

Characterisation of bacteria associated with the root nodules of *Hypocalyptus* and related genera

by

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Dedicated to my parents,
Hendrik and Lorraine.

Thank you for your unwavering support.

I certify that this dissertation hereby submitted to the University of Pretoria for the degree of *Magister Scientiae* (Microbiology), has not previously been submitted by me in respect of a degree at any other university.

Signature _____

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A characteristic trait of the plant family Leguminosae is their ability to form a mutualistic association with certain diazotrophic (nitrogen-fixing) Gram-negative soil bacteria. Informally these bacteria are called rhizobia and until about a decade ago were only found in the class Alphaproteobacteria. In 2001, nodulating isolates belonging to the genus *Burkholderia* (class Betaproteobacteria) were discovered. To differentiate between the two groups of rhizobia they are referred to as alpha- or beta-rhizobia. One of the first beta-rhizobia isolated, *B. tuberum*, associates with the indigenous fynbos legume *Aspalathus carnosa*, a relative of *Aspalathus linearis* from which ‘rooibos’ tea is made. This bacterium has also recently been shown to be able to nodulate a range of indigenous *Cyclopia* spp. Another two as yet undescribed *Burkholderia* species were found to nodulate the indigenous *Rhynchosia ferulifolia*. These three legumes all form part of the Papilionoideae, which is the largest subfamily of the Leguminosae. However, elsewhere in the world, especially in South America and Taiwan, the majority of beta-rhizobial *Burkholderia* species and isolates have been found in association with the genus *Mimosa* (subfamily Mimosoideae).

The work presented in this dissertation focused on the rhizobia associated with the indigenous papilionoid fynbos tribe, Hypocalypteae. This tribe contains only one genus, *Hypocalyptus*, which in turn consists of only three species, *H. sophoroides*, *H. oxalidifolius* and *H. coluteoides*. In order to investigate possible host associations at a broader scale, I also included rhizobial isolates from papilionoid legumes belonging to the tribe Podalyrieae, and focused specifically on three genera – *Podalyria*, *Virgilia* and *Cyclopia*. Rhizobial isolates from *Podalyria calyptrata*, *Virgilia oroboides* and a range of *Cyclopia* species were, therefore, included as these genera share certain characteristics with *Hypocalyptus*. Until this study the only information available with regards to the rhizobial associates of *Hypocalyptus* was the fact that *H. sophoroides* had been noted to possess nodules in the wild.

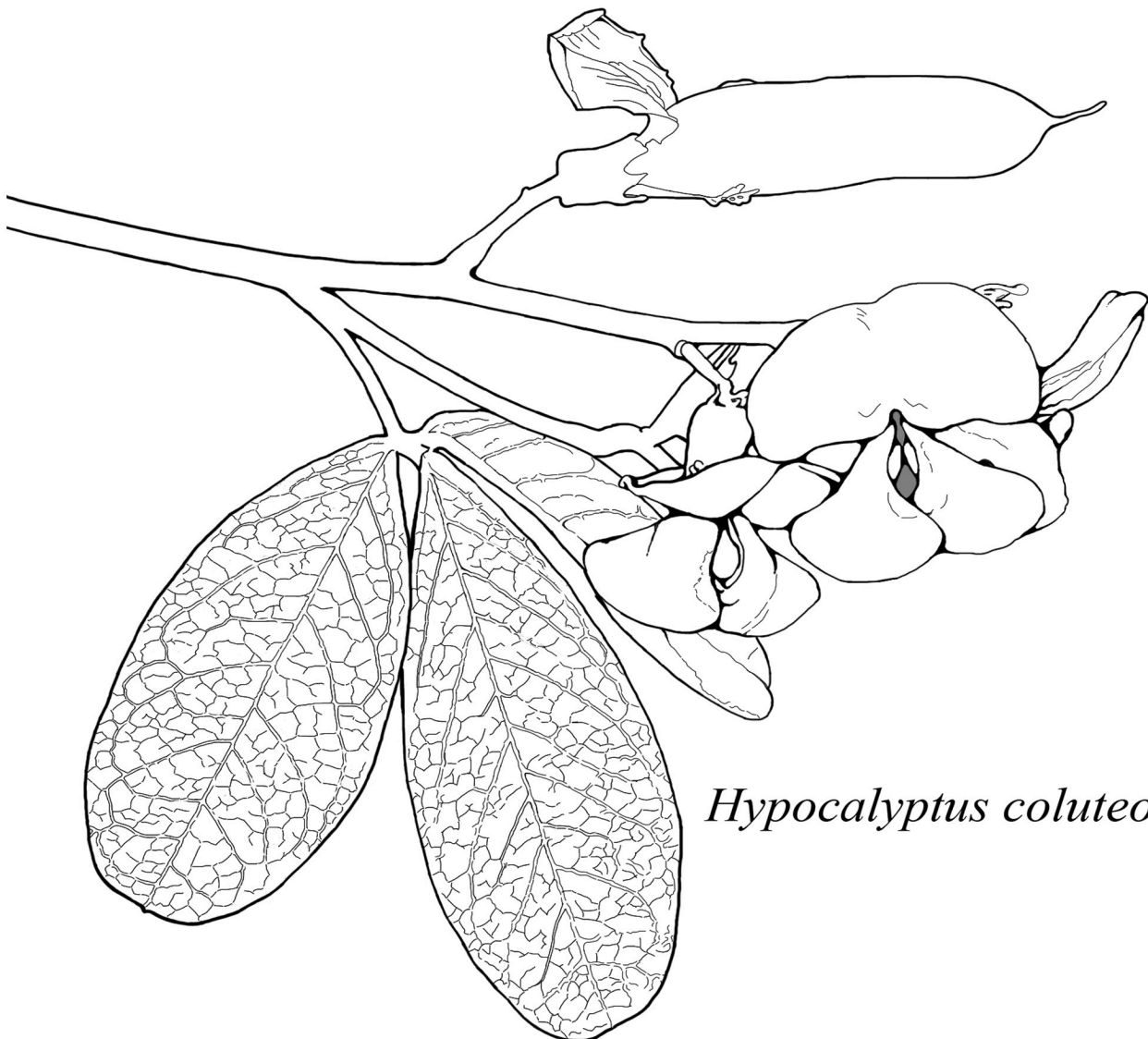
Chapter 1 is a review of the current literature, which focuses on the taxonomy of the alpha- and beta-rhizobia, as well as the genes and processes involved in nodulation and nitrogen-fixation. This chapter also considers the evolution of legumes and their rhizobial symbionts. Finally the chapter concludes with a discussion of the genus *Hypocalyptus* and its relatives, its taxonomic history and a short overview on the available information regarding its rhizobial symbionts.

In chapter 2 of this dissertation, I present the results of a study aimed at characterising the root-nodule bacteria associated with *Hypocalyptus* and its relatives. Details regarding the isolation and culturing of these indigenous bacteria, as well as the protocols followed to test their nodulation capabilities on the original host and the two promiscuous legumes, siratro (*Macroptilium atropurpureum*) and cowpea (*Vigna unguiculata*) are also included. The combined DNA sequence information for two housekeeping loci (i.e., the 16S rRNA and *recA* genes) were used to separate the isolates into groups or lineages, each of which potentially represent distinct species.

In Chapter 3, I considered the evolution of nitrogen-fixation and nodulation in my set of indigenous beta-rhizobia. For this purpose, the DNA sequence information for the common nodulation gene, *nodA*, as well as *nifH* which is one of the integral nitrogen-fixation genes, was used. As opposed to genes with housekeeping functions that are typically encoded on the core genome of bacterial species, the genes involved in the legume-rhizobium symbiosis are usually associated with the so-called accessory genomes of these bacteria. However, very little information is available regarding the evolution of these loci among our indigenous rhizobia. The primary aim of this chapter was thus to address their evolution on both local and global scales. At the local scale, inferred phylogenetic groups were analysed for their correspondence to host and/or geography. On a more global scale, phylogenetic trees were used to study the evolutionary history of the beta-rhizobia associated with the indigenous South African legumes.

Chapter 1

**Taxonomy, infection biology and evolution of rhizobia,
with special reference to those nodulating *Hypocalyptus***



Hypocalyptus coluteoides

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1.1 Introduction

The Gram-negative bacteria capable of fixing atmospheric nitrogen in association with plants, most specifically those in the family Leguminosae, are referred to as rhizobia (Markmann *et al.*, 2008; Soto *et al.*, 2006). During this symbiosis, a molecular crosstalk between the interacting partners results in the development of specialised organs, termed nodules, on the roots and/or stems of the host plant, in which atmospheric nitrogen is fixed (Liu *et al.*, 2005; Long, 2001; Wei *et al.*, 2009; Willems, 2006). Initially, it was thought that this nitrogen-fixing symbiotic ability was restricted to only a few bacteria (Sawada *et al.*, 2003). The advent of molecular biology and the subsequent dramatic advances in DNA-based technologies, however, facilitated the identification of this ability in numerous other bacteria. For example, the number of rhizobial species increased from 34 at the end of 2000 to 53 in the year 2006 (Willems, 2006). Also, it quickly became clear during this revival of rhizobiology that symbiotic nitrogen-fixation is not limited to the class Alphaproteobacteria, but that diverse members of the Betaproteobacteria also have this ability (Chen *et al.*, 2003; Moulin *et al.*, 2001). In 2001, it was estimated that the rhizobial symbionts of less than 10% of all legume genera had been completely characterised (Moulin *et al.*, 2001), suggesting that many more nodule-forming species remain to be discovered and described.

This nitrogen-fixing interaction is of importance to agriculture as it represents an alternative to the use of industrially produced nitrogen fertiliser (Carlsson and Huss-Danell, 2003). The Haber-Bosch process, which is responsible for producing inorganic nitrogen fertiliser, is very energy intensive and thus very costly due to the large amounts of fossil fuel it consumes (Cummings *et al.*, 2006). The reliance of this process on fossil fuels has also raised questions regarding its sustainability and concerns on its significant contribution to the production of CO₂ which in turn influences global warming (Cummings *et al.*, 2006; Jenkinson, 2001). Two different instances of the agricultural use of biologically fixed nitrogen can be found in South America and Australia (Cummings *et al.*, 2006). Legume crops are popular in South America and more specifically common bean (*Phaseolus vulgaris*) which is responsible for providing ~ 30% of the protein requirement of the South American population and in order to meet this requirement, while utilising nitrogen poor soils, this crop is inoculated with rhizobial symbionts (Cummings *et al.*, 2006; Hungria *et al.*, 2000). In Australia however non-legume crops such as cereals are planted in rotation with legume crops in order to enrich the nitrogen content of the soil (Crews and Peoples, 2004; Cummings *et al.*, 2006).

From a systematic point of view the rhizobia do not represent a monophyletic taxon (Masson-Boivin *et al.*, 2009). In fact, various phylogenetic studies have shown that many rhizobial groups are more closely related to bacteria that do not possess nodulation capabilities. For example, members of the rhizobial genus *Bradyrhizobium* are most closely related to species from non-rhizobial genera such as *Agromonas*, *Afipia*, *Nitrobacter* and *Rhodopseudomonas* (Garrity *et al.*, 2005). Currently, 94 species of rhizobia are known, and these are classified into 12 genera in the class Alphaproteobacteria, and four genera in the class Betaproteobacteria. For easier reference to the rhizobia in these two classes, the terms alpha- and beta-rhizobia have been introduced (Moulin *et al.*, 2001). The possibility of species from the Gammaproteobacteria being capable of nodulation has been mentioned (Benhizia *et al.*, 2004); however studies suggest that these bacteria are plant-growth promoting endophytes that mask the presence of the original rhizobia (Ibáñez *et al.*, 2009; Muresu *et al.*, 2008). A *Pseudomonas* strain has since, however, tested positive in nodulation tests and both nodulation and nitrogen-fixation genes could be amplified from this strain (Shiraishi *et al.*, 2010).

My main objective for this literature study is to review existing ideas on the development and evolution of the legume-rhizobium symbiosis, especially with reference to the legume tribe Hypocalyptae. To accomplish this, I will first provide an outline of contemporary rhizobial taxonomy and summarise recent knowledge on the genetic determinants of nodulation. I will then consider the evolution of the legume-rhizobium symbiosis and rhizobial nitrogen-fixation by taking into account current reviews on this issue and also incorporating relevant information for *Hypocalyptus* and its relatives.

1.2 The taxonomy of rhizobia

As mentioned above, in South America, rhizobial inoculums can provide legume crops with sufficient fixed nitrogen for growth and this has led to the development of commercial inoculant strains. Knowledge of the taxonomic position of these strains is important in order to ensure that the strain does in fact possess the potential to fix atmospheric nitrogen under the right environmental conditions as well as to ensure that it is capable of interacting with the selected plant host (Cummings *et al.*, 2006). ‘Rhizoagrin’ is one example of where taxonomic confusion led to the incorrect use of a ‘diazotrophic’ inoculant strain (Cummings *et al.*, 2006; Humphry *et al.*, 2007). The purpose of this free-living strain was to provide

wheat crops with fixed nitrogen, although a more in-depth study found this commercial strain to be most similar to *Rhizobium radiobacter* (formerly known as *Agrobacterium radiobacter*) (Cummings *et al.*, 2006; Humphry *et al.*, 2007). This particular strain does not possess the ability to fix nitrogen but does however produce the plant growth promoting agent, gibberelic acid, which could explain how its use led to a yield increase comparable to the addition of 30 kg/ha of nitrogen (Cummings *et al.*, 2006; Humphry *et al.*, 2007). Although the use of ‘Rhizoagrin’ did not result in any deleterious effects it still was not capable of accomplishing the function for which it was intended.

Initially, all rhizobia were classified into the single genus *Rhizobium* in the family Rhizobiaceae and class Alphaproteobacteria (Sawada *et al.*, 2003; Zakhia and de Lajudie, 2001). It was also presumed that these bacteria were able to form nodules on any legume host (Jordan, 1982; Sadowsky and Graham, 2006). Later studies, however, indicated that these bacterial strains could only interact with a specific range of host plants, which gave rise to the “cross inoculation” concept where strains were differentiated based on the specific legumes they nodulate (Sawada *et al.*, 2003; Willems, 2006; Zakhia and de Lajudie, 2001). This led to the recognition of 20 cross inoculation groups, some of which were also recognised as distinct *Rhizobium* species (Sadowsky and Graham, 2006). However, the cross inoculation concept came under scrutiny when tests revealed that this rhizobial grouping did not correspond to legume taxonomy (Broughton and Perret, 1999). The concept was dealt a final blow when it was shown that the genes responsible for symbiosis are encoded on mobile genetic elements that are subject to horizontal gene transfer (MacLean *et al.*, 2007; Sadowsky and Graham, 2006).

