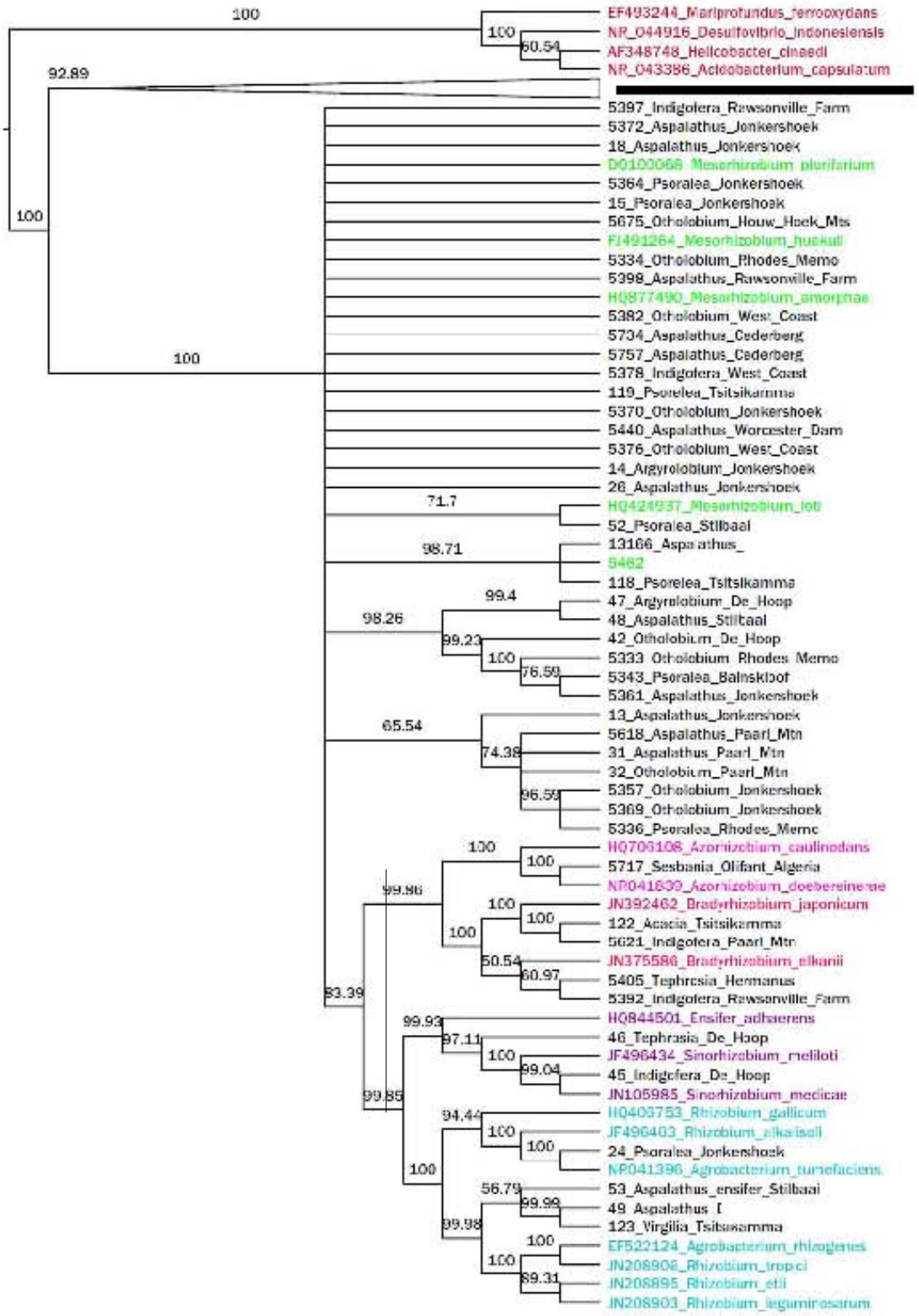

Figure 5. Bayesian 50% majority rule consensus tree showing the six main clades of the members of the alphaproteobacteria based on new CFR sequences and reference strains from the GenBank.

At the foot of the phylogenetic tree (Figure 5), *Rhizobium* or *Agrobacterium* (pp=100%) lineages were associated with four different host species (*Psoralea, Aspalathus and Virgilia*) found in different geographical areas namely Jonkershoek, Stibaai, De Hoop and Tsitsikama (Table 1).

A summary of the phylogenetic groupings of the isolates and their grow rate distribution is shown in Table 6. Both genera of the *Burkholderia* and *Achromobacter* lacked isolates with slow growth rate. Isolates associated with *Mesorhizobium* were distributed to all the three growth rate categories including fast, intermediate and slow. However, isolates associating with *Rhizobium*/*Agrobacterium* were either fast or intermediate whereas those associated with *Bradyrhizobium* were either slow or intermediate growth rate. All the isolates belonging to *Sinorhizobium*, *Azorhizobium* and *Mycobacterium* showed intermediate growth rates (Table 6).

Table 6. Distribution of the growth rates of the rhizobial isolates on different phylogenetic groupings

Genus association	Total no. of isolates	Number of isolates with growth rates that are:		
		fast	intermediate	Slow
Burkholderia	15	11	4	0
Achromobacter	1	1	0	0
Rhizobium/Agrobacterium	4	2	2	0
Sinorhizobium	2	0	2	0
Azorhizobium	1	0	1	0
Mycobaterium	1	0	1	0
Bradyrhizobium	5	0	3	2
Mesorhizobium	37	10	17	10

The results in Table 8 show a summary of the phylogenetic groupings of the isolates based on the tribe and genus of the host species. The *Podalyrieae tribe* were almost exclusively nodulated by *Burkholderia* with the exception of *V. divaricata* where *Rhizobium* was isolated (Table 8). Burkhoderia isolates were also obtained from species of *Rafnia*, *Aspalathus*, *Indigofera*, *Crotalaria* and *Bolusafra* (Table 8). On the other hand, members of Psoraleeae tribe were also almost exclusively nodulated by the *Mesorhizobium* with the exception of two species of *Psoralea* where *Rhizobium/Agrobacterium* and *Mycobaterium* were isolated (Table

8). Isolates from *Aspalathus* species were predominantly associated with *Mesorhizobium* that also infected some species of *Indigofera* and *Argyrolobium.* Species of *Indigofera* were also infected by *Bradyrhizobium* and *Sinorhizobium* among the α-rhizobia group. Species of *Tephrosia* and *Acacia* was also infected with *Bradyrhizobium* whereas that of *Sesbania* was infected by *Azorhizobium* (Table 8).

3.4. Authentication of rhizobia

Out of the 17 legume test species 16 were successfully nodulated by the rhizobial isolated Out of the 16 test species that nodulated 14 were the original hosts while *A. crenata* and *L. frutescens* (Table 7) was nodulation of the close relatives. All the uninoculated controls did not (Table 7). The seedlings that formed nodules grew much better than the uninoculated controls (Figures 6a - 6d). However, inoculating Aspalathus sp (AU11) with its rhizobial isolate number 48 (*Mesorhizobium*: Figure 4; Table 1) did not induce nodulation for a reason not known yet.

3.5. Distribution of the rhizobial isolates according to host ecology

Rhizobia isolates form legume nodules were widely distributed on various soil types of the CFR with *Mesorhizobium* and *Burkholderia* occurring on six out of the eight soil types collected and both absent in coastal sand and river sand. The highest occurrence of *Burkholderia* per soil type were isolated from alluvium (pH range 3.2-4.9; Table 1; Figure 7) and acid sand (pH range 3.7 – 4.6; Table 1; Figure 7) together constituting 10 of the total 15 *Burkholderia* isolates (Table 9). *Bradyrhizobia* were present in sandstone, granite, alluvium and river sand. *Rhizobium* or *Agrobacterium* were present in limestone, alluvium and river sand while *Sinorhizobium* were only present in limestone and coastal sand. *Mycobacterium* and *Achromobacter* were both isolated from sandstone and *Azorhizobium* from river sand (Table 9). Sandstone harboured the most diverse rhizobia associated with five distinct rhizobia genera (Table 9) followed by limestone and alluvium with four rhizobia genera. Only one rhizobia genus was obtained from coastal sand (Table 9).

Figure 6a. Seedling growth of *B. bituminosa* (C), inoculated (+) with their corresponding rhizobia isolate and uninoculated control (-). Figure A shows the nodules on the roots of the inoculated seedlings and B shows the absence of nodules on the uninoculated control.

Figure 6b. Seedling growth of *V. oroboides* (C), inoculated (+) with their corresponding rhizobia isolate and uninoculated control (-). Figure A shows the nodules on the roots of the inoculated seedlings and B shows the absence of nodules on the uninoculated control.