More recently, species of rhizobia are described and vindicated by means of a polyphasic procedure (Sadowsky and Graham, 2006). The intention of such a procedure is to make use of a range of different types of data including phenotypic, DNA-based and phylogenetic information, in order to obtain a consistent classification scheme for any given species (Gevers *et al.*, 2005; Vandamme *et al.*, 1996). To assign a new species by means of such a polyphasic approach, a set of criteria needs to be met. These are as follows: the isolates of the species must have consistent phenotypic characteristics; their 16S ribosomal RNA (rRNA) subunit sequences must be $\geq 97\%$ similar; and their DNA-DNA hybridisation values greater than 70%, especially in those cases where isolates share less than 97% 16S rRNA sequence similarity (Gevers *et al.*, 2005).

Most of the phylogenetic placements of described rhizobia have been accomplished solely on the basis of 16S rRNA gene sequence comparisons. However, it has been shown that a wider range of loci might be needed since 16S rRNA phylogenies differ from those of other housekeeping genes and there are also problems regarding the use of this gene in order to define new species or strains (Cummings *et al.*, 2006; Martens *et al.*, 2007; Martens *et al.*, 2008; van Berkum *et al.*, 2003). These problems include identical 16S rRNA sequences for genetically distinct organisms, especially when considering closely related species (Jaspers and Overmann, 2004; Martens *et al.*, 2007; Stępkowski *et al.*, 2003). DNA-DNA hybridisation is another standard method used during species descriptions and it determines genome-wide similarity between isolates based upon the percentage of reassociation which occurs between their genomes under tightly regulated conditions (Gevers *et al.*, 2005). This method might, however, soon be replaced due to the difficulty in obtaining standardised conditions instead another method known as multi-locus sequence analysis (MLSA), could be used as it relies heavily on the more standardised technology of DNA sequencing (Zeigler, 2003).

MLSA incorporates the DNA sequences of several housekeeping genes in order to genotypically distinguish between species even across the genus level (Gevers *et al.*, 2005). There are already phylogenetic studies on rhizobia which incorporate housekeeping genes other than the 16S rRNA and which could be combined for MLSA purposes (Martens *et al.*, 2007; Martens *et al.*, 2008). Examples of the products of such genes are: DnaK, a DNA chaperone; glutamine synthetase that is central to nitrogen assimilation; and citrate synthase that is important in controlling the amount of ATP produced within cells (Hernández-Lucas *et al.*, 2004; Stępkowski *et al.*, 2003; Turner and Young, 2000). Zeigler (2003) concluded that the efficiency of MLSA studies could be identical to or even exceed that of DNA-DNA hybridisation for measuring genome similarity (Martens *et al.*, 2008; Zeigler, 2003).

1.2.1 Alpha-rhizobia

Within the Alphaproteobacteria, species from several genera have been found to enter into a symbiotic association with legumes. These genera form part of the order Rhizobiales (Euzéby, 1997). The Alphaproteobacteria are known for their singular genome organisation and their frequently adopted intracellular lifestyles, which in certain instances have led to a reduction of their genome size while others instead have enlarged genomes (Williams *et al.*,

2007). These large genomes might be necessary for survival and competition in the rhizosphere (Young *et al.*, 2006).

Although all rhizobia were originally placed in the genus *Rhizobium*, the fast-growing and acid-producing (at least on yeast extract-mannitol agar) bacteria were later separated from those that produce an alkali and are slow-growing (Jordan, 1982). At the time, these slow-growing strains were moved to the novel genus *Bradyrhizobium* (Jordan, 1982; Sawada *et al.*, 2003). As taxonomic methods improved, more species were removed to other or new genera. In recent years this led to considerable controversy regarding the taxonomy of some rhizobial taxa. For example, the issues surrounding the genera *Agrobacterium/Rhizobium* and *Sinorhizobium/Ensifer* have been the focus of many debates with radical changes proposed for these genera (Farrand *et al.*, 2003; Sahgal and Johri, 2003; Willems *et al.*, 2003; Young *et al.*, 2001; Young, 2003). However, the classification of rhizobia (alpha and beta) is bound to remain dynamic and sometimes controversial, not only because of their relationships with non-symbionts (Sawada *et al.*, 2003; Young and Haukka, 1996; Fig. 1.1) and improvements in identification methods, but also because of the changing views on what bacterial species are and how they should be classified (Cohan, 2002; Cohan, 2006; Doolittle and Papke, 2006; Fraser *et al.*, 2009).

1.2.1.1 The genus *Rhizobium*

At present *Rhizobium* contains 50 described species (Euzéby, 1997; <http://www.bacterio.net>), of which the wide host range species, *R. leguminosarum*, is the type. Although most of the species in this genus possess the ability to form nodules and fix nitrogen (Table 1.1), there are some species which apparently lack this ability e.g. *R. selenireducens* (Hunter *et al.*, 2007). This is also true for those species previously classified as *Agrobacterium* that are now included in the genus (Euzéby, 1997; Farrand *et al.*, 2003; Young *et al.*, 2001, 2003). According to Young *et al.* (2001) the genus should also include *Allorhizobium undicola* that nodulates the aquatic legume *Neptunia natans* (de Lajudie *et al.*, 1998a).

1.2.1.2 The genus *Bradyrhizobium*

Currently this genus contains ten species (Euzéby, 1997), of which the type, *B. japonicum*, was initially assigned to the genus *Rhizobium* (Jordan, 1982). *Bradyrhizobium* is now

classified in the family Bradyrhizobiaceae that was created specifically to contain it (Sahgal and Johri, 2003). This genus also contains a subset of nodulating photosynthetic strains (Fleischman and Kramer, 1998; Molouba *et al.*, 1999; So *et al.*, 1994), which have only been encountered in *Aeschynomene* stem nodules and root nodules of *Lotononis bainesii* (Fleischman and Kramer, 1998).

Another species found to form part of *Bradyrhizobium* is *Blastobacter denitrificans*, an organism so named because of its ability to multiply by budding (Hirsch and Müller, 1985). *Blastobacter denitrificans* was proven able to nodulate *Aeschynomene indica* (van Berkum and Eardly, 2002), resulting in a proposal to accommodate it within the genus *Bradyrhizobium* (van Berkum *et al.*, 2006). It has recently been formally moved to the genus *Bradyrhizobium* and is now known as *Bradyrhizobium denitrificans* (Euzéby, 1997; Euzéby 2011; Table 1.1).

1.2.1.3 The genus *Mesorhizobium*

This genus was created by Jarvis *et al.* (1997) to contain the former *Rhizobium* species *R. loti*, *R. ciceri*, *R. mediterraneum*, *R. huakuii* and *R. tianshanense* following an extended 16S rRNA sequencing analysis. At present *Mesorhizobium* contains 22 species of which *M. loti* is the type (Euzéby, 1997; Table 1.1).

1.2.1.4 The genera *Ensifer* and *Sinorhizobium*

The genus *Sinorhizobium* was introduced to house the fast-growing soybean-nodulating bacteria *S. fredii* and *S. xinjiangensis* (Chen *et al.*, 1988), while *Ensifer* was first described to accommodate *Ensifer adhaerens*, a bacterial predator (Casida, 1982) apparently capable of nodulating legumes when it receives symbiotic plasmids from *Rhizobium tropici* (Rogel *et al.*, 2001). *Ensifer* and *Sinorhizobium* have been suggested to be synonymous (Willems *et al.*, 2003) but their taxonomic status is not yet resolved. Although the Judicial Commission of the International Committee on Systematics of Prokaryotes favours *Ensifer* (Euzéby, 1997; Table 1.1), there is still uncertainty regarding the placement of *E. adhaerens* and *S. morelense* (Lindström and Martínez-Romero, 2005; 2007; Martens *et al.*, 2007; Martens *et al.*, 2008; Wang *et al.*, 2002; Young, 2003). Therefore, *Ensifer* currently includes 13 species, while *Sinorhizobium* contains two i.e. *S. morelense* and *S. americanum* (Euzéby, 1997; Table 1.1).

1.2.1.5 The genus *Azorhizobium*

This genus contains two recognised species, *A. caulinodans* and *A. doebereineriae* (synonym *A. johannense*) (de Souza Moreira *et al.*, 2006). Both can nodulate legumes (de Souza Moreira *et al.*, 2006; Dreyfus *et al.*, 1988; Table 1.1). *Azorhizobium caulinodans* is the type species for the genus (Euzéby, 1997).

1.2.1.6 The genus *Ochrobactrum*

The genus *Ochrobactrum* includes 15 species of which *O. anthropi* is the type (Euzéby, 1997). Thus far three species capable of nodule formation have been described (Table 1.1). A possible fourth nodulating *Ochrobactrum* strain, was isolated from *Acacia mangium* root nodules (Ngom *et al.*, 2004), but it is not as yet described.

1.2.1.7 The genus *Methylobacterium*

This genus includes 35 described species (Euzéby, 1997), of which only one, *Methylobacterium nodulans*, is known to nodulate legumes (Jourand *et al.*, 2004; Sy *et al.*, 2001; Table 1.1). The type strain for this species was isolated from *Crotalaria podocarpa* and is non-pigmented (Jourand *et al.*, 2004; Sy *et al.*, 2001). *Methylobacterium* strains that have not been formally described and that are pink pigmented have also been isolated from the root nodules of *Lotononis* spp. (Jaftha *et al.*, 2002; Yates *et al.*, 2007). Their nodulation abilities appear to be species specific (Yates *et al.*, 2007).

1.2.1.8 The genus *Devosia*

The genus *Devosia* includes 11 described species (Euzéby, 1997). Of these, only one, *Devosia neptuniae*, is capable of nodulation (Rivas *et al.*, 2002; Rivas *et al.*, 2003; Table 1.1). Although a second species, *D. yakushimensis*, was extracted from the root nodules of *Pueraria lobata* in Japan both the amplification of symbiotic loci and nodulation tests failed (Bautista *et al.*, 2011).

1.2.1.9 The genera *Phyllobacterium* and *Labrys*

Phyllobacterium includes eight described species (Euzéby, 1997) of which three are associated with legume root nodules (Baimiev *et al.*, 2007; Mantelin *et al.*, 2006; Rasolomampianina *et al.*, 2005; Valverde *et al.*, 2005). *Labrys* includes seven species of which two, *L. neptuniae* and *L. okinawensis*, have been isolated from the root nodules of a

legume (Chou *et al.*, 2007; Euzéby, 1997; Islam *et al.*, 2007; Table 1.1). However, the symbiotic or nodulation capabilities of these *Phyllobacterium* and *Labrys* species must still be confirmed.

1.2.1.10 The genus *Shinella*

This genus was recently described (An *et al.*, 2006) and includes five recognised species (Euzéby, 1997; Table 1.1). Of these only one, *S. kummerowiae*, is associated with root nodules (Lin *et al.*, 2008). Although nodulation tests with this species on its original host have failed, it has been shown to harbour genes involved in nitrogen-fixation and nodulation (*nifH* and *nodD*; see below) (Lin *et al.*, 2008). Additionally, it should be noted that there is some confusion regarding the higher taxonomic grouping of this genus, as some authors recognise this genus as a member of the family Rhodocyclaceae (order Rhodocyclales, class Betaproteobacteria) (Euzéby, 1997). If this is true, then *S. kummerowiae* adds to diversity of the beta-rhizobia (see below).

1.2.2 Beta-rhizobia

The ability of members of the Betaproteobacteria to nodulate legumes and to fix nitrogen was only discovered in 2001 (Moulin *et al.*, 2001). The beta-rhizobia are found in the order Burkholderiales where they are separated in to four genera, three of which belong to the family Burkholderiaceae (*Burkholderia*, *Cupriavidus* and *Herbaspirillum*) (Barrett and Parker, 2006; Euzéby, 1997), while the last (*Achromobacter*) fall within the family Alcaligenaceae (Euzéby, 1997).

These beta-rhizobial genera have undergone extensive taxonomic changes over recent years (Gillis *et al.*, 1995; Vandamme and Coenye, 2004; Vaneechoutte *et al.*, 2004; Yabuuchi *et al.*, 1995). A good example of this would be the species *Ralstonia taiwanensis*, which is related to *Ralstonia eutropha* (Vaneechoutte *et al.*, 2004). To accommodate this group of related bacteria, Vaneechoutte *et al.* (2004), proposed the new genus *Wautersia*. However, Vandamme and Coenye (2004) later discovered that *Wautersia eutropha* (type species of the genus *Wautersia*) is conspecific with *Cupriavidus necator* (type species for the genus *Cupriavidus*). Consequently, all of the species previously recognised as belonging in the genus *Wautersia* were moved to *Cupriavidus* as this genus was described first (Vandamme and Coenye, 2004).

Rhizobia in the genera *Burkholderia* and *Cupriavidus* appear to be mostly associated with hosts from the legume genus *Mimosa* (Barret and Parker, 2005; Chen *et al.*, 2005a; Chen *et al.*, 2005b; Compant *et al.*, 2008). Research has shown that *Mimosa* species are nodulated by alpha-rhizobia as well, and that the two different rhizobial classes can occupy different nodules on the same plant (Barret and Parker, 2006; Elliott *et al.*, 2009). However, the beta-rhizobia, specifically those in the genus *Burkholderia*, out-compete those from the alpha-rhizobia (Elliott *et al.*, 2009). This competitive advantage of *Burkholderia* species seems linked to soil type and nitrogen availability (Elliott *et al.*, 2009).

1.2.2.1 The genus *Achromobacter*

Achromobacter contains six species (Euzéby, 1997) of which only, *A. xylosoxidans*, has thus far been found capable of nodulation (Benata *et al.*, 2008). This rhizobium nodulates *Prosopis juliflora* and both nodulation tests on the original host as well as amplification of the *nodC* gene were possible (Benata *et al.*, 2008).

1.2.2.2 The genus *Burkholderia*

The genus *Burkholderia* contains 70 described species (Euzéby, 1997; <http://www.bacterio.net>), of which six (*B. caribensis*, *B. mimosarum*, *B. nodosa*, *B. phymatum*, *B. sabiae* and *B. tuberum*) apparently possess the ability to establish a nitrogen-fixing symbiosis with legumes (Table 1.1). This genus was first described in 1992, and then only contained the seven species from *Pseudomonas* homology group II of which *B. cepacia* is the type species (Yabuuchi *et al.*, 1992). Later two of these species, *Burkholderia pickettii* and *Burkholderia solanacearum*, were moved by Yabuuchi *et al.* (1995) to the genus *Ralstonia*.

Burkholderia contains an astounding diversity of species occupying assorted habitats. Some species are both human and plant pathogens (e.g. *B. cepacia*; Chiarini *et al.*, 2006; Compant *et al.*, 2008), while others are endosymbionts of insects associated with plants (e.g. *B. cenocepacia*; Compant *et al.*, 2008; Santos *et al.*, 2004). Yet others have useful properties such as plant-growth promotion and a possible use as biocontrol agents against fungal phytopathogens (e.g. *B. bryophila*; Compant *et al.*, 2008; Vandamme *et al.*, 2007).

The nodulation ability of every presumptive nitrogen-fixer in the genus *Burkholderia* has not yet been confirmed. Vandamme *et al.* (2002) showed that *B. dolosa* (formerly known as *B. cepacia* genomovar VI; Vermis *et al.*, 2004) is capable of nodule formation on *Alysicarpus glumaceus*, while Coenye and Vandamme (2003) could not detect one of the common nodulation genes in this species. Therefore it is assumed that *B. dolosa* might have lost its symbiotic properties (Coenye and Vandamme, 2003). Similarly, *B. phymatum* was originally isolated from *Machaerium lunatum* nodules (Vandamme *et al.*, 2002), but later Elliott *et al.* (2007b) showed that this species cannot nodulate *M. brasilense* although it can nodulate a range of *Mimosa* species. This has led to the hypothesis that *B. phymatum* might in actual fact have been associated with a *Mimosa* host in the vicinity of the original *M. lunatum* plant (Elliott *et al.*, 2007a).

1.2.2.3 The genus *Cupriavidus*

At present the genus contains 12 described species (Euzéby, 1997). The type species is *C. necator* (Makkar and Casida, 1987). The only rhizobial species is *C. taiwanensis*, which nodulates *Mimosa pudica* (Chen *et al.*, 2001; Vandamme and Coenye, 2004). Intriguingly, this species was also isolated from the sputum of a cystic fibrosis patient (Chen *et al.*, 2001).

1.2.2.4 The genus *Herbaspirillum*

Herbaspirillum includes 11 described species (Euzéby, 1997) of which the type species, *H. seropedicae*, is a nitrogen-fixing cereal endophyte (Baldani *et al.*, 1986). Although there has been a report of *H. seropedicae* isolated from the roots of the legume *Cajanus cajan*, the possibility of maize roots in the original sample could not be ruled out and therefore additional research is needed to verify this association (Valverde *et al.*, 2003). *Herbaspirillum lusitanum* that nodulates *Phaseolus vulgaris* is thus then the only officially described rhizobial species for this genus (Valverde *et al.*, 2003; Table 1.1).

1.3 Rhizobia and nodulation

The ability of rhizobia to nodulate plants requires the products of the nodulation (*nod*) genes that are carried on mobile genetic elements such as plasmids or transposable infection islands (Flores *et al.*, 2005; MacLean *et al.*, 2007; Mylona *et al.*, 1995; Wernegreen and Riley, 1999). Based on previous research it is now known that nodulating species from the

following genera carry their symbiotic loci on plasmids: *Rhizobium* (Wang *et al.*, 1999b), *Sinorhizobium/Ensifer* (Wang *et al.*, 1999b), *Phyllobacterium* (Valverde *et al.*, 2005), *Ochrobactrum* (Trujillo *et al.*, 2005; Zurdo-Piñeiro *et al.*, 2007), and *Devosia* (Rivas *et al.*, 2002) as well as *Burkholderia* and *Cupriavidus* (Amadou *et al.*, 2008; Chen *et al.*, 2003). Symbiotic loci of the genera *Bradyrhizobium* (Wang *et al.*, 1999b), *Azorhizobium* (Lee *et al.*, 2008) and *Methylobacterium* are apparently located on mobile chromosomal elements (Renier *et al.*, 2008). For the most part *Mesorhizobium* species also carry their symbiotic machinery on the chromosome (Wang *et al.*, 1999b), although some species such as *M. amorphae* (Wang *et al.*, 1999b) and *M. huakuii* (Xu and Murooka, 1995) carry theirs on plasmids.

The process of nodule formation revolves around the exchange of signal molecules between the host plant and its specific rhizobial partner/s (Schultze and Kondorosi, 1998). A variety of flavonoid signal molecules released by the plant roots are responsible for attracting the correct rhizobial partner and activating the transcription of their nodulation genes (Cooper, 2007; Perret *et al.*, 2000). Expression of these genes leads to the formation of the nodulation signal molecules or Nod factors (NFs) (Perret *et al.*, 2000; Schultze and Kondorosi, 1998). Through an intricate process the NFs are recognised by the plant (Crespi and Gálvez, 2000; Goormachtig *et al.*, 2004; Limpens and Bisseling, 2003) and subsequently have far reaching effects in the plant, ranging from altering plant root hair morphology to the induction of the plant nodulin genes responsible for the formation of the nodule (Cooper, 2007; Gage and Margolin, 2000; Mulder *et al.*, 2005).

There are different infection routes which the rhizobia can use upon recognition of the NFs by the plant (Brewin, 1991; Goormachtig *et al.*, 2004; Sprent and de Faria, 1988). Of these the best studied example is by means of root hairs, which appears to be a two-step process (Gage, 2004; Hirsch, 1992; Rodríguez-Navarro *et al.*, 2007; Skorupska *et al.*, 2006). The first step involves the protein rhicadhesin, which can bind to both plant root hairs and bacterial cell poles in the presence of calcium (Gage, 2004; Hirsch, 1992; Rodríguez-Navarro *et al.*, 2007). An alternative to rhicadhesin is the binding of legume lectins to surface polysaccharides of their corresponding rhizobial symbiont (Hirsch, 1999; Rodríguez-Navarro *et al.*, 2007). The second step in the attachment process involves cellulose fibrils or fimbriae (Gage, 2004; Hirsch, 1992; Rodríguez-Navarro *et al.*, 2007) which results in a permanent bond. Once the rhizobia are attached to the plant cells, the root hairs undergo

morphological changes which results in the formation of a “shepherd’s crook” (Esseling *et al.*, 2003; Gage, 2004; Schmidt and Panstruga, 2007). Within the shepherd’s crook the plant cell wall is degraded and the cytoskeleton then exerts an inwards drag on the cell wall leading to the formation of an inward growing cell wall tunnel (Brewin, 2004; Gage, 2004; Schultze and Kondorosi, 1998). The bacteria on the other hand exert pressure by their repeated division and growth resulting in an inward growing tube filled with rhizobia which is called the infection thread (Brewin, 2004; Gage, 2004).

The rhizobial NFs are also responsible for the formation of the nodule primordium by triggering cell division within the root cortex (Brewin, 2004; Yang *et al.*, 1994). In the formation of determinate nodules, cell division occurs in the outer cortex after which the nodule primordium quickly loses its meristematic function thus resulting in a spherical shape (Foucher and Kondorosi, 2000; Hayashi *et al.*, 2000; Hirsch, 1992; Figs. 1.2 and 1.3). In contrast, the nodule primordium for indeterminate nodules is formed within the inner cortex and possess an apical meristem (Crespi and Gálvez, 2000; Foucher and Kondorosi, 2000), which results in an elongated nodule (Fig. 1.2). As soon as development of the nodule primordium has been completed, the rhizobia are enveloped by the plant cell membrane and released from the infection thread in a process similar to endocytosis (Mergaert *et al.*, 2006; Parniske, 2000). This results in the formation of the so-called symbiosome with a peribacteroid membrane in which the bacteria differentiate (Franche *et al.*, 2009; Mergaert *et al.*, 2006; Parniske, 2000; Schultze and Kondorosi, 1998). In the case of indeterminate nodules, this differentiation process involves genome duplication without entering cell division (i.e. endoreduplication), which results in elongated bacteroid cells (Mergaert *et al.*, 2006). In determinate nodules however, the differentiation process does not involve such endoreduplication (Mergaert *et al.*, 2006). As a result bacteroids from indeterminate nodules are unable to resume autonomous growth again, while those from determinate nodules still possess growth abilities similar to rhizospheric bacteria (Mergaert *et al.*, 2006). In parallel, several rounds of endoreduplication of the infected plant cells results in enlarged cells to ensure a suitable habitat with increased metabolic activity to support efficient nitrogen-fixation by the increasing rhizobial inhabitants (Mergaert *et al.*, 2006; Trevaskis *et al.*, 2002; Vinardell *et al.*, 2003).

1.3.1 The genetic determinants of nodulation

The nodulation genes can be divided into two primary groups – the common *nod* genes and the host-specificity *nod* (*hsn*) genes which are responsible for host recognition (Dénarié *et al.*, 1992; Hirsch, 1992; Menna and Hungria, 2011). The *hsn* genes include the *nol*, *nod* and *noe* genes (Spaink, 2000); although not all rhizobia encode the same sets of *hsn* genes (see below). The common *nod* genes (*nodABC* and *nodD*) are encoded by the majority of alpha- and beta-rhizobia studied thus far (Chen *et al.*, 2003; Fujishige *et al.*, 2008; Long, 2001). These genes are relatively conserved, so much so that they are often interchangeable between different bacteria without affecting the effectiveness of the symbiosis or the host specificity of the bacterium (Broughton and Perret, 1999; Hirsch, 1992; Peters, 1997). However, studies have shown that the substrates used by NodA and NodC as well as allelic variation within the genes do, in fact, affect host specificity (Kamst *et al.*, 1997; Peters, 1997; Roche *et al.*, 1996; Schultze and Kondorosi, 1998; Spaink, 2000). Together the proteins encoded by the common *nod* genes are responsible for the NF (Fujishige *et al.*, 2008; Fig. 1.4). The main lipochitooligosaccharide (LCO) core structure of the NF (Soto *et al.*, 2006) is provided by a chitooligosaccharide synthase NodC, the acetyl group is removed from the nonreducing end of the LCO by the NodB deacetylase and an acyl chain is added to the now vacant position on the LCO by the NodA acyltransferase (Broughton *et al.*, 2000; Peters, 1997; Schultze and Kondorosi, 1998). The fourth common *nod* gene, *nodD*, encodes the transcriptional activator NodD responsible for the expression of the *nod* genes (see below; Chen *et al.*, 2003; Dénarié *et al.*, 1992; Perret *et al.*, 2000).

Surprisingly, a small group of rhizobia, the so-called photosynthesising bradyrhizobia, have been shown not to possess either the *nodA* or *nodC* genes (Giraud *et al.*, 2007). These bacteria are stem-nodulators commonly associated with the aquatic legume genera *Aeschynomene* and *Sesbania* (Fleishman and Kramer, 1998). The fact that these rhizobia apparently lack NodA and NodC suggests the existence of signalling molecules other than the normal NFs for establishing the legume-rhizobium symbiosis (Masson-Boivin *et al.*, 2009). It has been suggested that this group might have included the common ancestor to all rhizobia because they possess both the ability to photosynthesise and to fix nitrogen and since they encode a potentially more primitive signalling molecule than contemporary NFs (Giraud *et al.*, 2007). The idea that these photosynthesising bradyrhizobia are simply

following a different evolutionary trajectory can however not be excluded (Giraud *et al.*, 2007).

The *hsn* are not functionally conserved among different species of rhizobia (Hirsch, 1992). The products of these genes are responsible for modification of the NF core so that it becomes recognisable by the specific plant host (Brewin, 1991; Downie and Walker, 1999; Peters, 1997; Spaink, 2000). Therefore, exchange of certain *hsn* genes among rhizobia and *hsn* gene mutations has been shown to not only lead to loss of function, but in some cases alteration of rhizobial host range (Hirsch, 1992; Mergaert *et al.*, 1997). The NF modifications brought about by *hsn* gene products vary a great deal among strains in terms of their specific positions along the length of the LCO, the types of fatty acid incorporated into the LCO and the number and type of substitutions found on the LCO (Cooper, 2007; Mergaert *et al.*, 1997). Examples of *hsn* genes include *nodEF* whose products determine the character of the *N*-linked acyl substituent that gets added onto the NF backbone by NodA (Peters, 1997) and *nolK* whose product might be responsible for adding a D-arabinose substitution onto the NF core structure of *Azorhizobium caulinodans* (Mergaert *et al.*, 1993). Not all *hsn* genes, however, are involved in NF structure as some of them seem to be responsible for transporting the NF out of the rhizobial cell e.g. *nolF*, *nolG*, *nolH* and *nolI* (Spaink, 2000).

1.3.2 Regulation of nodulation and the *nod* genes

Several different levels of specificity or control are encountered during the establishment of a root nodule. At the highest level there is specificity between the plant and bacterium which need to recognise each other in order to start interacting. The bacterium is responsible for the correct NF structure, which is determined by the products of the *nod* and *hsn* genes. The plant, on the other hand, is responsible for the release of the chemical signalling molecules (flavonoids) that interact with the bacterial regulator NodD (see below) to bring about expression of the nodulation genes (Geurts and Bisseling, 2002; Gibson *et al.*, 2008; Mergaert *et al.*, 1997; Perret *et al.*, 2000).

The next level of control is encountered at the level of the *nod* genes. The protein encoded by *nodD* plays a vital role in activating the transcription of those sets of *nod* genes responsible for the formation of NFs (Chen *et al.*, 2003; Dénarié *et al.*, 1992; Perret *et al.*,

2000). This gene is expressed continuously and suppresses the expression of all the genes in the *nod* operon unless plant inducer molecules, such as flavonoids, are present (Cooper, 2007; Hirsch, 1992). Situated within the *nod* operons are regulatory sections of DNA known as the *nod* boxes (Hirsch, 1992; Perret *et al.*, 2000), and it is to these motifs that NodD binds. Although flavonoids do not need to be present in order for this binding-process to take place, it is thought that a proper flavonoid-NodD combination can bind more securely to the *nod* boxes (Hirsch, 1992; Perret *et al.*, 2000), thus leading to the expression of the appropriate *nod* genes. The C-terminal of NodD is involved in the recognition of the specific host flavonoids while the N-terminal binds to the conserved *nod* boxes (Dénarié *et al.*, 1992; Hirsch, 1992). Certain rhizobial species possess in addition to *nodD* genes another symbiotic gene regulator (SyrM) which apparently functions without flavonoid induction (Hanin *et al.*, 1998; Spaink, 2000).