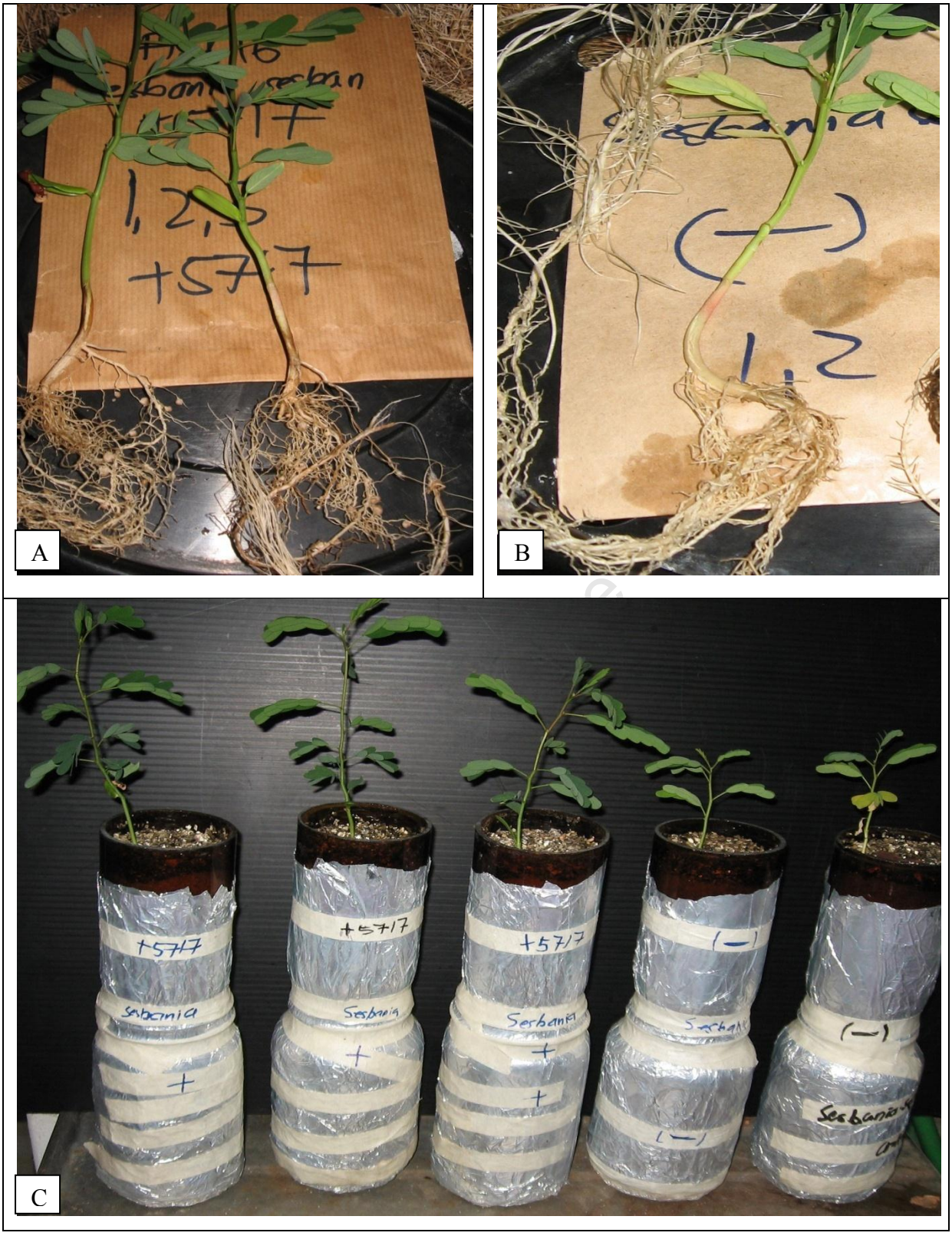

Figure 6c. Seedling growth of *S. punicea* (C), inoculated (+) with their corresponding rhizobia isolate and uninoculated control (-). Figure A shows the nodules on the roots of the inoculated seedlings and B shows the absence of nodules on the uninoculated control.

Figure 6d. Seedling growth of *P. calyptrata* (C), inoculated (+) with their corresponding rhizobia isolate and uninoculated control (-). Figure A shows the nodules on the roots of the inoculated seedlings and B shows the absence of nodules on the uninoculated control.

Table 7. **Nodulation status of legumes species and rhizobia isolates that were assessed for** authentication. All species except A. crenata and L. frutescens were inoculated by isolates from original host. Symbols: "-" = no nodulation and "+" = nodulation. NA = Not assessed because the seedling died.

Tribe	Species	Collector	Athentication no.	Innoculum & Voucher ID.		Innoculated			Control	
						1	2	3	1	2
Crotalarieae	*Crotararia sp.*	Oscar	AU05	*Burkholderia*	120	+	+	+	-	-
Crotalarieae	*Aspalathus sp.*	Muthama	AU10	*Mesorhizobium*	26	+	+	**NA**	-	-
Crotalarieae	*Aspalathus sp.*	Muthama	AU11	*Mesorhizobium*	48	-	-	-	**NA**	-
Crotalarieae	*Aspalathus callosa* L.	Silverhill seeds	AU12	*Burkholderia*	5477	+	+	+	-	-
Crotalarieae	*Aspalathus crenata* L.	Silverhill seeds	AU13	*Mesorhizobia*	13166	+	+	+	-	-
Crotalarieae	*Rafnia sp*.	Oscar	AU15	*Burkholderia*	28	+	+	+	-	-
Galegeae	*Lessertia frutescens* L.	Silverhill seeds	AU04	*Sinorhizobium*	46	+	+	+	-	-
Genisteae	*Argyrolobium sp.*	Oscar	AU08	*Mesorhizobium*	14	+	+	**NA**	**NA**	**NA**
Indigofereae	Indigofera sp.	Oscar	AU14	*Burkholderia*	5878	+	+	+	-	-
Millettieae	*Tephrosia capensis* (Jacq.) Pers.	Oscar	AU02	*Bradyrhizobium*	5405	+	+	**NA**	-	-
Phaseoleae	*Bolusafra bituminosa* (L.) Kuntze	Muthama	AU17	*Burkholderia*	29	+	+	+	-	-
Podalyrieae	*Virgilia divaricata* Adamson	Muthama	AU06	*Rhizobium*	23	+	+	+	-	-
Podalyrieae	*Virgilia oroboides* (Berg.) Salter	Silverhill seeds	AU07	*Burkholderia*	5366	+	+	**NA**	-	-
Podalyrieae	*Podalyria calyptrata* (Retz) Willd.	Muthama	AU09	*Burkholderia*	5337	+	+	+	-	-
Psoraleeae	*Psoralea pinnata* L.	Muthama	AU01	*Mesorhizobium*	5336	+	+	**NA**	-	-
Psoraleeae	*Otholobium hirtum* (L.) C.H. Stirt.	Silverhill seeds	AU03	*Mesorhizobium*	32	+	+	**NA**	-	-
Sesbanieae	*Sesbania punicea* (cav.) Benth.	Muthama	AU16	*Azorhizobium*	5717	+	+	+	-	-

Table 8. The number of rhizobial isolates per their phylogenetic grouping according to host of isolation.

		β-rhizobia			α-rhizobia				
Tribe	Genus	Burkholderia	Achromo-bacter	Meso-rhizobium	Brady-rhizobium	Rhizobium/ Agrobacterium	Sino-rhizobium	Azo-rhizobium	Myco-bacterium
Indigofereae	*Indigofera*	2	1	2	3	-	1	-	-
Crotalarieae	*Aspalathus*	2	-	15	-	2	-	-	-
	Crotalaria	1	-	-	-	-	-	-	-
	Rafnia	3	-	-	-	-	-	-	-
Podalyrieae	*Amphithalea*	1	-	-	-	-	-	-	-
	Podalyria	4	-	-	-	-	-	-	-
	Virgilia	1	-	-	-	1	-	-	-
Phaseoleae	*Bolusafra*	1	-	-	-	-	-	-	-
Psoraleeae	*Otholobium*	-	-	10	-	-	-	-	-
	Psoralea	-	-	8	-	1	-	-	1
Genisteae	*Argyrolobium*	-	-	2	-	-	-	-	-
Millettieae	*Tephrosia*	-	-	-	1	-	-	-	-
Acacieae	*Acacia*	-	-	-	1	-	-	-	-
Galegeae	*Lessertia*	-	-	-	-	-	1	-	-
Sesbanieae	*Sesbania*	-	-	-	-	-	-	1	-
	Total 67	**15 (21%)**	**1(2%)**	**37 (58%)**	**5 (6%)**	**4 (4%)**	**2 (3%)**	**1(2%)**	**1(2%)**

Other isolates: *Bacillus*: (*Aspalathus* 3; *Indigofera* 1; *Rafnia* 1; *Rhynchosia* 1), *Brevi-bacilus:* (*Aspalathus* 1), *Serratia*: (*Psoralea*)

Table 9. The number of rhizobial isolates per their phylogenetic grouping according to host legume soil type.