Some rhizobia have only one copy of *nodD* (e.g. *Rhizobium trifolii*), while others have multiple copies (e.g. *Bradyrhizobium japonicum* and *Ensifer meliloti*) (Dénarié *et al.*, 1992; Peck *et al.*, 2006). Those with multiple copies of *nodD* seem capable of recognising a variety of different flavonoids, thus potentially increasing the host range of that particular bacterium (Horvath *et al.*, 1987; Peck *et al.*, 2006; Perret *et al.*, 2000). Some bacteria might contain supplementary regulatory molecules other than the NodD. An example of this occurs in *B. japonicum* which also makes use of NodV and NodW proteins, which interact with one specific iso-flavonoid (Loh *et al.*, 1997; Loh and Stacey, 2003; Schultze and Kondorosi, 1998; Spaink, 2000). The presence of NodV which can recognise other signal molecules, than the flavonoids recognised by NodD, should also increase the possible host range of the bacterium (Loh *et al.*, 1997; Loh and Stacey, 2003). In such instances where a bacterium possesses multiple copies of *nodD*, regulation of this gene can become quite complicated as in some species the different copies are capable of regulating their own expression either by means of autoregulation or by regulation through yet another set of separate proteins (Loh and Stacey, 2003; Spaink, 2000). In *B. japonicum*, for instance, the function of the Nola protein is regulation of the *nodD2* gene (Loh *et al.*, 1999; Perret *et al.*, 2000; Schultze and Kondorosi, 1998; Spaink, 2000), while the expression of *nodD2* leads to the suppression of all the other *nod* genes (Loh and Stacey, 2003).

Negative regulation of *nodD* expression has been encountered in some rhizobia (e.g. *E. meliloti*), and appears to be necessary for efficient nodulation (Schultze and Kondorosi,

1998; Spaink, 2000). In *E. meliloti* the protein NolR was found to suppress the expression of *nodD* and therefore also those common *nod* genes responsible for the formation of NFs (Perret *et al.*, 2000; Spaink, 2000). The suppression of these genes is necessary before rhizobial differentiation into bacteroids can take place and nitrogen-fixation can commence. NolR appears to be present in several species within the genera *Sinorhizobium/Ensifer* and *Rhizobium*, but has not been found in species from *Bradyrhizobium*, *Azorhizobium* and *Mesorhizobium*, which indicates that a different method of negative regulation might exist in these genera (Kiss *et al.*, 1998).

1.4 Rhizobia and nitrogen-fixation

Biological nitrogen-fixation involves the production of ammonium from atmospheric nitrogen by the oxygen-sensitive enzyme, nitrogenase (Crespi and Gálvez, 2000; Dixon and Kahn, 2004; Franche *et al.*, 2009; Mylona *et al.*, 1995). Due to the toxicity of ammonium to the plant, it quickly assimilates this compound into amino acids (Crespi and Gálvez, 2000). Previous research has shown that the two nodule types differ in terms of amino acid export. Determinate nodules primarily export ureides such as allantoin and allantamide which are the products of purine oxidation (Todd *et al.*, 2006), while indeterminate nodules export amides in the form of glutamine and asparagine (Crespi and Gálvez, 2000; Ludwig and Poole, 2003; Prell and Poole, 2006).

There are different types of nitrogenases; the most abundant however, is the Mo-nitrogenase whose cofactor contains molybdenum while the others contain either vanadium or iron only (Franche *et al.*, 2009; Masson-Boivin *et al.*, 2009). Mo-nitrogenase consists of two protein subunits, the first consists of molybdenum iron (the MoFe-protein) and the second of only iron (the Fe-protein) (Rees *et al.*, 2005). The Fe-protein is responsible for providing the MoFe-protein with the necessary energy for the reduction of atmospheric nitrogen (Rees *et al.*, 2005).

Due to the oxygen-sensitivity of nitrogenase, oxygen is excluded from the nodule. This is accomplished by the structure of the nodule parenchyma which is characterised by a few small intercellular spaces to restrict the diffusion of oxygen from the environment into the nodule (Dixon and Kahn, 2004; Mylona *et al.*, 1995). Free oxygen in the symbiosome is further restricted by the presence of leghaemoglobin (an example of a nodulin), which is

encoded by plant symbiotic haemoglobin genes (Gualtieri and Bisseling, 2000; Perazzolli *et al.*, 2004; Trevaskis *et al.*, 1997). These genes differ from those encoding non-symbiotic haemoglobins which occur across the floral kingdom in both nitrogen-fixing and non-fixing plants alike (Perazzolli *et al.*, 2004; Trevaskis *et al.*, 1997), and can be classified based on their mode of induction, affinity for oxygen and nucleotide sequence (Garrocho-Villegas and Arredondo-Peter, 2008; Perazzolli *et al.*, 2004). Non-symbiotic haemoglobins are probably involved in signalling under adverse environmental conditions and during normal root development (Parent *et al.*, 2008), because they are able to scavenge the messenger molecule nitric oxide (NO) (Perazzolli *et al.*, 2004). In contrast the symbiotic plant haemoglobins are only produced within effective nodules (Gualtieri and Bisseling, 2000). It is thought that leghaemoglobin evolved from the non-symbiotic haemoglobin, since only three changes in the tertiary structure of non-symbiotic haemoglobin can result in the formation of leghaemoglobin (Garrocho-Villegas and Arredondo-Peter, 2008).

Despite sensitivity to oxygen, the reduction of nitrogen by the bacteroids is entirely dependent upon the energy created by oxidative phosphorylation or respiration (Mylona *et al.*, 1995; Preisig *et al.*, 1996). The carbon needed for bacteroid respiration is provided by the plant which receives fixed nitrogen in return (Lodwig and Poole, 2003). In order to accomplish the generation of sufficient energy for the process under a very low oxygen concentration rhizobia make use of a customised electron transport pathway that ends with a cytochrome oxidase, *cbb₃*. This oxidase has an elevated affinity for oxygen (Preisig *et al.*, 1996) and is coded for by the *fixNOQP* genes of which only *fixQ* seems to be redundant (Preisig *et al.*, 1996; Zufferey *et al.*, 1996).

1.4.1 The genetic determinants of nitrogen-fixation

The two classes of genes involved in fixing atmospheric nitrogen into a form usable by the plant are the *nif* and *fix* genes. The *nif* genes are found in all diazotrophic organisms whether they are symbiotic or not and are responsible for the formation, structure and regulation of nitrogenase (Dixon and Kahn, 2004). The *fix* genes, however, are only encountered in symbiotic nitrogen-fixers and could possibly be involved in functions such as the development of bacteroids (Fischer, 1994), although homologues of certain *fix* genes have also been found in non-diazotrophs (Dixon and Kahn, 2004). Both classes are tightly packed on mobile genetic elements that may or may not be the same as those harbouring the *nod*

genes. For example, the *nif* genes are carried on single plasmids in *Rhizobium leguminosarum* (Long, 2001), *Ensifer meliloti* (Barnett *et al.*, 2001) and *Cupriavidus taiwanensis* (Amadou *et al.*, 2008), and on the same chromosomal island in *Bradyrhizobium japonicum* (Kuykendall, 2005a). In certain cases such as *Mesorhizobium loti*, the symbiosis loci are spread out across plasmids and/or chromosomes (Chen *et al.*, 2005c; Long, 2001).

There is an unexpected variation in the number of *nif* genes between rhizobial genera (Masson-Boivin *et al.*, 2009). *Rhizobium leguminosarum* bv. *viciae* encodes only eight *nif* genes while *E. meliloti* (still referred to as ‘*S. meliloti*’ by the authors) encodes nine, and these two rhizobia possess the lowest number of *nif* genes for all described nitrogen-fixers up to date (Masson-Boivin *et al.*, 2009). More *nif* genes are found in the *Sesbania rostrata* nodulator, *Azorhizobium caulinodans*, and the informal grouping of photosynthetic bradyrhizobia who both contain 15 genes (Masson-Boivin *et al.*, 2009). This is not so different from the 20 genes first described in the most studied diazotrophic bacterium *Klebsiella pneumoniae*, but the loss of so many genes from the previously discussed bacteria could either mean that novel genes are fulfilling the traditional roles or that these bacteria are in fact streamlining their nitrogen-fixing process (Masson-Boivin *et al.*, 2009).

Nitrogenase is composed of the products of the *nifH* gene that encodes the iron protein and *nifDK* that encodes the MoFe-protein (Fisher, 1994; Franche *et al.*, 2009; Mylona *et al.*, 1995). The active form of this enzyme requires binding of a MoFe cofactor which is constructed out of the products of the *nifENB* genes (Fisher, 1994). As the cofactor becomes irreversibly denatured in the presence of oxygen, inactivation of the enzyme is prevented by the exclusion of oxygen from the nodule (Mylona *et al.*, 1995). Leghaemoglobin is of primary importance, however, in delivering oxygen to the bacteroids at tensions sufficiently low enough to prevent denaturation of oxygen-sensitive nitrogen-fixation molecules, while maintaining the function of other oxidative metabolic processes (Pawlowski, 2008).

1.4.2 Regulation of nitrogen-fixation and the genes involved

Regulation of the nitrogen-fixation process and the genes which form part of it occurs primarily at the transcriptional level (Dixon and Kahn, 2004). It is a tightly controlled process, not only due to the large energy input required, but also due to the slow production rate of the enzyme and the oxygen sensitivity of the whole system (Dixon and Kahn, 2004;

Fischer, 1994). Although there is some variation in the regulatory pathway among rhizobia, NifA and sigma factor 54 (σ^{54}) (also known as RpoN, NtrA or σ^N) are central to all of them (Dixon and Kahn, 2004). The σ^{54} protein binds to the bacterial RNA polymerase, thus directing the transcription machinery to the promoters of the nitrogen-fixation genes (Buck *et al.*, 2000). However, initiation of transcription by the σ^{54} bound RNA polymerase depends on activation by NifA, which is a member of the enhancer-binding protein family (Buck *et al.*, 2000; Dixon and Kahn, 2004).

Free-living diazotrophic bacteria regulate the expression of their nitrogen fixation genes in response to oxygen and nitrogen levels, while symbiotic nitrogen-fixers only react to oxygen levels (Dixon and Kahn, 2004; Fischer, 1994; Masson-Boivin *et al.*, 2009). Obviously the symbiotic diazotrophs would require a system that could detect when enough nitrogen has been fixed. Some rhizobia have specific genes to achieve this goal (Arcondéguy *et al.*, 2001; Dixon and Kahn, 2004; Michel-Reydellet and Kaminski, 1999).

To detect low oxygen-levels, some rhizobia make use of the FixL-FixJ regulatory system to activate transcription of *nifA* (Masson-Boivin *et al.*, 2009). In this regulatory system, FixL acts as an oxygen sensor. When bound to oxygen this protein cannot activate FixJ, while in low-oxygen conditions FixL leads to the formation of phosphorylated FixJ, which facilitates transcription of both *nifA* and *fixK* (Dixon and Kahn, 2004; Foussard *et al.*, 1997; Hérouart *et al.*, 2002; Mylona *et al.*, 1995). FixK activates expression of the genes encoding cytochrome oxidase *cbb₃* (Fischer, 1994; Foussard *et al.*, 1997), as well as FixT, which is involved in the regulation of the expression of the FixL-FixJ system (Foussard *et al.*, 1997; Garnerone *et al.*, 1999). Some of the proteins might fulfil slightly altered roles in different rhizobia, such as FixK which in *Azorhizobium caulinodans* activates *nifA* expression instead of FixJ (Bobik *et al.*, 2006; Dixon and Kahn, 2004).

Some rhizobia such as *Bradyrhizobium japonicum* sense redox conditions through a system that incorporates the proteins RegR-RegS (Bauer *et al.*, 1998; Dixon and Kahn, 2004; Lindemann *et al.*, 2007). The objective of this system is to control the expression of the *fixR-nifA* operon (Emmerich *et al.*, 2000), which has two overlapping but differentially regulated promoters – *fixRp₁* and *fixRp₂* (Emmerich *et al.*, 2000; Lindemann *et al.*, 2007; Sciotti *et al.*, 2003). The first promoter, *fixRp₁*, is automatically switched on by NifA (thereby NifA indirectly directs its own expression) only under conditions of low oxygen concentration and

is recognised by σ^{54} (Emmerich *et al.*, 2000; Lindemann *et al.*, 2007). The second promoter, *fixRp₂*, forms part of the RegR-RegS system and remains constantly active (under both aerobic and anaerobic conditions) (Emmerich *et al.*, 2000). This promoter requires the binding of RegR to an upstream activating sequence. If this protein cannot bind to the upstream activating sequence, expression of *fixR-nifA* under aerobic conditions no longer takes place, while expression under anaerobic conditions become severely diminished (Emmerich *et al.*, 2000). The function of the RegS protein in this regulatory system is to phosphorylate the RegR protein, after which the DNA binding capacity of RegR increases dramatically (Emmerich *et al.*, 2000; Lindemann *et al.*, 2007).

The alpha-rhizobium *Azorhizobium caulinodans* is capable of fixing nitrogen during symbiosis as well as when it is free-living (Michel-Reydellet and Kaminski, 1999). During growth in the rhizosphere nitrogen-fixation is linked to ammonia assimilation, although the link between these two processes becomes unnecessary during symbiosis (Michel-Reydellet and Kaminski, 1999). In this bacterium two P_{II}-like proteins are responsible for controlling the link between nitrogen-fixation and ammonia assimilation. It is thought that P_{II} proteins are fundamental in processing the cell signals responsible for reporting the cell's carbon and nitrogen condition, which in turn determines nitrogen assimilation of the bacterium (Ninfa and Atkinson, 2000). They probably accomplish this by assuming specific conformations under specific conditions, thus allowing recognition by the appropriate receptors and facilitating the required cellular outcomes (Ninfa and Atkinson, 2000). Two genes with P_{II}-like protein products have been found in *A. caulinodans*. They encode GlnB whose gene is transcribed together with the glutamine synthetase - *glnA* involved in nitrogen assimilation, and GlnK whose gene is co-transcribed with a likely ammonium transporter gene, *amtB* (Michel-Reydellet and Kaminski, 1999). If ammonia is present then either one of these proteins are able to repress nitrogen-fixation or in an environment suitable to nitrogen-fixation they are responsible for deactivating glutamine synthetase, thereby halting ammonium assimilation (Michel-Reydellet and Kaminski, 1999).

1.5 The evolution of the legume-rhizobium symbiosis

Hypotheses on the evolution of the legume-rhizobium symbiosis require consideration of the legumes and their evolution; rhizobia and their evolution and also the evolution of their interaction. It is possible that low nitrogen conditions (Sprent, 2007) resulted in the

development of a predisposition towards the formation of a nitrogen-fixing symbiosis in the last common ancestor of the plants capable of the nodule-forming symbiosis (Soltis *et al.*, 1995; Soltis *et al.*, 2000). This predisposition in the legume hosts most probably also involved incorporation of elements from the older and more wide-spread arbuscular endomycorrhiza (Gualtieri and Bisseling, 2000; Lum *et al.*, 2002). Because the loci responsible for these nodule-forming and nitrogen-fixing abilities are transferable among similar genomic frameworks, the legume-rhizobium symbiosis has undoubtedly influenced the evolution of the rhizobia extensively (Bailly *et al.*, 2007; Crossman *et al.*, 2008; Moulin *et al.*, 2004).

1.5.1 Legume evolution

Legumes are not the only plants which enter into a nitrogen-fixing symbiosis with rhizobia. Plants in the genus *Parasponia* (family Ulmaceae) may also be nodulated by rhizobia (Gualtieri and Bisseling, 2000; Sprent, 1994; Sprent, 2007). This is also true for the so-called actinorhizal plants that represent a varied collection of genera from eight families, that can enter into symbiosis with species from the gram-positive bacterial genus *Frankia* (Gualtieri and Bisseling, 2000; Markmann and Parniske, 2008). However, all of these diverse hosts form part of the Rosid I clade of angiosperms. It is therefore, speculated that the most recent ancestor of this group developed a predilection towards nodulation (Soltis *et al.*, 1995; Soltis *et al.*, 2000), which probably involved some form of genetic innovation that would have served as the source of evolution for all subsequent root nodule endosymbioses (Markmann and Parniske, 2008). According to this hypothesis, all root nodule forming symbioses would share certain genetic traits (Markmann and Parniske, 2008). Indeed, experimental results now confirm the presence of shared genetic traits between the plants that establish symbiosis with rhizobia, *Frankia* species and arbuscular mycorrhiza (see below) (Banba *et al.*, 2008; Gherbi *et al.*, 2008; Markmann *et al.*, 2008; Markmann and Parniske, 2008). Among the descendants of this ancestor the process of nodulation probably evolved multiple times independently in different orders within the Rosid I lineage (Gualtieri and Bisseling, 2000; Markmann and Parniske, 2008; Soltis *et al.*, 1995). In many instances, this ability was subsequently lost, leading to the occurrence of non-nodulating species within these mainly nodulating orders of Rosid I (Gualtieri and Bisseling, 2000; Markmann *et al.*, 2008).

Using the fossil record to estimate the temporal and geographic origins of legumes has proven problematic (Doyle and Luckow, 2003; Herendeen *et al.*, 1992; Sprent, 2007). This is because legumes only became abundant in fossils during the Eocene or middle of the Tertiary period around 30-40 million years ago (mya) (Doyle and Luckow, 2003; Herendeen *et al.*, 1992). There is also a clear geographical bias in the abundance of legumes from the different regions, since the fossils from Africa and South America show much less diversity than those from North America and Europe (Doyle and Luckow, 2003), which may reflect genuine legume diversity at that time or alternatively inferior preservation or less thorough sampling (Doyle and Luckow, 2003).

Currently it is most widely believed that legumes evolved near the beginning of the Tertiary period around 60 mya (Lavin *et al.*, 2005; Sprent, 2007). This was followed by a swift diversification and at around 50 mya the three subfamilies (Caesalpinioideae, Mimosoideae and lastly Papilionoideae) were already established (Doyle and Luckow, 2003; Herendeen *et al.*, 1992; Lavin *et al.*, 2005; Lewis and Schrire, 2003; Sprent, 2007). This is in contrast to previous suggestions that the three subfamilies evolved in the sequence: Caesalpinioideae, Mimosoideae and lastly Papilionoideae (Lavin *et al.*, 2005; Sprent, 2007). The fact that the occurrence of nodulation follows this same pattern, with nodulation becoming more abundant with a progression from Caesalpinioideae to Papilionoideae, has been seen as indirect support for this order of evolution. However, the most recent data suggest that within the space of 1 to 2 million years after the emergence of their ancestor, legumes with traits of the Caesalpinioideae and Papilionoideae respectively emerged, while the mimosoid legumes split off from those with caesalpinoid traits about 40 million years later (Lavin *et al.*, 2005; Sprent 2007), making the Mimosoideae the youngest subfamily.

There are a couple of theories regarding the dispersal of the legumes from their centre of origin. According to the Gondwanan hypothesis, legumes had an African origin and subsequently moved to South America and from there to North America (Doyle and Luckow, 2003). This would have been during the late Cretaceous (65 to 145 mya), although more recent legume phylogenies and continental drift data do not support this hypothesis (Doyle and Luckow, 2003; Pennington *et al.*, 2006). Accordingly, the alternative boreotropical hypothesis was introduced (Doyle and Luckow, 2003) which suggests that the Leguminosae originated at the northern side of the Tethys Sea (Schrire *et al.*, 2005; Sprent, 2007) which existed during the Eocene (35 to 55 mya) within the vast and uninterrupted land

mass consisting of Europe, North America and Africa (Doyle and Luckow, 2003). The increasingly cooler climate and eventual break up of this large land mass resulted in the current legume distribution (Doyle and Luckow, 2003; Sprent, 2007). Although this hypothesis seems plausible, it does not explain the disjoint geographic distribution patterns of certain legume groups that apparently depend more strongly on climatic factors such as temperature and rainfall (Sprent, 2007). Some authors therefore invoke additional dispersal mechanisms such as tropical storms (Nathan, 2006; Sprent, 2007), sea and/or wind currents across oceans (Pennington *et al.*, 2006; Renner, 2004; Sprent, 2007) to explain the current legume distribution.

1.5.2 Evolution of the rhizobia

As mentioned in the introduction, the nodule-forming bacteria or rhizobia by no means form a monophyletic group (Amadou *et al.*, 2008; Sawada *et al.*, 2003). The majority of the rhizobial genera include species capable of nodulating legumes in addition to species that lack this ability, many of which are pathogens or of some biochemical importance (Coenye and Vandamme, 2003; Sawada *et al.*, 2003; van Berkum and Eardly, 2002; Young *et al.*, 2006; Young and Haukka, 1996). The genus *Burkholderia*, for instance, contains species capable of nodulation, nitrogen-fixation, as well as plant and animal pathogenicity while others also have plant-growth promoting activities (Coenye and Vandamme, 2003).

Estimations based on phylogenetic analyses of the glutamine synthetases genes suggests that the fast- and slow-growing rhizobia split from each other 500 mya (Hirsch *et al.*, 2001; Turner and Young, 2000); in other words, bacteria from the slow-growing genus *Bradyrhizobium* appeared around this time. Then at about 200-300 mya, the fast-growing rhizobia such as *Sinorhizobium/Ensifer* and *Rhizobium* split (Hirsch *et al.*, 2001; Turner and Young, 2000). Therefore, the Alpha- and Betaproteobacteria had to have separated even earlier than 500 mya (Young *et al.*, 2006). However, all these estimates are based upon the assumption that nodule-associated nitrogen-fixation first occurred within bacteria of the order Rhizobiales. The idea that a “rhizobial predisposition” appeared in other bacteria from which it was subsequently lost cannot be excluded.

As with other bacteria, horizontal gene transfer (HGT) has played an important role in the evolution of rhizobia (Bailly *et al.*, 2007; Moulin *et al.*, 2004; Steenkamp *et al.*, 2008;

Sullivan and Ronson, 1998). HGT refers to the transfer of genetic information between dissimilar or unrelated organisms, which is facilitated by mobile genetic elements such as transposons or plasmids (Gibson *et al.*, 2008). Not surprisingly, the genetic information determining the legume-rhizobium symbiosis is carried either on such transmissible plasmids or as symbiosis islands imbedded in the bacterial chromosome. These symbiotic plasmids or islands typically form part of the accessory genome and have marked differences when compared to the core genome (Young *et al.*, 2006), both in terms of gene and nucleotide content as well as in the rate of evolution (Crossman *et al.*, 2008). Consequently, the accessory component may undergo faster evolutionary changes, thus enabling bacteria with an initial standard complement of genetic information to adapt to and occupy a variety of environmental niches, including the establishment of different symbioses with diverse hosts (Crossman *et al.*, 2008). One example of a bacterium that is thought to have received its diazotrophic and nodulating capabilities by means of HGT is the beta-rhizobium *Cupriavidus taiwanensis* LMG 19424 (Amadou *et al.*, 2008).

Of interest is that there seems to be a significant association between *nod* genotypes and host plant groupings. This is quite logical since the *nod* genotypes confer host specificity (Wernegreen and Riley, 1999). Consequently, the term “biovar” was introduced to distinguish bacteria with different symbiotic capabilities within a particular species (Wang *et al.*, 1999a). Thus different biovars of the same species, while possessing similar chromosomal backgrounds exhibit differences in their symbiotic plasmids or chromosomal elements that radically influence their respective host ranges. Such instances occur frequently in the genus *Rhizobium*. For example *R. gallicum* bv. *gallicum* and *R. gallicum* bv. *phaseoli* differ in their ability to effectively nodulate *Leucaena* and *Phaseolus* spp. respectively (Amarger *et al.*, 1997). HGT of these mobile symbiotic elements could therefore lead to the development of novel biovars and plant host ranges.

1.5.3 Evolution of the nodulation symbiosis

Several possible origins of nodule formation have been suggested. One is that nodulation might have stemmed from processes involved in the formation of arbuscular endomycorrhiza (AM) (Gualtieri and Bisseling, 2000; Lum *et al.*, 2002; Szczyglowski and Amyot, 2003). The AM symbiosis is formed between fungi of the phylum Glomeromycota (Hibbett *et al.*, 2007) and approximately 80% of higher plants (Gualtieri and Bisseling, 2000). This

association appears to have been present as early as the most primitive land plants (Brundrett, 2002; Markmann *et al.*, 2008). There is a strong likelihood that the two symbiotic interactions are related as they both utilise similar host genes to establish the respective symbioses (Albrecht *et al.*, 1999; Gianinazzi-Pearson, 1996; Gualtieri and Bisseling, 2000; Harrison, 1997; Hirsch *et al.*, 2001; van Rhijn *et al.*, 1997).

A second possibility is that two separate nodulation situations concurrently gave rise to the whole spectrum of nodulation and infection processes seen today (Sprent, 1994). The first of these would involve infection of a wound on the stem of a tropical legume by a *Bradyrhizobium*-like ancestor with the ability to photosynthesise, as well as, nodulate (Sprent, 1994). This interaction probably did not involve the formation of infection threads (Sprent, 1994; Sprent, 2008). The second situation would have been more parasitic in nature and would have occurred between a *Rhizobium*-like ancestor and the roots of a legume, eventually leading to the formation of infection threads (Sprent, 1994; Sprent, 2008; Fig. 1.5).

Sprent (2007) suggests that the evolution of the legume-rhizobium symbiosis was driven by a shortage in available nitrogen. At around 55 mya there was a radical increase in humidity and temperature coupled with the large-scale release of carbon dioxide and methane from the sea-floor into the atmosphere (Sprent, 2007). This could have caused a corresponding decrease in the available nitrogen, thus leading to the development of nodulation in rhizobia at that time. According to Sprent (2007) this scenario seems probable for the evolution of the dalbergioids, genistoids and nodulating groups of the Caesalpinioideae.

1.6 Taxonomy of the legume genus *Hypocalyptus*

Hypocalyptus represents a plant genus in the subfamily Papilionoideae and larger family Leguminosae (Lewis and Schrire, 2003). Its members are restricted to the Eastern and Western Cape areas in the Cape Floristic Region (CFR) (Schutte and van Wyk, 1998a). Only three *Hypocalyptus* species are known (*H. oxalidifolius*, *H. coluteoides* and *H. sophoroides*), and all three form part of the fynbos vegetation of this biodiversity hotspot (Schutte and van Wyk, 1998a). All of them species have trifoliolate leaves and magenta-pink flowers with yellow nectar guides, intrusive calyx bases and fused stamens (Schutte and van Wyk, 1998a; Fig. 1.6).

1.6.1 The tribe *Hypocalypteae*

Classification of the genus *Hypocalyptus* has proven to be quite difficult. Originally it was placed within the subtribe Genistinae of the tribe Loteae by Bentham when he first described the Papilionoideae in 1873 (Schutte and van Wyk, 1998a). However, he later changed the taxonomy of the tribe Loteae so that *Hypocalyptus* formed part of the subtribe Cytisinae within the tribe Genisteae (Schutte and van Wyk, 1998a), although Harvey did away with the “subtribe” ranks of the tribe Genisteae in 1862. More than a century later, Hutchinson also recognised *Hypocalyptus* as part of the subtribe Cytisinae, which he elevated to tribal status Harborne, 1969; Schutte and van Wyk, 1998a). In 1981 the genus was moved to the tribe Lipariae by Polhill, which was subsequently separated into two divisions, with one representing the subtribe Lipariinae and the other the subtribe Hypocalyptinae that included the genus, *Hypocalyptus* (Schutte and van Wyk, 1998a). Finally, in 1998 the genus was moved to the tribe Hypocalypteae (Yakovlev) A.L. Schutte of which it is the only member (Schutte and van Wyk, 1998a).

1.6.1.1 *Hypocalyptus* as a member of the tribe Cytiseae

Hutchinson recognised *Hypocalyptus* as a member of the tribe Cytiseae after he divided the genera in the tribe Genisteae into four tribes: the Cytiseae, Laburneae, Genisteae and Lupineae (Harborne, 1969). This classification scheme of Hutchinson was not readily accepted because of anomalies concerning the distribution and morphology of the tribes. Following a study of their chemical characteristics, Harborne (1969), suggested that the genera *Loddigesia* and *Hypocalyptus* should be removed from the Genisteae group and instead be integrated into the genus *Crotalaria* in the tribe Crotalarieae. This was because neither *Loddigesia* nor *Hypocalyptus* possess flavones and isoflavones although these are present in most of the remaining Genisteae (Harborne, 1969). The above two genera, as well as the genus *Crotalaria*, were also the only genera of the 22 examined that tested positive for leucoanthocyanidin. However, Harborne’s suggestions were never put into practice and *Hypocalyptus* was never formally transferred to the tribe Crotalarieae.