Soil type	β-rhizobia		α-rhizobia					
	Burkhol-deria	Achromo-bacter	Meso-rhizobium	Brady-rhizobium	Rhizobium/ Agrobacterium	Sino-rhizobium	Azo-rhizobium	Myco-bacterium
Sandstone	2	1	5	2	-	-	-	1
Granite	1	-	7	1	-	-	-	-
Shale	1	-	5	-	-	-	-	-
Limestone	1	-	4	-	2	1	-	-
Alluvium	5	-	14	1	1	-	-	-
Coastal sand	-	-	-	-	-	1	-	-
Acid sand	5	-	2	-	-	-	-	-
River sand	-	-	-	1	1	-	1	-
Total	15	1	37	5	4	2	1	1

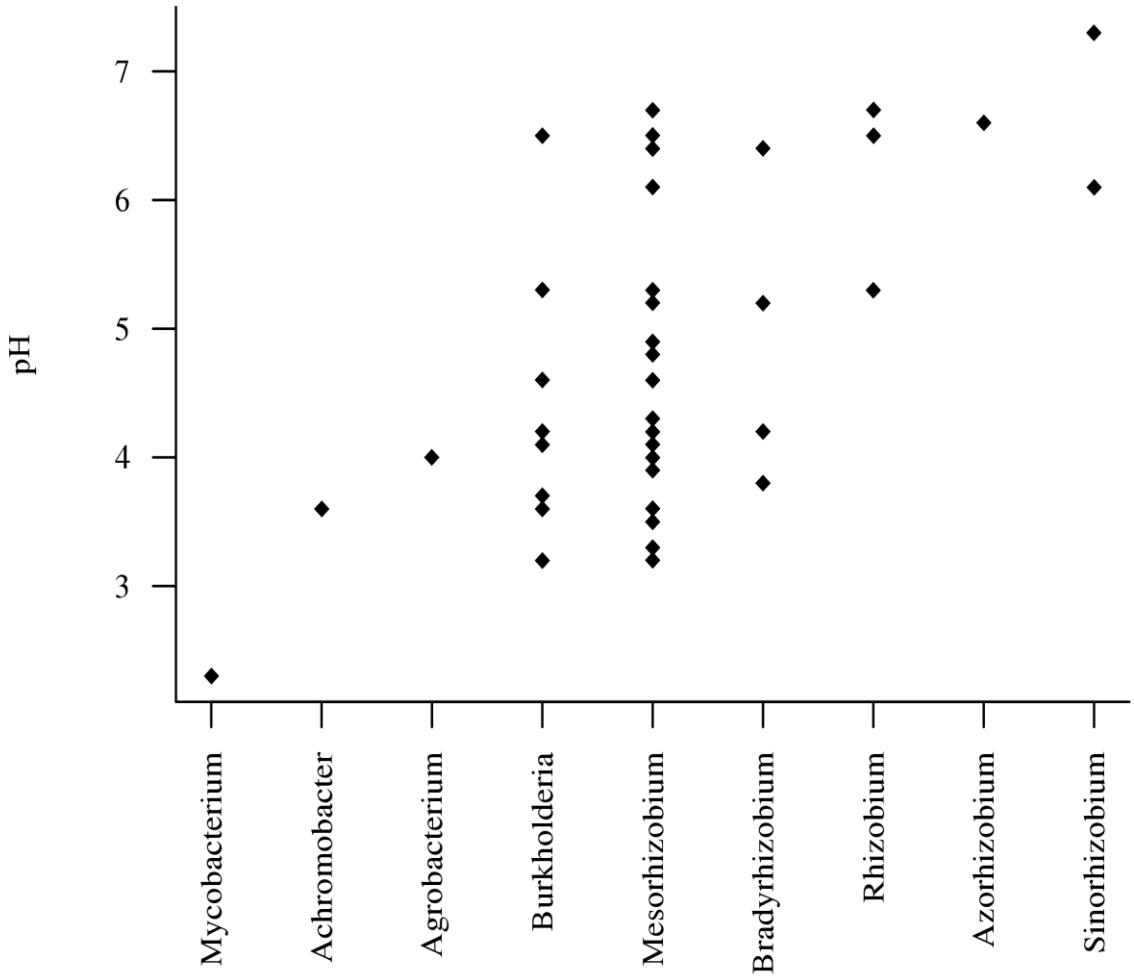

Figure 7. The distribution of CFR rhizobia isolates according to soil pH.

4.0. Discussion

4.1. Phylogenetic characterization of the CFR rhizobia isolates

Phylogenetic characterization of rhizobial isolates revealed very high genetic diversity among symbionts associated with CFR legumes belonging to seven distinct genera in both alpha and beta classes of Proteobacteria. Betaproteobacteria class consisted of two genera namely *Burkholderia* and *Achromobacter*, while Alphaproteobacteria comprised of the five main rhizobia genera: *Azorhizobium, Agrobacterium/Rhizobium, Bradyrhizobium, Mesorhizobium* and *Sinorhizobium* (Marsudi et al., 1999). Such a finding was not surprising as earlier studies that focussed at CFR rhizobia at particular host legumes genera have reported these genera individually as legume symbionts (Hassen et al. 2011; Phalane et al. 2008; Elliott et al. 2007; Kock 2004; Le Roux 2003). There were no isolates that formed membership with the *Cupriavidus* lineage suggesting that CFR plant species have low affinity for *Cupriavidus* or lack of these rhizobia in the CFR soil. The genus *Cupriavidus* has recently been reported to be the common symbiont of *Mimosa diplotricha* in subtropical southern China (Liu et al., 2012). However, Bontemps et al. (2010) in an effort to show that *Burkholderia* were old symboints of the *Mimosa* species in the Cerrado and Caantiga biomes of Brazil, also reported no isolates associated with *Cupriavidus* supporting the view that the genus may either be very specialised or localized in Southern China. The isolation of Mycobaterium from a nodule is very interesting because it belongs to the phylum Actinobacteria, Gram-positive soil bacteria. It was also not possible in this study to auntheticate the *Mycobacterium* isolate due to lack of seeds of the host species, isolate may be just a plant endophyte rather than a nitrogen-fixing symbiont. Similarly, unless the Achromobacter isolate can be authenticated, it could also be considered a plant endophyte because it is not recognized as a legume symbiont and nitrogen fixer.

The findings in this study revealed that there were some degrees of legume and rhizobia specificity at the generic and even tribal level in that some legumes had exclusivity to particular symbionts. Selective preference was earlier reported by Odee et al. (1997) who indicated that herbaceous legumes used in their study as trap hosts showed preference to nodulate with particular rhizobia. For example, they pointed out that *Phaseolus vulgaris* nodulated with the only homogeneous *Rhizobium* group and *Macroptilium atropurpureum* and *Vigna unguiculata* nodulated mainly with *Bradyrhizobium*. In this study, *Burkholderia* lineages seemed to be the most preferred symbiont of the Podalyrieae and in some genera of Crotalarieae including *Rafnia* and *Aspalathus*. Nodulation of *Aspalathus* by the members of

Burkholderia corroborates the earlier novel finding by Moulin et al. (2001) who reported *Aspalathus carnosa* as the first species which was known to be nodulated by members of the betaproteobacteria. It was also identified (Figure 4) that *Aspalathus callosa* (isolate 5477) together with *A. carnosa* (isolate 5496) were hosts to *Burkholderia* which formed a clade with *Burkholderia turberum* (pp=100 %). The finding supported earlier results associated with the two species and *Aspalathus linearis* with *Burkholderia* symbiosis (Hassen et al. 2011; Elliott et al. 2007; Moulin et al. 2001). Nodulation of the Podalyrieae genus *Cyclopia* by *Burkholderia* was earlier reported by Elliott et al. (2007) and is consistent with our findings that the Podalyrieae were almost exclusively nodulated by *Burkholderia*. Interestingly these rhizobia did not form a clade with *Burkholderia tuberum* suggesting that members of *Burkholderia* associated with legumes in this study could be different.

Considering that Podalyrieae and Crotalarieae are among the oldest Cape floral clades with stem node age between 44−46 mya (Edwards and Hawkins, 2007) and their association with *Burkholderi,* Podalyrieae and basal lineages of Crotalarieae, it could be hypothesized that *Burkholderia* were the ancient symbionts of the legumes in the CFR. Other studies have reported *Burkholderia* to be very old symbionts of specific legume hosts in other regions (Chen et al., 2003; Bontemps et al., 2010). In addition, South Africa being one of the *Burkholderia* centres of diversity (Mishra et al., 2012), it is conceivable that *Burkholderia* is possibly the oldest symbiont of legumes in the CFR.

Among the Alpha rhizobia, *Mesorhizobium* lineages are the main preferred symbionts of the Psoraleeae and for most members of the Crotalarieae (Figure 5). However, *Mesorhizobium* formed a polytomy on the tree, suggesting that our data was insufficient to produce a well resolved phylogeny. It also suggest that members of the *Mesorhizobium* were very closely related to each other, such that variations in 16S rRNA sequences within species was as much as variations between species (Li et al., 2009). On the other hand, there is a possibility that the *Mesorhizobium* lineage could be diverse because there were three clades within the polytomy (Figure 5). The existence of *Mesorhizobium* in the region was not news as previous studies had earlier reported it (Phalane et al., 2008; Hassen et al., 2011). However, the report that *Mesorhizobia* is the most common and widely distributed rhizobia among the alpha bacteria in the soils of the CFR is novel. In literature, consistent with our results, was the finding of Bala et al. (2002) where members of the *Mesorhizobium* group accounted for 92 %

of all isolates covering five different soils from Kenya, Malawi and Zambia. The reason for the complete dominance of *Mesorhizobium* in the study by Bala et al. (2002) was unclear, but attributed it to better adaptation to the soils or of their homologous hosts. In addition, Han et al. (2008) also reported the *Rhizobium* and *Mesorhizobia* to be the predominant species of rhizobia associated with wild legumes native to Xinjiang, China which they also attributed to the rhizobial and host species adaptation to the local environment. Contrary to the findings of this study, other studies from areas of similar nutrient poor soils such as Western Australia reported rather *Bradyrhizobium* to be the predominant symbionts (Stępkowski et al., 2012).

However, some of the results of this study revealed that there was both host and rhizobia promiscuity. Rhizobia promiscuity can be defined as the ability of a rhizobium lineage to posses diverse strains that can selectively nodulate host species of various taxa regardless of endemism(De Meyer et al., 2011). Promiscuity has been reported in literature to be the main explanation to why many legumes grow successful in many soils (Bala, 2003a). In addition, it also allows the conducive hosts to spread into new habitats (De Meyer et al., 2011). On the other hand host promiscuity could be referred to as the ability of the legume host to nodulate with many different rhizobia types (Bala and Giller, 2006). The two genera *Indigofera* and *Aspalathus* were the most diversely nodulated (Table 8) by different rhizobia genera and are among the largest genera in the CFR (Table 3). Our results were in agreement with the hypothesis that the success of many legume hosts in terms of species numbers and their wide distribution in nature is a result of their relative permissiveness to nodulate with diverse rhizobia (De Meyer et al. 2011; Bala and Giller 2006; Wolde-meskel et al. 2004; Bala et al. 2003b).

4.2. Distribution of rhizobial isolates according to ecology

The CFR rhizobial isolates phylogenetic groupings were associated with legume hosts which occurred on eight different soil types (Table 9) with a very wide distribution indicating that rhizobia distribution was not a limiting factor in most soils of the CFR. The host distribution was rather the main factor that seems to influence rhizobia distribution. *Burkholderia* and *Mesorhizobia* lineages were present in all soil types with an exception of coastal sand and river sand. The distribution of the symbionts based on host legume distribution as opposed to soil type was not a new phenomenon because Bala et al. (2003a) focusing on the distribution and diversity of rhizobia associated with agroforestry legumes in soils from three continents in the tropics had a similar result. Also common to both *Burkholderia* and *Mesorhizobia*

lineages was that these two were the most widely distributed rhizobia symbionts of the CFR, and both were similarly rare in coastal sand and river sand (Table 9) indication that, to some extent, some rhizobia were exclusive in particular habitats. The effect of habitat conditions were reported by Odee et al. (1997) who showed that predominant rhizobia varied from one locality to the other. Five out of eight soil types were associated with at least three rhizobial lineages indicating diversity of symbionts in CFR soil. Sandstone isolates were the most diverse harbouring five rhizobial lineages and this was attributed to the fact that sandstone is the most widely distributed substrate in the CFR. Generally there is no clear distinction in the role played by various soil types in influencing rhizobia distribution and diversity in the CFR. And this result was in agreement with Bala et al. (2003b) who also found no differences in rhizobial diversity between different soil types tested in their study.

The highest numbers of rhizobial isolates per their phylogenetic groupings per given soil types among the two predominant groups (*Burkholderia* and *Mesorhizobia*) was associated with their adaptation to soils of a wide pH range (pH 3.8–5.4 for *Burkholderia* and 3.2–6.8 for *Mesorhizobia*). *Mesorhizobium* lineages in sandstone, granite and alluvium had the highest numbers suggesting that the predominant CFR symbionts were adapted to the acidic CFR soils. In the case of *Burkholderia* lineages, 11/15 rhizobial isolates were isolated from very acidic soils of pH 3.2–4.8. However, *Sinorhizobium* lineages were associated with soils of pH close to neutral values (pH 6.7–7.3) and were rare in relatively high acidic soils. The occurrence of *Sinorhizobium* associated with relatively high pH was consistent with literature that have reported the predominance of *Sinorhizobium* with decreasing soil acidity (Bala and Giller 2006). Other studies have reported soil pH to be a very important factor affecting rhizobia distribution and diversity (Bontemps et al. 2010; Bala and Giller 2006; Bala et al. 2003a; Woomer et al. 1988). Association of *Burkholderia* occurrence and diversity with acidic soils is widely known in literature (Sprent 2012) and current findings colloborate the association of *Burkholderia* with mostly acidic soils. On the other hand *Burkholderia* has also been reported to occur in alkaline soils of pH 8–9 (Mishra et al. 2012; Sprent 2012; Talbi et al. 2010; Chen et al. 2003). Similarly, one of the *Burkholderia* isolates was isolated from almost neutral pH soil (isolate 55; pH 6.5) which belonged to the clade that formed membership with *Burkholderia phymatum* (pp=100 %) and was of intermediate growth rate. The isolation of the *Burkholderia* isolate was attributed to adaptation of the host (*Rafnia triflora)* which was profusely nodulated on limestone soils of Stilbaai in Agulhas Plain. The adaptability of the host to the various soil types of the region and environmental conditions

was reported to be correlated with that of their compatible rhizobia (Marsudi et al., 1999; Garau et al., 2005; Stępkowski et al., 2012). In another study, Talbi et al. (2010) also reported *Burkholderia phymatum* isolated from a crop legume growing on alkaline soils (pH 8.1) in Morocco which was inconsistent with the general view that *Burkholderia* were particularly adapted to acid infertile soils. Therefore, *Burkholderia* seem to be widespread in nature and maybe adapted to various environmental factors including varying pH. Although *Burkholderia* have been frequently isolated from acidic soils, there is now evidence to show that they are not restricted to only acidic conditions (Mishra et al. 2012; Sprent 2012; Talbi et al. 2010; Chen et al. 2003).

4.3. Morphological traits of CFR rhizobia isolates

The growth rates, colony shape and types observed in this study (Table 4) were consistent with those in literature (Odee et al., 1997; Bala et al., 2004). The results showed that there were generally no restrictions on the distribution of rhizobial isolates on various soils of the CFR based on their growth rates (Table 5). However, there were some relationship between the growth rates and rhizobial genera (Table 6). Generally, rhizobia of fast growth rate were traditionally associated with the genus *Rhizobium* (Odee et al., 1997; Graham, 2008) and *Burkholderia* (Moulin et al., 2001; Elliott et al., 2007), whereas isolates of intermediate growth type were associated with *Mesorhizobium* (Young and Haukka, 1996), and *Bradyrhizobium* with slow growth rate (Boone et al., 1999). In addition, fast growers were reported to be associated with acidic soils, while slow growers are associated with alkaline conditions (Graham 2008; Bala and Giller 2006; Menna et al. 2006; Hungria et al. 2001). The reports on *Burkholderia* are consistent with the findings of this study where the isolates were predominantly fast growers, and *Rhizobium* also showed fast to intermediate growth rates (Table 6). Marsudi et al. (1999) reported that *Burkholderia* lineages were mostly fast growing a phenomena consistent with most *Burkholderia* isolates in this study and other reports in literature (Liu et al., 2007; Garau et al., 2009; Bontemps et al., 2010). *Bradyrhizobium* isolates also showed a trend of slow growth as reported in literature (Boone et al. 1999) although some recorded intermediate growth stages (Table 6). On the contrary, other studies have shown that *Bradyrhizobium* is not always a slow grower (Stępkowski et al., 2012) as it can differentially adapt to different environmental conditions. In addition, the reports of *Mesorhizobium* being traditionally associated with intermediate growth rate (Young and Haukka, 1996) was not consistent with the results of this study because *Mesorhizobium* isolates were very diverse belonging to all three growth types regardless of soil type and pH

(Table 6 and Figure 6). *Mesorhizobia* that had a fast growth type were isolated from Psoraleae (*Psoralea* or *Otholobium)* while the slow growers were isolated from *Aspalathus* (Table 1). *Aspalathus* species in general have in the past been reported to be associated with *Bradyrhizobium* based solely on slow growth rate on growth media in vitro (Boone et al., 1999), which was falsified by the findings of Hassen et al. (2011) on the characterization of rhizobia isolates from *Aspalathus linearis* using molecular techniques. They reported rather that *A. linearis* was associated with diverse rhizobia but predominated by *Mesorhizobium* (53 %) and *Bradyrhizobium* was the least at 2 %. Their finding is in agreement with the results of this study that *Mesorhizobium* were the predominant symboints of *Aspalathus* species (Table 8) and the isolates were in all the growth rate categories (Table 6).