1.6.1.2 *Hypocalyptus* as a member of the tribe Lipariae

Polhill (1981) later recognised *Hypocalyptus* as a member of the tribe Lipariae. Even though he recognised differences between *Hypocalyptus* and all other genera in the Lipariae, he still believed that this genus was the closest to those in the combined Lipariae

and Podalyrieae. Later, Schutte and van Wyk (1998b) analysed chemical, morphological and cytological data for *Hypocalyptus* and the genera contained within the southern African tribes Liparieae and Podalyrieae. They found that the Liparieae and Podalyrieae are monophyletic and proposed that their respective genera be grouped together within the Podalyrieae. However, they concluded that the genus *Hypocalyptus* could not form part of this grouping due to morphological discrepancies (Schutte and van Wyk, 1998b). For example, flower colouring among the genera of the Liparieae and Podalyrieae is diverse, ranging from yellow, orange-red, white, pink to purple with white or a deep violet nectar guide. In contrast, all *Hypocalyptus* species have magenta pink flowers and a yellow nectar guide (Schutte and van Wyk, 1998b).

As far as chemical characteristics are concerned, *Hypocalyptus* is the only genus in this group that accumulates the amino acid canavanine in its seed coat, while it and *Cyclopia* are the only genera that do not produce alkaloids (Schutte and van Wyk, 1998b). Also, the genera *Hypocalyptus*, *Cyclopia* and *Virgilia* are all exceptions when considering chromosome base numbers in the tribes Liparieae and Podalyrieae (Schutte and van Wyk, 1998b). While the rest of the genera have a base number of $x = 9$, *Hypocalyptus* possesses a number of $x = 10$, *Virgilia* has a number of $x = 27$ and *Cyclopia* a number of $x = 18$.

1.6.1.3 *Hypocalyptus* as a member of the tribe Hypocalypteae

In 1998, Schutte and van Wyk (1998a) suggested the removal of *Hypocalyptus* from the Liparieae. Analysis of secondary metabolites, revealed the presence of minute quantities of 'unique' flavonoids in *Hypocalyptus* seeds that were not encountered in any of the 87 genistoid species they studied. Another similar study focusing specifically on seed flavonoids further found that *Hypocalyptus* possessed none of the main seed flavonoids found in the Podalyrieae or Liparieae (De Nysschen *et al.*, 1998). An earlier study by van Wyk *et al.*, (1995) of anthocyanin (pink and purple flower pigments) production also revealed the unique nature of *Hypocalyptus*; all the Liparieae genera contained cyaniding-3-glucoside, whereas *Hypocalyptus* contained malvidin-3-glucoside.

The phylogeny of the tribes contained within the genistoid alliance was investigated by Crisp *et al.* (2000), using ribosomal RNA internal transcribed spacer (ITS) regions and the chloroplast gene *rbcL* that encodes the ribulose-1-5-bisphosphate carboxylase/oxygenase (Doyle *et al.*, 1997). The tribes in this alliance are largely from the southern hemisphere and

are presumed to be related (i.e., Genisteae, Crotalariae, Podalyrieae, Liparieae, Hypocalypteae, Thermopsidae, Euchresteae, Bossiaeeae, Mirbelieae and Brongniartieae) (Crisp *et al.*, 2000). The results of this study showed that the genistoid genera are separated into four large clades (Crisp *et al.*, 2000; Fig. 1.7). The species of the Hypocalypteae formed a monophyletic group separate from all other genistoids, and was placed as a sister group to the tribe Indigofereae and the ‘inverted repeat loss’ or IRL clade (Crisp *et al.*, 2000; Lavin *et al.*, 1990). These findings further emphasise that the genus *Hypocalyptus* does indeed fit best into a tribe all of its own: the Hypocalypteae (Schutte and Van Wyk, 1998a).

1.6.2 The rhizobia of the Hypocalypteae and its relatives

Despite substantial research on the systematics of *Hypocalyptus* and the genera in the allied tribe Podalyrieae (Schutte and van Wyk, 1998a; van der Bank *et al.*, 2002), very little is known about the rhizobia of these plants. Grobbelaar and Clarke (1972) observed that *H. sophoroides* species are nodulated, but the possible identity of their nodulating symbionts was not reported. More recent information is available on the rhizobia of species in the tribe Podalyrieae, however. The genus *Cyclopia* is nodulated by diverse rhizobia from the genus *Burkholderia* (Kock, 2003; Spriggs and Dakora, 2007), and certain genera such as *Bradyrhizobium* and *Rhizobium* in the Alphaproteobacteria (Kock, 2003). Interestingly, both *Aspalathus* and *Cyclopia* are nodulated by *Burkholderia tuberum* (Elliott *et al.*, 2007a), suggesting that their rhizobial symbionts are capable of cross-nodulation. Although a complete picture regarding the rhizobial symbionts of legumes in the tribes Hypocalypteae and Podalyrieae has yet to emerge, my present study aims to form the basis for future work in this interesting field.

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1.8 Tables

Table 1.1 Taxonomic placement of alpha- and beta-rhizobia

Class	Order	Family	Genus	Genus and species	Host Plant	References
Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	<i>Azorhizobium</i>	<i>A. caulinodans</i>	<i>Sesbania rostrata</i>	Dreyfus <i>et al.</i> , 1988
Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	<i>Azorhizobium</i>	<i>A. doebereineriae</i>	<i>Sesbania virgata</i>	de Souza Moreira <i>et al.</i> , 2006
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. canariense</i>	<i>Chamaecytisus proliferus</i> subsp. <i>proliferus</i> var. <i>palmensis</i>	Vinuesa <i>et al.</i> , 2005
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. denitrificans</i>	<i>Aeschynomene indica</i>	van Berkum and Eardly, 2002
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. elkanii</i>	<i>Glycine</i> spp., <i>Macroptilium atropurpureum</i> , <i>Vigna unguiculata</i> , <i>V. radiata</i> , non-nodulating <i>rjlrjI</i> soybean	Kuykendall, 2005a
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. iriomotense</i>	<i>Macroptilium atropurpureum</i> , <i>Entada koshunensis</i>	Islam <i>et al.</i> , 2008
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. japonicum</i>	<i>Glycine</i> spp., <i>Macroptilium atropurpureum</i>	Kuykendall, 2005a
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. japonicum</i> bv <i>genistearum</i>	<i>Lupinus</i> spp., <i>Adenocarpus</i> spp., <i>Teline</i> spp., <i>Chamaecytisus proliferus</i> , <i>Spartocytisus supranubius</i> , <i>Ornithopus</i> spp.	Vinuesa <i>et al.</i> , 2005
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. japonicum</i> bv <i>glycinearum</i>	<i>Glycine</i> spp.	Vinuesa <i>et al.</i> , 2005
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. jicamae</i>	<i>Pachyrhizus erosus</i> , <i>Lespedeza</i> spp.	Ramírez-Bahena <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. liaoningense</i>	<i>Glycine max</i>	Xu <i>et al.</i> , 1995
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. pachyrhizi</i>	<i>Pachyrhizus erosus</i>	Ramírez-Bahena <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. yuanmingense</i>	<i>Lespedeza cuneata</i> , <i>Vigna unguiculata</i> , <i>Glycyrrhiza uralensis</i>	Yao <i>et al.</i> , 2002
Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	<i>Devosia</i>	<i>D. neptuniae</i>	<i>Neptunia natans</i>	Rivas <i>et al.</i> , 2003
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. arboris</i>	<i>Acacia senegal</i> , <i>Prosopis chilensis</i>	Nick <i>et al.</i> , 1999
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. fredii</i>	<i>Glycine max</i> , <i>G. soja</i> , <i>Vigna unguiculata</i> , <i>Cajanus cajan</i>	Scholla and Elkan, 1984; Chen <i>et al.</i> , 1988
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. fredii</i> bv <i>mediterraneanse</i>	<i>Phaseolus vulgaris</i>	Mnasri <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. garamanticus</i>	<i>Argyrolobium uniflorum</i> , <i>Medicago sativa</i>	Merabet <i>et al.</i> , 2010
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. kostiensis</i>	<i>Acacia senegal</i> , <i>Prosopis chilensis</i>	Nick <i>et al.</i> , 1999
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. kummerowiae</i>	<i>Kummerowia stipulacea</i>	Wei <i>et al.</i> , 2002

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Class	Order	Family	Genus	Genus and species	Host Plant	References
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. medicae</i>	<i>Medicago truncatula</i> , <i>M. orbicularis</i> , <i>M. polymorpha</i> , <i>M. rugosa</i>	Rome <i>et al.</i> , 1996
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. meliloti</i>	<i>Melilotus</i> spp., <i>Medicago</i> spp., <i>Trigonella</i> spp., <i>Glycine max</i>	de Lajudie <i>et al.</i> , 1994; Kuykendall <i>et al.</i> , 2005b
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. meliloti</i> bv <i>acaciae</i>	<i>Acacia tortilis</i>	Ba <i>et al.</i> , 2002
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. meliloti</i> bv <i>ciceri</i>	<i>Cicer arietinum</i>	Maâtallah <i>et al.</i> , 2002
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. meliloti</i> bv <i>lancerottense</i>	<i>Lotus lancerottensis</i> , <i>L. corniculatus</i> , <i>L. japonicus</i>	León-Barrios <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. meliloti</i> bv <i>medicaginis</i>	<i>Medicago laciniata</i>	Villegas <i>et al.</i> , 2006
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. meliloti</i> bv <i>mediterraneanse</i>	<i>Phaseolus vulgaris</i>	Mnasri <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. meliloti</i> bv <i>meliloti</i>	<i>Medicago sativa</i> , <i>M. truncatula</i>	Villegas <i>et al.</i> , 2006
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. mexicanus</i>	<i>Acacia angustissima</i> , <i>A. cochliacantha</i> , <i>Leucaena leucocephala</i> , <i>Phaseolus vulgaris</i>	Lloret <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. mexicanus</i> bv <i>acaciellae</i>	<i>Acaciella angustissima</i>	Rogel <i>et al.</i> , 2011
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. numidicus</i>	<i>Lotus creticus</i> , <i>Argyrolobium uniflorum</i>	Merabet <i>et al.</i> , 2010
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. saheli</i>	<i>Sesbania</i> spp., <i>Acacia seyal</i> , <i>Leucaena</i> <i>leucocephala</i> , <i>Neptunia oleracea</i>	de Lajudie <i>et al.</i> , 1994
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. terangae</i>	<i>Sesbania</i> spp., <i>Acacia</i> spp., <i>Leucaena</i> <i>leucocephala</i> , <i>Neptunia oleracea</i>	de Lajudie <i>et al.</i> , 1994
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Ensifer</i>	<i>E. xinjiangensis</i>	<i>Glycine max</i>	Chen <i>et al.</i> , 1988
Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	<i>Labrys</i>	<i>L. neptuniae</i>	<i>Neptunia oleracea</i>	Chou <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	<i>Labrys</i>	<i>L. okinawensis</i>	<i>Entada phaseoloides</i>	Islam <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. albiziae</i>	<i>Albizia kalkora</i>	Wang <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. alhagi</i>	<i>Alhagi sparsifolia</i>	Chen <i>et al.</i> , 2010
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. amorphae</i>	<i>Amorpha fruticosa</i>	Wang <i>et al.</i> , 1999b
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. australicum</i>	<i>Biserrula pelecinus</i>	Nandasena <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. camelthorni</i>	<i>Alhagi sparsifolia</i>	Chen <i>et al.</i> , 2011
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. caraganae</i>	<i>Caragana microphylla</i> , <i>C. intermedia</i> , <i>Glycyrrhiza uralensis</i> , <i>Astragalus adsurgens</i> , <i>Phaseolus vulgaris</i>	Guan <i>et al.</i> , 2008

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Class	Order	Family	Genus	Genus and species	Host Plant	References
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. chacoense</i>	<i>Prosopis alba</i>	Velázquez <i>et al.</i> , 2001
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. ciceri</i>	<i>Cicer arietinum</i>	Nour <i>et al.</i> , 1994; Jarvis <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. ciceri</i> by <i>biserrulae</i>	<i>Biserrula pelecinus</i> , <i>Astragalus membranaceus</i>	Nandasena <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. gobiense</i>	<i>Oxytropis glabra</i> , <i>Glycyrrhiza uralensis</i> , <i>Lotus corniculatus</i> , <i>Robinia pseudoacacia</i>	Han <i>et al.</i> , 2008a
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. huakuii</i>	<i>Astragalus sinicus</i>	Chen <i>et al.</i> , 1991; Jarvis <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. loti</i>	<i>Lotus corniculatus</i> , <i>L. tenuis</i> , <i>L. japonicus</i> , <i>L. krylovii</i> , <i>L. filicalius</i> , <i>L. schoelleri</i>	Jarvis <i>et al.</i> , 1982; Jarvis <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. mediterraneum</i>	<i>Cicer arietinum</i>	Nour <i>et al.</i> , 1995; Jarvis <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. metallidurans</i>	<i>Anthyllis vulneraria</i>	Vidal <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. opportunistum</i>	<i>Biserrula pelecinus</i>	Nandasena <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. plurifarum</i>	<i>Acacia senegal</i> , <i>A. tortilis</i> subsp. <i>raddiana</i> , <i>A. nilotica</i> , <i>A. seyal</i> , <i>Leucaena leucocephala</i> , <i>Neptunia oleracea</i>	de Lajudie <i>et al.</i> , 1998b
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. robiniae</i>	<i>Robinia pseudoacacia</i>	Zhou <i>et al.</i> , 2010
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. septentrionale</i>	<i>Astragalus adsurgens</i>	Gao <i>et al.</i> , 2004
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. shangrilense</i>	<i>Caragana bicolor</i> , <i>C. erinacea</i> , <i>C. microphylla</i> , <i>C. intermedia</i> , <i>Glycyrrhiza uralensis</i> , <i>Astragalus adsurgens</i> , <i>Vigna unguiculata</i> , <i>V. radiata</i> , <i>Phaseolus vulgaris</i>	Lu <i>et al.</i> , 2009b
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. tarimense</i>	<i>Lotus corniculatus</i> , <i>Glycyrrhiza uralensis</i> , <i>Oxytropis glabra</i> , <i>Robinia pseudoacacia</i>	Han <i>et al.</i> , 2008a
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. temperatum</i>	<i>Astragalus adsurgens</i>	Gao <i>et al.</i> , 2004
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. tianshanense</i>	<i>Glycyrrhiza pallidiflora</i> , <i>G. uralensis</i> , <i>Sophora alopecuroides</i> , <i>Caragana polourensis</i> , <i>Halimodendron holodendron</i> , <i>Swainsonia salsula</i> , <i>Glycine max</i>	Chen <i>et al.</i> , 1995; Jarvis <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. tianshanense</i> by <i>ciceri</i>	<i>Cicer arietinum</i>	Rivas <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	<i>Methylobacterium</i>	<i>M. nodulans</i>	<i>Crotalaria glaucoides</i> , <i>C. podocarpa</i> , <i>C. perrottetii</i>	Jourand <i>et al.</i> , 2004
Alphaproteobacteria	Rhizobiales	Brucellaceae	<i>Ochrobactrum</i>	<i>O. ciceri</i>	<i>Cicer arietinum</i>	Imran <i>et al.</i> , 2010
Alphaproteobacteria	Rhizobiales	Brucellaceae	<i>Ochrobactrum</i>	<i>O. cytisi</i>	<i>Cytisus scoparius</i>	Zurdo-Piñeiro <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Brucellaceae	<i>Ochrobactrum</i>	<i>O. lupini</i>	<i>Lupinus albus</i> , <i>L. honoratus</i>	Trujillo <i>et al.</i> , 2005