Sinorhizobium was of intermediate growth type which was in agreement with the results of Bala et al. (2004). On the contrary, de Lajudie et al. (1994) reported fast growth rates in their description of the *Sinorhizobium* species. In other studies, *Sinorhizobium* were reported to belong to either fast or slow depending on soil pH adaptation of its compatible host (Bala and Giller 2006;Garau et al. 2005). Therefore, the host adaptability to soils of varying pH and other edaphic factors could explain the variation of the growth rates of the rhizobia. In another example, *Achromobacter* was reported for the first by Benata et al. (2008) as a symbiont of *Prosopis juliflora*, a host adapted to saline soils. In contrast, *Achromobacter* isolated from *Indigofera* species in this study was obtained from very acidic soils (Table 1; pH 3.6). The isolation of *Achromobacter* from acidic soil could be attributed to adaptation of the host legume (*Indigofera* species) to the acidic conditions.

4.4. Conclusion

In this study, the endosymbionts present in the nodules of CFR legumes revealed a large diversity of rhizobia belonging to both Alpha and Beta proteobacteria. The most predominant rhizobia among the CFR isolates were identified as *Mesorhizobia*. In addition, the *Mesorhizobia* strains predominated as symbionts nodulating the Psoraleeae and Crotalarieae (*Aspalathus*). The isolates identified as *Burkholderia* were the second most abundant rhizobial group and almost exclusively nodulated the Podalyrieae and Crotalarieae. In terms of rhizobia ecology the results of this study showed that generally soil type did not limit the distribution of rhizobia in the CFR. Rather the CFR rhizobia isolates showed to be widespread occurring in almost all soil types with a wide pH range.

The increase in the number of studies focused on rhizobia biogeography has changed traditional views by providing evidence that show that there are many dynamics that determine host infection and growth behaviour of rhizobia (Stępkowski et al. 2012; Bala and Giller 2006; Garau et al. 2005; Bala et al. 2003b). The ability of hosts to adapt to different environmental conditions has been reported to be correlated with adaptations of their compatible rhizobia symbionts, a concept that others have termed differential adaptation (Garau et al., 2005).

CHAPTER 3

Do Cape legumes nodulate in soils where they do not naturally grow?

3.1. Introduction

The distribution patterns of plants all over the planet has been one of the most intriguing research topics of much scientific research the world over in an effort to understand factors influencing plant growth and distribution. Uncovering factors limiting plant growth and distribution is key to plant scientists in their effort to understand species occurrence, productivity, biodiversity and their conservation (Salisbury, 1926; Küper et al., 2006). It is suggested that no one factor could be singled out to independently influence distribution patterns of plants rather interaction of factors ranging from climatic, edaphic, topographic and biotic (Salisbury, 1926; Billings, 1952; Grace, 1987; William and Pilmanis, 1998; Essl et al., 2009; Reed et al., 2009).

Soil can be defined as the substratum upon which the plants grow and from which they derive their mineral nutrients and most of their water supply (Rajakaruna, 2004). Cain (1944) ranked edaphic factors second to climatic factors as the major environmental determinants of plant distribution. Plant environment being the summation of all external forces and matter affecting the growth, structure, and reproduction of that plant (Billings, 1952). The variation in physical, chemical and biological properties of soil may promote growth of one plant while at the same time suppressing the growth of the other resulting in variations in the distribution of plants (Billings, 1952; William and Pilmanis, 1998; Rajakaruna, 2004). Some studies have reported that variation in edaphic factors resulted in variations in plant distribution, abundance and diversity (Toledo et al. 2012; Gregoire 2010; Arshad et al. 2008; Rajakaruna 2004; D. B. Clark et al. 1998). Arshad et al. (2008) particularly reported that salinity, organic matter, and ionic concentration (Na, P, and K) seemed to have been the ecological characteristics responsible for plant distribution in Cholistan desert.

In the CFR, rainfall varies distinctly according to topography. In the lowlands it ranges between 300-500 mm, but over 1000 mm in the mountains because of the persistence of clouds and fog and snow that falls in winter (Linder 2003; Goldblatt and Manning 2000; Bond and Goldblatt 1984; Goldblatt 1978). The three main vegetation types of the CFR fynbos were noted for their topographic distribution comprising of Mountain fynbos, False fynbos and Coastal fynbos (Bond and Goldblatt 1984). Although, the fynbos vegetation is characterized by the presence of species of the families Ericaceae, Restionaceae and Proteaceae, Fabaceae is the second largest family to Asteraceae in the CFR (Goldblatt and Manning 2000). One characteristic of the CFR legumes is often their appearance after

disturbance on tributaries, roadsides and after fire (Power et al. 2010; Linder 2003; Van der Bank et al. 1999). Unique about the CFR legumes was their occurrence in distinct patches intermixed with other vegetation types and habitats ranging from water seeps, river valleys, and mountain slopes regardless of topography. The distribution pattern of the legumes in the CFR is such that some species form distinct populations restricted to one locality while others are widespread. It is however not understood why CFR legumes occur in patches and there has not been studies to explore the role of symbiotic rhizobia to the unique pattern. Several studies have reported the diversity and abundance of rhizobia symbionts in the CFR (Phalane et al., 2008; Hassen et al., 2011; Rodríguez-Echeverría et al., 2011; Gerding et al., 2012), but the impact of these bacteria on the ecosystem processes and legume distribution is poorly understood if known at all.

Since Larfay and Buddon (1998) report that there were fewer studies on the ecology of rhizobia legume symbionts and their geographic distribution in relation to their host, several studies have focused on the subject. Some studies have reported edaphic factors particularly pH to be central to the rhizobial ecological adaptations (Odee et al., 1997; Bala et al., 2002, 2004; Bala, 2003a; Benata et al., 2008; Bontemps et al., 2010). Other studies have also focused on the distribution of rhizobia in various soil types and phylogenetic groupings were found to be independent of the site of isolation (Bala and Giller 2006; Bala et al. 2003b). In those studies, different rhizobia groups formed symbiosis with each host across the soils from distinctly separated geographical regions. However, Bontemps et al. (2010) attributed the governance of *Burkholderia* niches to physical factors rather than plant species. In addition, the results from the study by Bala et al. (2003b) suggested that legumes exhibit better nodulation ability in soils from its centre of diversity than to soils it was introduced. In that case, the presence of rhizobia in a given locality presupposes favourable conditions for the growth and establishment of a legume for that species and the opposite may also be true for its absence. Therefore, for a legume to form nodules and fix nitrogen, its compatible symbiont should be present in soil. Thus, the ability of a legume to form nodules and fix nitrogen in symbiosis with rhizobia could be a function of the extent and distribution of its compatible rhizobia. The distribution of CFR legumes is such that some species are restricted to certain areas while others are widespread all over the region. However there has not been a study to investigate the likely effect of rhizobia symbionts on the variations in the CFR legume distribution. The objective of this chapter was to assess the absence or presence of rhizobia in soils influences the distribution of legumes in the CFR. It was hypothesized that

the legume plant distribution in the CFR is constrained by the presence of their compatible rhizobia.