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Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Phyllobacterium</i>	<i>P. ifriqiense</i>	<i>Astragalus algerianus</i> , <i>Lathyrus numidicus</i>	Mantelin <i>et al.</i> , 2006
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Phyllobacterium</i>	<i>P. leguminum</i>	<i>Argyrolobium uniflorum</i> , <i>Astragalus algerianus</i>	Mantelin <i>et al.</i> , 2006
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Phyllobacterium</i>	<i>P. trifolii</i>	<i>Trifolium pratense</i> , <i>T. repens</i> , <i>Lupinus albus</i>	Valverde <i>et al.</i> , 2005
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. alarii</i>	<i>Medicago ruthenica</i>	Berge <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. alkalisoli</i>	<i>Caragana intermedia</i> , <i>C. microphylla</i> , <i>Phaseolus vulgaris</i> , <i>Vigna radiata</i>	Lu <i>et al.</i> , 2009a
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. cellulosilyticum</i>	<i>Medicago sativa</i>	García-Fraile <i>et al.</i> , 2007
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. daejeonense</i>	<i>Medicago sativa</i>	Quan <i>et al.</i> , 2005
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. etli</i>	<i>Phaseolus vulgaris</i>	Segovia <i>et al.</i> , 1993
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. etli</i> bv <i>mimosae</i>	<i>Mimosa affinis</i> , <i>Phaseolus vulgaris</i> , <i>Leucaena leucocephala</i>	Wang <i>et al.</i> , 1999a
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. fabae</i>	<i>Vicia faba</i>	Tian <i>et al.</i> , 2008
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. galegae</i> bv <i>officinales</i>	<i>Galega officinalis</i>	Lindström, 1989; Radeva <i>et al.</i> , 2001
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. galegae</i> bv <i>orientalis</i>	<i>Galega orientalis</i>	Lindström, 1989; Radeva <i>et al.</i> , 2001
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. gallicum</i> bv <i>gallicum</i>	<i>Phaseolus</i> spp., <i>Leucaena leucocephala</i> , <i>Macroptilium atropurpureum</i> , <i>Onobrychis viciifolia</i>	Amarger <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. gallicum</i> bv <i>phaseoli</i>	<i>Phaseolus</i> spp., <i>Macroptilium atropurpureum</i>	Amarger <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. giardinii</i> bv <i>gallicum</i>	<i>Phaseolus vulgaris</i> , <i>Leucaena leucocephala</i>	Amarger <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. giardinii</i> bv <i>giardinii</i>	<i>Phaseolus</i> spp., <i>Macroptilium atropurpureum</i> , <i>Leucaena leucocephala</i>	Amarger <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. giardinii</i> bv <i>phaseoli</i>	<i>Phaseolus</i> spp., <i>Macroptilium atropurpureum</i>	Amarger <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. hainanense</i>	<i>Desmodium</i> spp., <i>Arachis hypogaea</i> , <i>Stylosanthes guyanensis</i> , <i>Centrosema pubescens</i> , <i>Tephrosia candida</i> , <i>Acacia sinuata</i> , <i>Zornia diphylla</i> , <i>Uraria crinita</i> , <i>Macroptilium lathyroides</i>	Chen <i>et al.</i> , 1997
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. huautlense</i>	<i>Sesbania herbacea</i> , <i>Leucaena leucocephala</i>	Wang <i>et al.</i> , 1998
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. indigoferae</i>	<i>Indigofera</i> spp.	Wei <i>et al.</i> , 2002

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Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. leguminosarum</i> bv <i>phaseoli</i>	<i>Phaseolus vulgaris</i> , <i>P. angustifolius</i> , <i>P. multiflorus</i>	Jordan, 1984; Sawada <i>et al.</i> , 2003
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. leguminosarum</i> bv <i>trifolii</i>	<i>Trifolium</i> spp.	Jordan, 1984; Sawada <i>et al.</i> , 2003
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. leguminosarum</i> bv <i>viceae</i>	<i>Lathyrus</i> spp., <i>Lens</i> spp., <i>Pisum</i> spp., <i>Vicia</i> spp.	Jordan, 1984; Sawada <i>et al.</i> , 2003
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. loessense</i>	<i>Astragalus complanatus</i> , <i>A. scobwerrimus</i> , <i>A. chrysopterus</i>	Wei <i>et al.</i> , 2003
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. lusitanum</i>	<i>Phaseolus vulgaris</i>	Valverde <i>et al.</i> , 2006
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. mesosinicum</i>	<i>Albizia julibrissin</i>	Lin <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. miluonense</i>	<i>Lespedeza chinensis</i> , <i>L. davidii</i> , <i>L. formosa</i> , <i>L. cuneata</i> , <i>L. pilosa</i> , <i>L. virgata</i> , <i>L. cyrtobotrya</i> , <i>L. bicolor</i> , <i>L. tomentosa</i>	Gu <i>et al.</i> , 2008
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. mongolense</i> bv <i>orientale</i>	<i>Medicago ruthenica</i>	van Berkum <i>et al.</i> , 1998; Silva <i>et al.</i> , 2005
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. multihospitium</i>	<i>Robinia pseudoacacia</i> , <i>Lotus frondosus</i> , <i>L. tenuis</i> , <i>Astragalus betetovii</i> , <i>A. aksuensis</i> , <i>Alhagi toum</i> , <i>Halimodendron halodendron</i> , <i>Oxytropis meinshausenii</i> , <i>O. glabra</i> , <i>Caragana jubata</i> , <i>Lathyrus odoratus</i> , <i>Vicia</i> <i>hirsuta</i> , <i>Sophora alopecurioides</i>	Han <i>et al.</i> , 2008b
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. oryzae</i>	<i>Phaseolus vulgaris</i> , <i>Glycine max</i>	Peng <i>et al.</i> , 2008
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. phaseoli</i>	<i>Phaseolus vulgaris</i> , <i>Trifolium repens</i>	Ramírez-Bahena <i>et al.</i> , 2008
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. pisi</i>	<i>Pisum sativum</i> , <i>Phaseolus vulgaris</i> , <i>Trifolium repens</i>	Ramírez-Bahena <i>et al.</i> , 2008
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. sullae</i>	<i>Hedysarum coronarium</i>	Squartini <i>et al.</i> , 2002
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. tibeticum</i>	<i>Trigonella archiducis-nicolai</i> , <i>T. foenum-</i> <i>graecum</i> , <i>Medicago lupulina</i> , <i>M. sativa</i> , <i>Melilotus officinalis</i> , <i>Phaseolus vulgaris</i>	Hou <i>et al.</i> , 2009
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. tropici</i>	<i>Phaseolus vulgaris</i> , <i>Leucaena esculenta</i> , <i>L.</i> <i>leucocephala</i>	Martínez-Romero <i>et al.</i> , 1991
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. tubonense</i>	<i>Oxytropis glabra</i> , <i>Medicago sativa</i> , <i>Vigna unguiculata</i>	Zhang <i>et al.</i> , 2011

1.8 Tables

Class	Order	Family	Genus	Genus and species	Host Plant	References
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. undicola</i>	<i>Neptunia natans</i>	de Lajudie <i>et al.</i> , 1998a; Young <i>et al.</i> , 2001
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. vignae</i>	<i>Vigna radiata</i> , <i>Astragalus dahuricus</i> , <i>A. oxyglottis</i> , <i>Desmodium microphyllum</i>	Ren <i>et al.</i> , 2011
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	<i>R. yanglingense</i>	<i>Gueldenstaedtia multiflora</i> , <i>Coronilla varia</i> , <i>Amphicarpaea trisperma</i>	Tan <i>et al.</i> , 2001
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Shinella</i>	<i>S. kummerowiae</i>	<i>Kummerowia stipulacea</i>	Lin <i>et al.</i> , 2008
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Sinorhizobium</i>	<i>S. americanum</i>	<i>Acacia</i> spp., <i>Leucaena leucocephala</i> , <i>Phaseolus vulgaris</i>	Toledo <i>et al.</i> , 2003
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	<i>Sinorhizobium</i>	' <i>S. morelense</i> '	<i>Leucaena leucocephala</i>	Wang <i>et al.</i> , 2002
Betaproteobacteria	Burkholderiales	Alcaligenaceae	<i>Achromobacter</i>	<i>A. xylosoxidans</i>	<i>Prosopis juliflora</i>	Benata <i>et al.</i> , 2008
Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>B. caribensis</i>	<i>Mimosa diplotricha</i> , <i>M. pudica</i>	Vandamme <i>et al.</i> , 2002
Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>B. mimosarum</i>	<i>Mimosa pigra</i> , <i>M. scabrella</i>	Chen <i>et al.</i> , 2006
Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>B. nodosa</i>	<i>Mimosa bimucronata</i> , <i>M. scabrella</i>	Chen <i>et al.</i> , 2007
Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>B. phymatum</i>	<i>Machaerium lunatum</i>	Vandamme <i>et al.</i> , 2002
Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>B. sabiae</i>	<i>Mimosa caesalpinifolia</i>	Chen <i>et al.</i> , 2008
Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>B. tuberum</i>	<i>Aspalathus carnososa</i>	Vandamme <i>et al.</i> , 2002
Betaproteobacteria	Burkholderiales	Burkholderiaceae	<i>Cupriavidus</i>	<i>C. taiwanensis</i>	<i>Mimosa pudica</i>	Chen <i>et al.</i> , 2001; Vandamme and Coenye, 2004
Betaproteobacteria	Burkholderiales	Oxalobacteraceae	<i>Herbaspirillum</i>	<i>H. lusitanum</i>	<i>Phaseolus vulgaris</i>	Valverde <i>et al.</i> , 2003

1.9 Figures

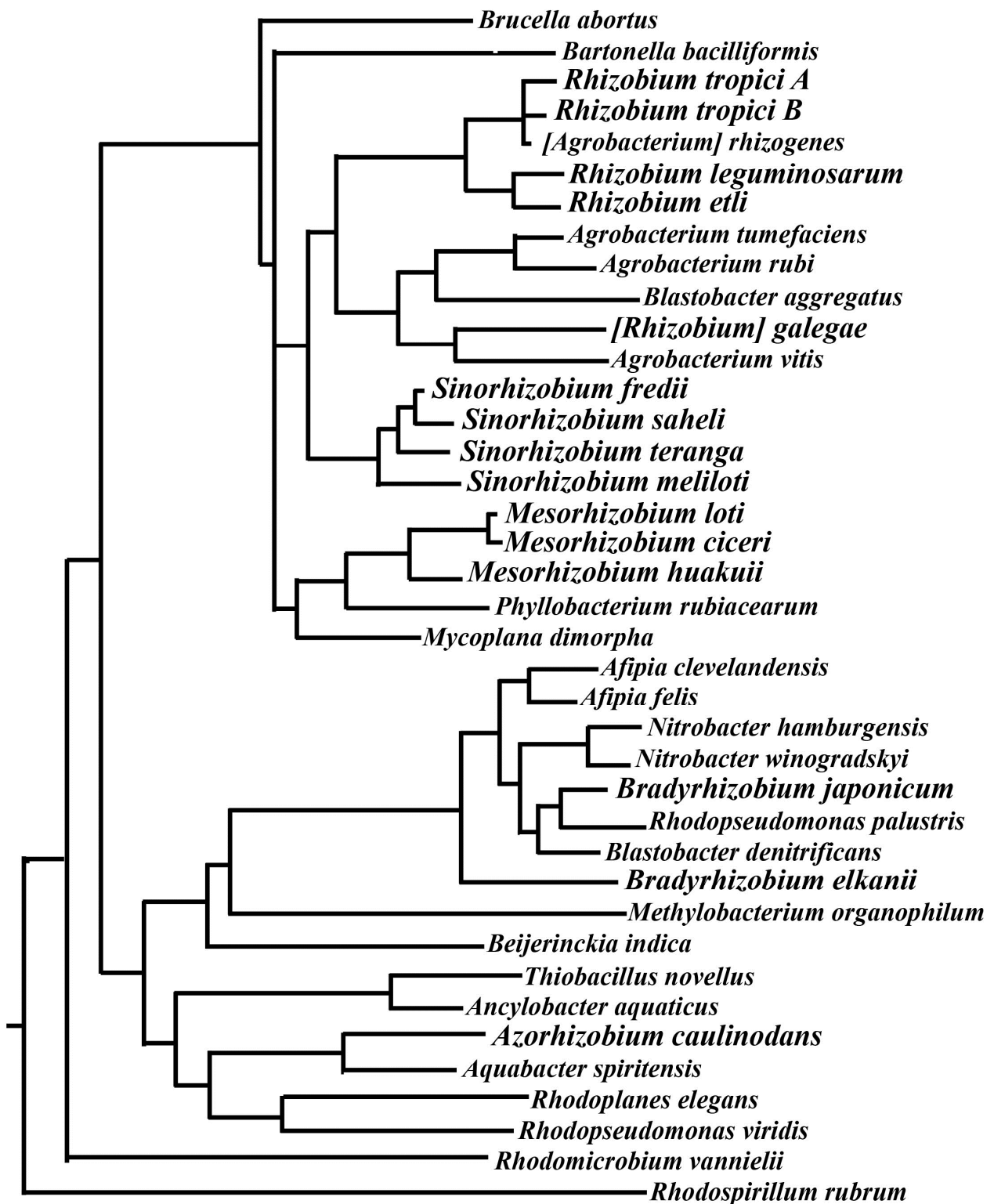


Fig. 1.1 Phylogenetic groupings formed by rhizobia and non-symbiotic microorganisms using a Neighbour-Joining phylogenetic analysis based upon near full-length 16S rRNA sequences (source: Young and Haukka, 1996).