3.2. Materials and Methods

Plant growth, inoculation and harvesting

A total of 19 legume species (Table 3) were selected according to their distribution in the CFR (Goldblatt and Manning 2000) and also to cover phylogenetic diversity of the CFR legumes. Five main soil types of the CFR identified as shale, sandstone, granite, limestone and coastal sand collected from the field within legume stands were used as rhizobia inoculants of the 19 legume species. Legume seeds were obtained from Silverhill Seed Company (Kenilworth, Cape Town, South Africa) and Kirstenbosch Gardens Seeds Unit (South African National Biodiversity Institute (SANBI),, Kirstenbosch). Scarification of seed was done in concentrated sulphuric acid for 15 minutes (Moulin et al., 2001; Benata et al., 2008; Estrella et al., 2009) and seeds were thereafter systematically rinsed in at least six changes of sterile distilled water and soaked overnight in sterile distilled water containing smoke extract (CAPE Seed Primer, SANBI Kirstenbosch)(Power et al., 2010). Seeds were germinated in vermiculite and watered with N-free solution (Vincent 1970). Seedlings were transplanted to 18 cm diameter pots filled with about 3kg of acid washed silica sand. The procedure of transplanting was such that the seedling was positioned centrally and 100 g of soil inoculum was poured around the roots before it was covered with the sand and then watered to field capacity. Each soil type was used to inoculate the 19 legume species and there were two replicate pots for each species, and uninoculated plants for each species were used as controls. Modified N-free solution originally formulated at the University of Western Australia (UWA) containing (μM): $Ca(NO_3)_2$, 400; K_2SO_4, 200; $MgSO_4$, 54; $MnSO_4$, 0.24; $ZnSO_4$, 0.10; $CuSO_4$, 0.02; H_3BO_3, 2.4; $NaMoO_4$, 0.03; Fe-EDTA was supplied to the plants at 50 to 70 percent field capacity twice a week for two weeks. After the two week period, $Ca(NO3)_2$ was replaced by $CaSO_4$ in the nutrient solution to encourage nodulation and N-fixation. The plants were allowed to grow for up to 60 days in the glasshouse in a completely randomised design.

The plants were harvested for nodule collection after 60 days. Soil around the roots was carefully removed with water. Roots were checked for nodules and the presence and absence of the nodules was recorded. Selected nodules were checked for active N-fixation by checking the presence of leghemoglobin in sliced nodules (dos Reis et al. 2010). The shoot

and roots were packaged separately, dried and stored as voucher specimens. Nodules were collected, stored in vials for rhizobia isolation following the procedure as described in Chapter 2.

Genomic DNA Extraction of cultivated legume species

Out of the 19 glasshouse legume species, eight species were selected for rhizobia isolation and DNA extraction based on their distribution in the CFR and also according to taxa they represented in the field study. The selection of only eight species was due to budgetary constraints. A total of 24 rhizobial isolates were obtained from the selected species as indicated in (Table 1). DNA was extracted and amplified using the standard protocol described in Chapter 2 and sequencing was performed at the Stellenbosch University sequencing unit. The phylogenetic tree re-construction was done using the standard protocol described in Chapter 2.

Table 3.1. Selected legume species from the glasshouse (GH) study for rhizobia isolation, their geographical distribution, soil type and area of collection and voucher number. n/a = nodules not selected

Tribe	Species	Distribution	Soil type and Origin				
			Sandstone (Silvermine)	Granite (Jonkershoek)	Shale (Rhodes Memorial)	Limestone (De Hoop)	Coastal Sand (Koppie Alleen)
Acacieae	*Acacia karoo*	NW, SW, KM, LB, SE	GH175	GH41	GH40	GH173	n/a
Crotalarieae	*Lebeckia sericea*	NW, SW, KM, SE	GH181	GH96	GH95	GH179	n/a
Psoraleeae	*Otholobium striatum*	NW, SW, KM	n/a	n/a	n/a	GH188	GH190
Podalyrieae	*Virgilia oroboides*	SW, LB, SE	GH166	GH93	GH92	n/a	GH165
Crotalarieae	*Aspalathus linearis*	NW, SW	GH157	n/a	(GH38)	n/a	n/a
Podalyrieae	*Podalyria calyptrata*	SW	n/a	GH76	GH75	GH167	GH168
Psoraleeae	*Psoralea pinnata*	SW	n/a	GH61	GH60	n/a	n/a
Millettieae	*Tephrosia grandiflora*	SE	GH178	GH70	n/a	n/a	n/a

3.3. Results

Distribution of rhizobia on various soil types of the CFR

A total of 19 legume species growing either as widely distributed or localized in the CFR were inoculated with five different soil types of the CFR in a glasshouse experiment. Majority of legume species that were widely distributed in the CFR were observed to nodulate with different types of soil of different pH (Table 3.1). For instance, seedlings of *Acacia karoo*, *Lebeckia sericea*, *Hypocalyptus sophoroides* and *Podalyria myrtillifolia*, species that are widely spread to atleast four phytogeographical regions of the CFR were nodulated by all five or four different soil types used in the study (Table 3.1). In addition, some species such as *Cyclopia pubescens*, *C. sessiflora*, *Liparia vestita*, *P. calyptrata* and *Otholobium fruticans* that are restricted to one or two phytogeographical regions of the CFR were also nodulated by all five or four different soil types. However, species of *Lessertia frutiscens*, *Tephrosia grandiflora* and *P. sericea* which were also restricted to one or two phytogeographical regions were observed to be soil specific because they nodulated with only one or two soil types. The soil collected from shale and granite derived substrates formed nodules in many of the legume species recorded at 84 % and 95 % respectively. The least nodulation was recorded with soil from limestone (42 %) and coastal sand (32 %) inoculated plants. *Crotalaria capensis* formed no nodules when inoculated with any of the soil types (Table 3.3).

Characterization of isolates by growth rate and colony morphology

The 24 rhizobial isolates selected from the 8 select legume species also formed three growth categories based on growth rate which included fast (colony appearance within 3 days), intermediate (colony appearance between 3 to 6 days) and slow (colony appearance after seven days) (Table 3.2). Colony shape was either raised or semi-raised by the intermediate and slow growth rates while it was raised for fast growers. The colony types such as creamy translucent and watery were common in all the three growth rates making it difficult to differentiate between isolates. However, the intermediate and slow growth rates isolates did not possess white opaque colony types. On the other hand the slow growth had no creamy opaque which was present in the other two growth rate types. Furthermore, the intermediate growth rate differed from the rest because its isolates didn't show any milky translucent colony type (Table 3.2).

Table 3.2. Growth rates and colony shape and types of rhizobial isolates from the selected legume species and grown on YEM agar.

Growth rate	Shape	Colony type	Propotion of Isolates (%)
Fast (<3days)	raised	white opaque, creamy opaque, creamy translucent, watery	38
Intermediate (4,5&6days)	raised, semi-raised	creamy opaque, creamy translucent, watery	54
Slow (>7days)	raised, semi-raised	creamy translucent, watery	8

Phylogenetic characterization of rhizobial isolates from the glasshouse study

The 25 rhizobia isolates obtained from selected species of the glasshouse inoculation study were sequenced using the 16S rRNA to determine whether the species were nodulated by the same rhizobia in the different soil types. The results showed that the isolates belonged to the two Proteobacteria classes: Alphaproteobacteria and Betaproteobacteria (Figure 3.1) that were present in all the soil types tested (sandstone, granite, shale, limestone and coastal sand). Isolates from *P. calyptrata* and *Virgilia oroboides* (Podalyrieae) formed a clade with *Burkholderia with* Posterior Probability support (PP) =100 %. Regardless of the soil types plants grew in, the members of the Podalyrieae were associated with the *Burkholderia* lineage. It was also evident from the phylogenetic tree (Figure 1) that the *Burkholderia* species could possibly be varied between soil types because isolates from *V. oroboides* from sandstone, coastal sand and shale (GH166, GH165, and GH92) formed a clade, and this clade was sister to another clade with isolates from *V. oroboides* from granite soil (GH93).

Table 3.3. Nodulation of legumes by rhizobia from different soil types of varying pH and the select sequenced isolates CFR. +/- Shows presence or absence of nodules. The number in the parenthesis indicates glass house and collection number of the nodules that were selected for gene sequencing.

Tribe	Genus	Species	Distribution	Soil type, area of collection and pH				
				Sandstone (Silvermine)	Granite (Jonkershoek)	Shale (Rhodes Memorial)	Limestone (De Hoop)	Coastal sand (Koppie Alleen)
				3.73	4.88	5.31	6.39	7.32
Acacieae	*Acacia*	*karoo* Hayne	NW, SW, KM, LB, SE	**+(GH175)**	**+(GH41)**	**+(GH40)**	**+(GH173)**	-
Crotalarieae	*Aspalathus*	*linearis* Burm. f.	NW,SW	**+(GH157)**	+	**+(GH38)**	-	-
	Crotalaria	*capensis*	SE	-	-	-	-	-
	Lebeckia	*sericea* Thurnb	NW,SW,KM,SE	**+(GH181)**	**+(GH96)**	**+(GH95)**	**+(GH179)**	-
Galageae	*Lessertia*	*frutiscens* (L.)	NW	-	+	-	-	-
Hypocalypteae	*Hypocalyptus*	*sophoroides* (P.J. Burgius) Baill	NW, SW, KM, LB, SE	+	+	+	-	-
Millettieae	*Tephrosia*	*grandiflora (*L'Her.) Pers	SE	**+(GH178)**	**+(GH70)**	-	-	-
Phaseoleae	*Erythrina*	*caffra*	NW,SW,KM,LB	+	+	+	-	-
Podalyrieae	*Cyclopia*	*puberscens* Eckl. & Zeyh.	KM,LB	+	+	+	+	-
	Cyclopia	*sessiflora* Eckl. & Zeyh	LB,SE	-	+	+	+	+
	Cycopia	*subternata* Vogel	SE	+	+	+	-	-
	Liparia	*vestita* Thunb.	SW,AP	+	+	+	+	-
	Podalyria	*myrtillifolia* Willd.	NW, SW, AP, LB, SE	+	+	+	-	+
	Podalyria	*sericea* R.Br.	NW,SW,LB	-	+	+	-	-
	Podalyria	*calyptrata* (Retz) Willd.	SW	+	**+(GH76)**	**+(GH75)**	**+(GH167)**	**+(GH168)**
	Virgilia	*oroboides* (P.J. Burgius) T.M. Salter	SW, LB, SE	**+(GH166)**	**+(GH93)**	**+(GH92)**	-	**+(GH165)**
Psoraleeae	*Otholobium*	*striatum* (Thunb.) C.H. Stirt.	NW,SW,KM	-	+	-	**+(GH188)**	**+(GH190)**
	Otholobium	*fruticans* (L.) C.H. Stirt.	SW	+	+	+	+	+
	Psoralea	*pinnata* L.	SW	-	**+(GH61)**	**+(GH60)**	-	-

NW (North West), SW (South West), KM (Karoo Mountain), SE (South East), LB (Langeberg Mt), AP (Agulhus Plain)

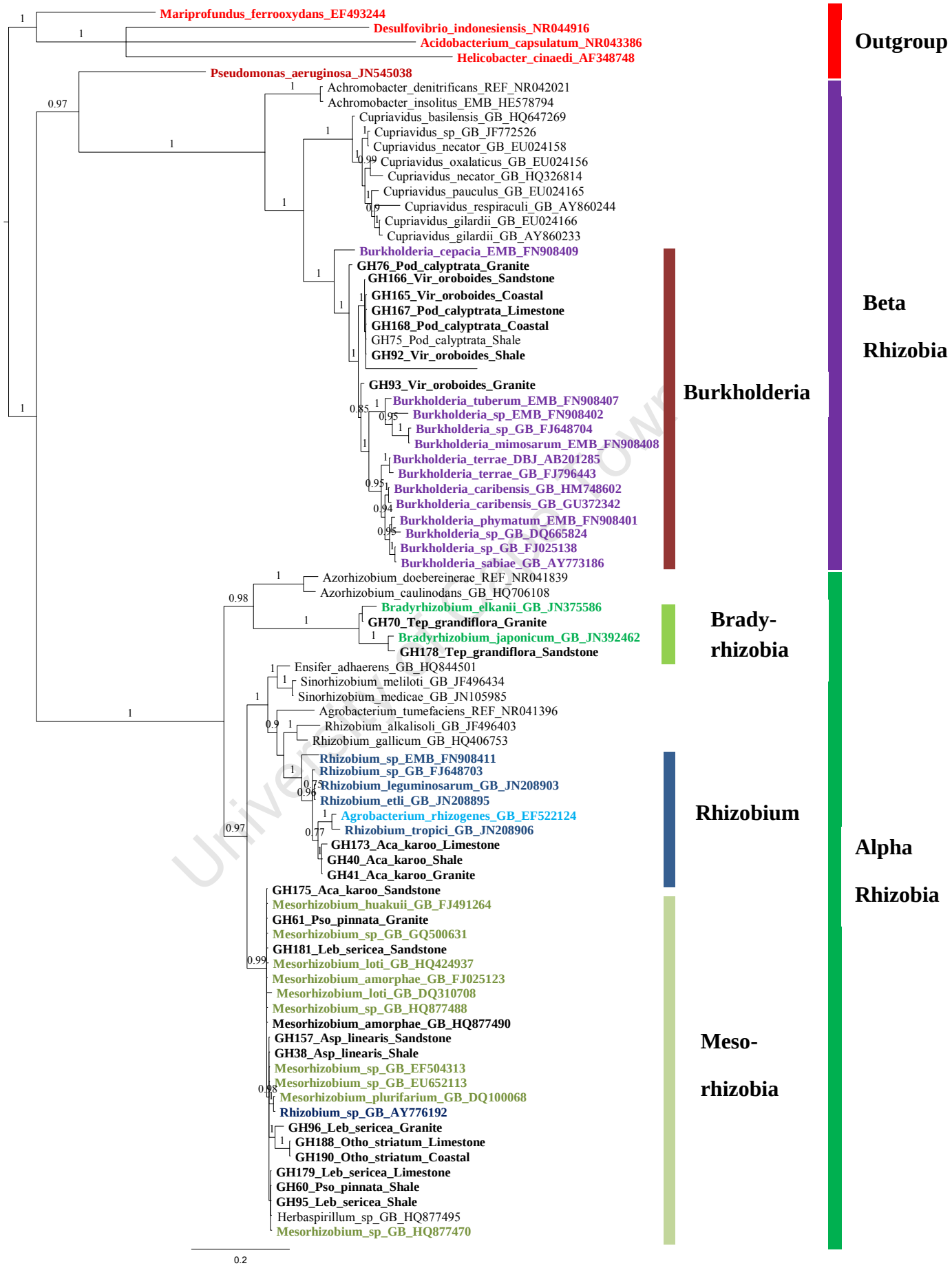

Figure 3.1. Bayesian 50% majority rule consensus tree showing the five main clades comprising beta and alpha proteobacteria based on CFR sequences and reference strains from the GenBank.

Isolates that were associated with Alphaproteobacteria formed clades belonging to three rhizobia lineages: *Bradyrhizobium, Rhizobium* and *Mesorhizobium* that were also distributed in all the soil types tested in this study. For example, isolates from *L. sericea*, *A. linearis* and *P. pinnata* were associated with members of the *Mesorhizobium* lineage regardless of the type of the soil used to inoculate them. Similarly, *T. grandiflora* isolates from both sandstone and granite derived soils were associated with *Bradyrhizoum* (Figure 3.1). However, the isolates of *A. karoo* inoculated with sandstone soil was in a clade with *Mesorhizobia* which was different from those inoculated with soil from granite, shale and limestone (GH41, GH40 and GH173), and with those in a clade associated with *Rhizobium* (Figure 3.1).

3.4. Discussions

The effect of rhizobia on the distribution of legumes

The 19 legume species tested in the glass house experiment for nodulation by five different soil types of the CFR revealed that the legume species were nodulated with rhizobia from more than one soil type and with soil from areas they do not naturally grow implying that soil rhizobia do not limit the distribution of the host. Regardless of the fact that *Podalyria calyptrata* and *Otholobium fruticans* distribution was restricted to South Western (SW) region of the CFR, they nodulated with all soil types including limestone and coastal sand from South East region (SE) where they do not naturally occur. Furthermore, *A. karoo* was noted to nodulate with rhizobia associated with *Mesorhizobium* (from sandstone soil) and *Rhizobium* (from granite, shale and limestone) soil indicating that this legume species was promiscuous. Other studies have also reported results in agreement with our findings and they attributed this to the promiscuity of legumes host (Bala and Giller 2006; Bala, et al. 2003a; Bala and Giller 2002; Staley 1999; Woomer et al. 1988). These results further indicate that the success of legumes to spread widely is generally attributed to their ability to nodulate with more than one rhizobia strain. Evidence from a study by Bala, et al. (2003b) suggested that a legume exhibited better nodulation ability in soils from its centre of diversity and its symbiont was most diverse in its host's centre of diversity. Their findings were in support of the proposal by Lie, et al. (1987) that the centres of diversity of rhizobia coincided with those of their specific legume symbionts. However, other studies have attributed the ability of legume host to nodulate in soils they do not naturally occur to rhizobia promiscuity (Odee et al., 1997; De Meyer et al., 2011). Some rhizobia types are said to be capable of nodulating host species of various taxa irrespective of endemism. Pérez-Fernández and Lamont (2003)

reported indigenous Australian rhizobia that were effective to nodulate exotic legumes as well as the native legumes.

The ability by CFR legume host that is restricted phytogeographical area to nodulate in soils from which they do not naturally occur could also be attributed to rhizobia being cosmopolitan. As early as 1934, the Dutch microbiologist L. M. G. Baas-Becking was reported to be the first to address the issue of bacteria biogeography (Staley, 1999). He hypothesized that bacteria was everywhere and supported it by pointing out that bacteria were readily dispersed from one area on Earth to another by both abiotic and biotic means. In the late 1990s, with the advent of developed molecular phylogenetic methods, certain cyanobacteria species have been reported cosmopolitan based on 16S rDNA sequence analysis (Staley, 1999). This view was supported in this study by the observation that similar rhizobia were isolated from different soils. For example, the *Rhizobium* isolated from different plants of *A. karoo* inoculated by different types of soil including granite, shale and limestone were similar because they formed one clade (Figure 3.1). Similarly, *T. grandiflora* was nodulated by *Bradyrhizobium* isolates from sandstone and granite soil that also formed one clade. Among the *Burkholderia* lineage, isolates from *P. calyptrata* inoculated with soil from granite, shale, limestone and coastal sand formed one clade that was shared by isolates of *V. oroboides* inoculated with soil from sandstone, shale and coastal sand.

On the other hand, some studies have shown that rhizobia can limit the distribution of its host due to failure of the rhizobia to establish in the soil (Thrall et al. 2011; Bala and Giller 2006; Essl et al. 2009; Han et al. 2008). Factors limiting rhizobia establishment could vary from soil, climatic and geographic distance. Some studies have reported soil pH to be a key factor affecting rhizobia diversity and distribution (Bontemps et al. 2010; Garau et al. 2005; Bala, et al. 2003a). In this study, *Bradyrhizobium* was isolated from the more acidic soils with pH range 3.73−4.88 (sandstone and granite soils) but not in shale, limestone and coastal sand inoculum soil with pH range of 5.31−7.32). However, the pH range of 3.7−7.3 did not seem to affect the distribution of *Burkholderia* and *Mesorhizobium* which were isolated in all the soil types tested. The isolation of *Mesorhizobium* and *Burkholderia* from all the five soil types tested was not surprising because their legume hosts were also observed to be widely distributed in the CFR (Chapter 2).

3.5.Conclusion

In this study, it was found out that rhizobia had no effect in the non-occurrence of legume host in areas they do not naturally grow in the CFR. This was revealed by the ability of CFR legume host to form nodules in soils they do not naturally occur in a glasshouse experiment. These results also suggest that rhizobia could be cosmopolitan in the region.

Chapter 4

General Discussion and Synthesis

Fabaceae (legumes) is ranked as the second largest plant family in the CFR comprising of an amazing 760 species of which 627 are endemic (Goldblatt and Manning 2000). Their ability to fix nitrogen in symbiosis with rhizobia in their root nodules has been reported as one main unique reason that can help explain their success in terms of species richness and distribution (Bank et al., 2002; Raychaudhuri et al., 2007; Sprent, 2007). However the effects of diversity and distribution of the rhizobia in the region on their host legume distribution was unknown. Therefore, the main focus in this study was to target the entire legume family so as to assess how rhizobia diversity could be influencing the distribution of legumes on various soil types of the CFR. It was hypothesized that the rhizobia isolates from indigenous legumes of the CFR would cluster phylogenetically according to soil types (Chapter 2.) The results of this study revealed that there was no link between rhizobia phylogeny and soil type thereby nullifying the above mentioned hypothesis.

This study revealed a very high rhizobia diversity nodulating CFR legume. The CFR rhizobia isolates include most species commonly known to cause nodules and fix nitrogen in symbiosis with legumes belonging to both Alphaproteobacteria (*Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium*) and Betaproteobacteria (*Burkholderia* and *Achromobacter*, with the later not commonly known as rhizobia). The high diversity of the CFR isolates was expected considering that the previous studies focused on a few genera revealed such high diversity (Hassen et al. 2011; Phalane 2008; Elliott et al. 2007; Kock 2004; Spriggs 2004; Le Roux 2003).

The observation that *Mesorhizobium* was the predominant rhizobia, in diverse species rich legume lineages, isolated in the CFR was novel and contrary to the expectation because the edaphic factors of the region implicated *Burkholderia*. The soils of the CFR are predominantly sandstone (60 %), nutrient poor (Goldblatt and Manning 2000) and highly acidic (Table 1 Chapter 2). Other studies on rhizobia diversity in other regions of similar soil factors such as southwest Australia (Sprent, 2012) have revealed predominance of *Bradyrhizobium* instead, and is consistent with *Bradyrhizobium* being traditionally associated with acidity and nutrient poor soils (Pérez-Fernández and Lamont 2003; Marsudi et al. 1999; Lafay and Burdon 1998). On the other hand, in recent studies (Sprent 2012; Bontemps et al. 2010) legume nodulation by beta rhizobia have also been reported to be characteristic of acid low nutrient soils. In addition, the CFR has been identified as one of *Burkholderia* centres of diversity (Mishra et al., 2012) and that is why it is surprising that *Burkholderia* is not the

predominant rhizobia species in the CFR as would have been expected given the conditions mentioned above.

The predominance of *Mesorhizobium* in the region is perhaps conceivable because they seemed to be the preferred symbiont of species in the tribe Crotalarieae which is the largest among the CFR legumes comprising of 297species with over 257 of these endemic (Table 3 Chapter 2). In addition, the Psoraleeae which is also endemic to the CFR (Goldblatt and Manning 2000) was exclusively nodulated by *Mesorhizobium*. This could mean that the *Mesorhizobium* might be playing a crucial role in the success of the legumes in terms of species occurrence and species-richness. On the other hand *Podalyria*, also an endemic genus comprising of about 20 species is exclusively nodulated by *Burkholderia* which also happens to be the second most common symbiont of the CFR legumes. So while rhizobia phylogeny does not show a link with soil type, its relative specificity at general level was apparent and this could possibly explain the success of some legume hosts as compared to others

The biogeography of the legume plants in the CFR is well documented and reveals that there exist variations in their distribution. The distribution of legumes in the region is such that some species are widespread occurring in most of the six phytogeographic (Goldblatt and Manning 2000) divisions that exist while some are restricted to one or more divisions but completely absent in others. In a study by Barraclough (2006) some of the reasons summarised as the possible explanation of the distribution of the flora and vegetation in the CFR included topographical factors, edaphic factors, pollinator specialization, fire and short dispersal distances. However there is no mention of the role the microbes could be playing to contribute to these complex plant distribution patterns. It was therefore hypothesized in this study (Chapter 3) that the legume plant distribution in the CFR is constrained by the presence of their compatible rhizobia. However the results of this study showed that rhizobia are not limiting in the majority of the CFR soils because some hosts species restricted to one phytogeographic area or soil type were nodulated by soil inoculants collected from areas they do not naturally occur. A typical example in this study is that of *Podalyria calyprata* which nodulated with all five soil types even though it is known to naturally grow in only one of them (Table 3.3, Chapter 3). In particular, there has not been any observation made or reported of *Podalyria calyptrata* growing naturally in Limestone soils. Therefore, there exist other reasons to why a particular species would not naturally grow in soils that posses their compatible rhizobia. These factors could include soil pH, nutritional status, soil texture or

depth, or prevailing local temperature of an area. For example, the pH in limestone soil (pH 6.5; Table 1) is considerably high compared to that of Sandstone (pH as low as 3; Table 1) on which *Podalyria calyprata* naturally grows.

Conclusion

The overall objective of this study was to assess the diversity and phylogenetic relationship of rhizobia in the CFR and their role in the ecology of their legume host. A weak link between rhizobia phylogeny and the biogeography of their legume host exist as revealed by rhizobia selective preference and the success of specific legume hosts in terms of species numbers in the CFR. However neither the occurrence of rhizobia nor its diversity is correlated to soil type. The distribution of legume hosts in the CFR on the other hand seems to be controlled by some factors other than the occurrence of their compatible rhizobia in the various soil types. The results of this study reveal that rhizobia are present in most CFR soils.

Future Research

This study forms part of the baseline studies required in an effort to uncover areas of further investigation as its contribution to science and these areas of further study include:

Rhizobia diversity

- Sequencing of more genes such as the *Nif*-genes, NodA genes so as to understand the nodulation potential of the rhizobia isolates in the CFR.
- Use of gene markers other than 16S rRNA in the study of rhizobia diversity so as to get resolution for closely related rhizobia lineages such as *Mesorhizobium* that form a polytomy in this study (Chapter 2 Figure 4)
- Inclusion of more than one nodule colony per nodule, and more than one nodule per per plant in diversity studies so as to be able to identify multiple rhizobia occupancy per nodule and per plant.

Rhizobia ecology

- Comparison of nodulation and nitrogen fixation efficiency by various rhizobia types
- Cross inoculation studies to determine nodulation and nitrogen fixation by promiscous rhizobia on different legume species.
- Evolutionary studies on the association of specific rhizobia types to specific legume genera or species for example the exclusive nature of nodulation of the Podalyrieae by *Burkholderia*.

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