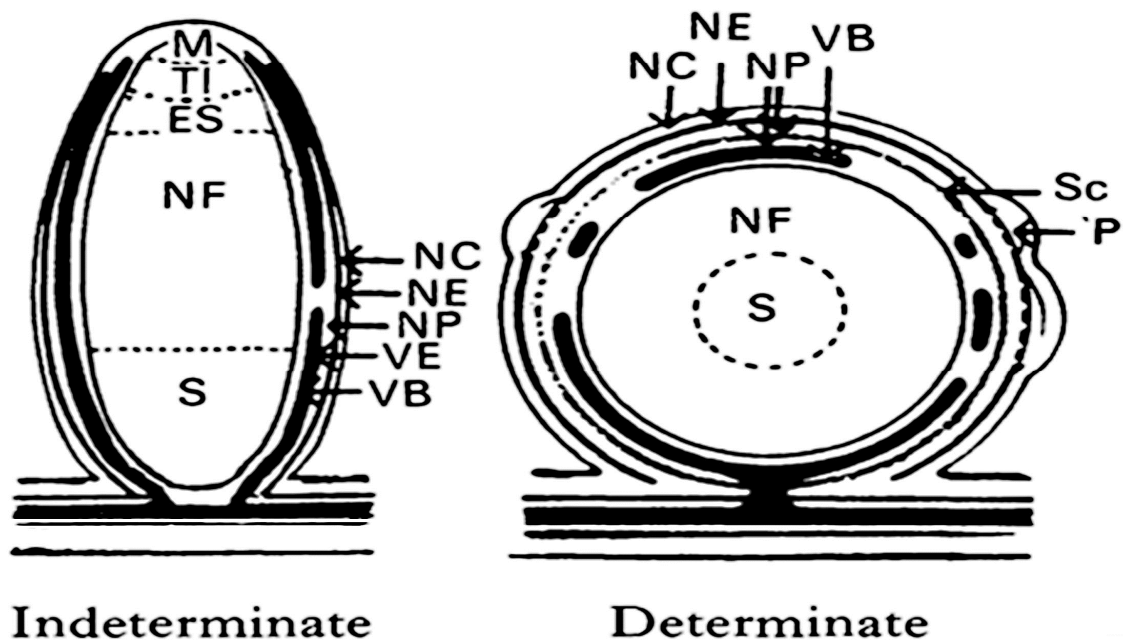


Fig. 1.2 Differences in shape and internal structure of indeterminate and determinate nodules (source: Hirsch, 1992). Abbreviations: ES – early symbiotic zone; M – meristem; NC – nodule cortex; NE – nodule endodermis; NF – nitrogen-fixing zone; NP – nodule parenchyma; P – periderm; S – senescent zone; Sc – sclerenchyma; TI – thread invasion zone; VB – vascular bundle; VE – vascular endodermis.

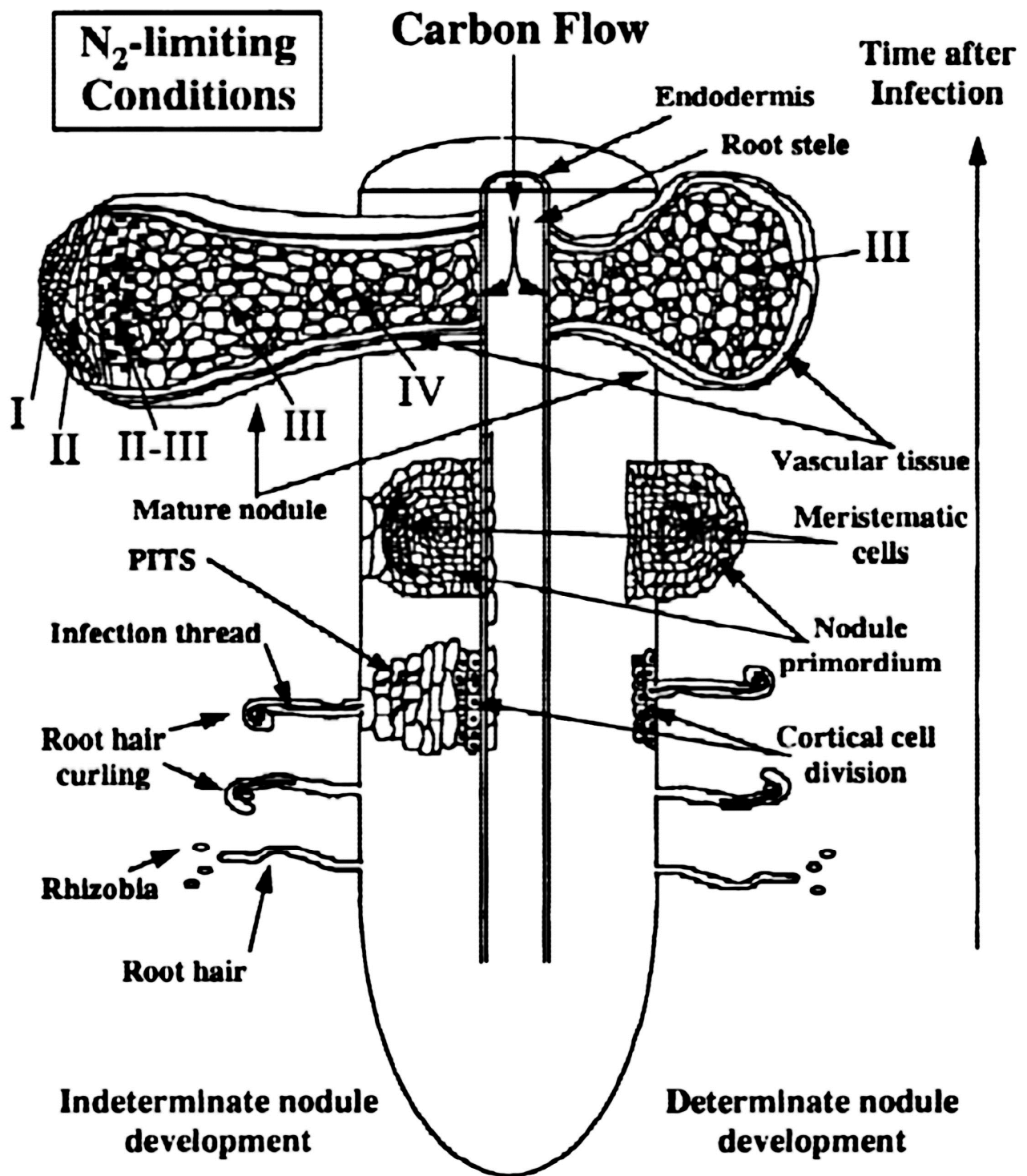


Fig. 1.3 Differences between indeterminate and determinate nodule formation (source: Crespi and Gálvez, 2000). The different developmental zones which are formed in the indeterminate nodules are discussed in detail within the text. Abbreviations: PITS – preinfection threads.

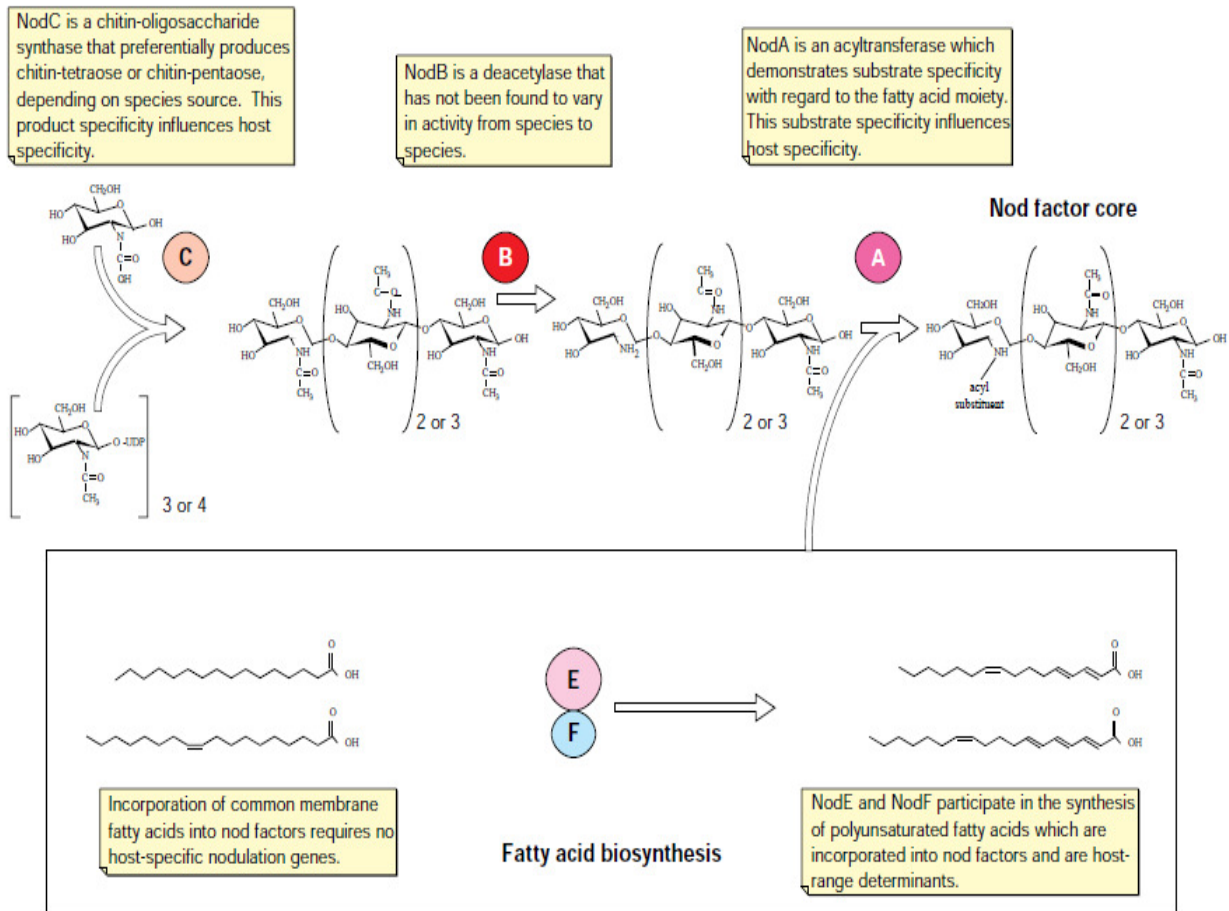


Fig. 1.4 A representation of the formation of a Nod factor (NF) (source: Peters, 1997). The function of each of the common nodulation genes (*nodABC*) in the production of the backbone of the NF is indicated as well as the functions of *nodEF* which are the host specific nodulation genes responsible for the addition of specific fatty acid groups onto the NF backbone.

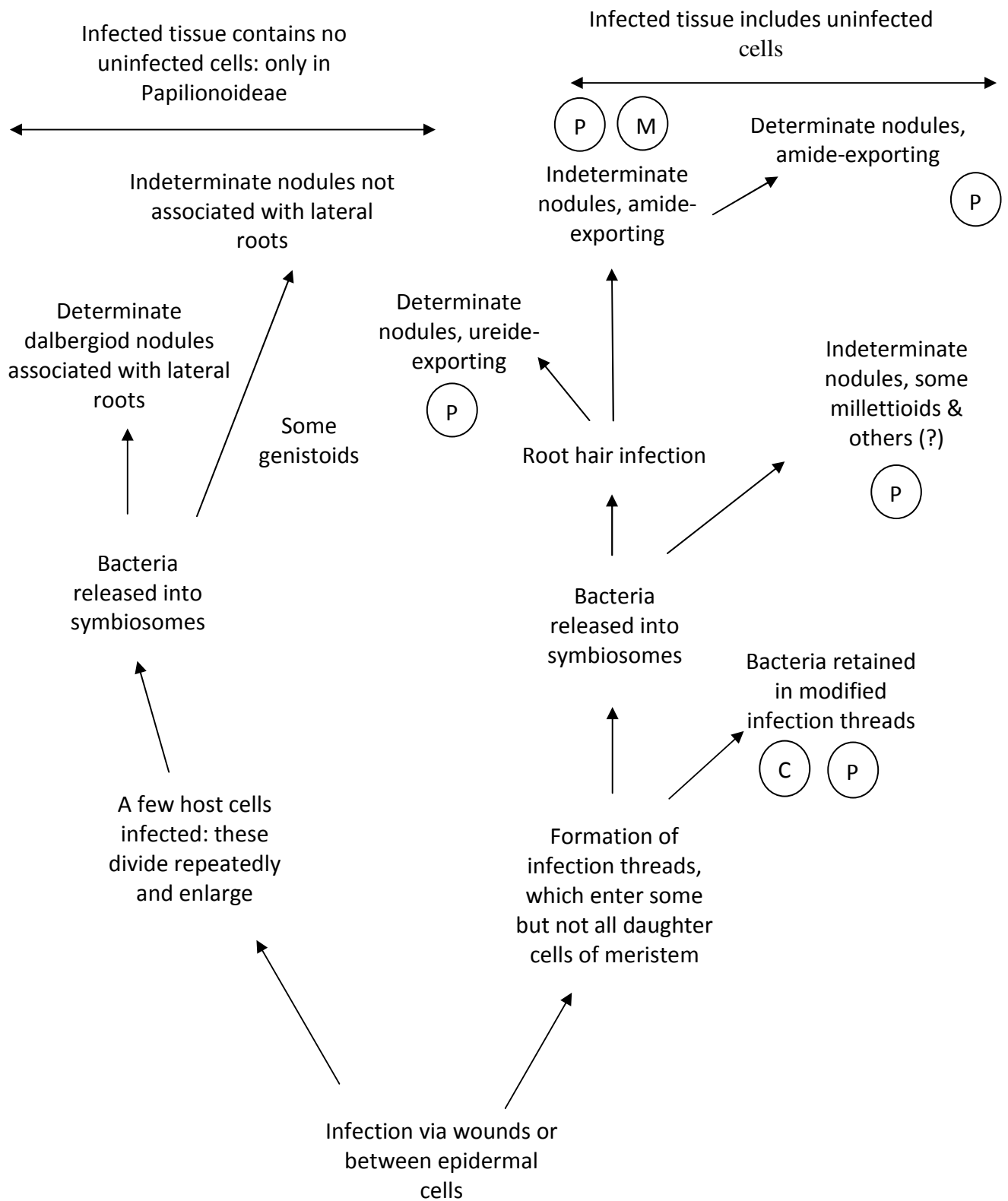


Fig. 1.5 Possible stages in the evolution of present day legume nodules (source: Sprent, 2007). Events limited to certain subfamilies are indicated by the encircled letters: P – Papilionoideae, M – Mimosoideae; C – Caesalpinioideae.

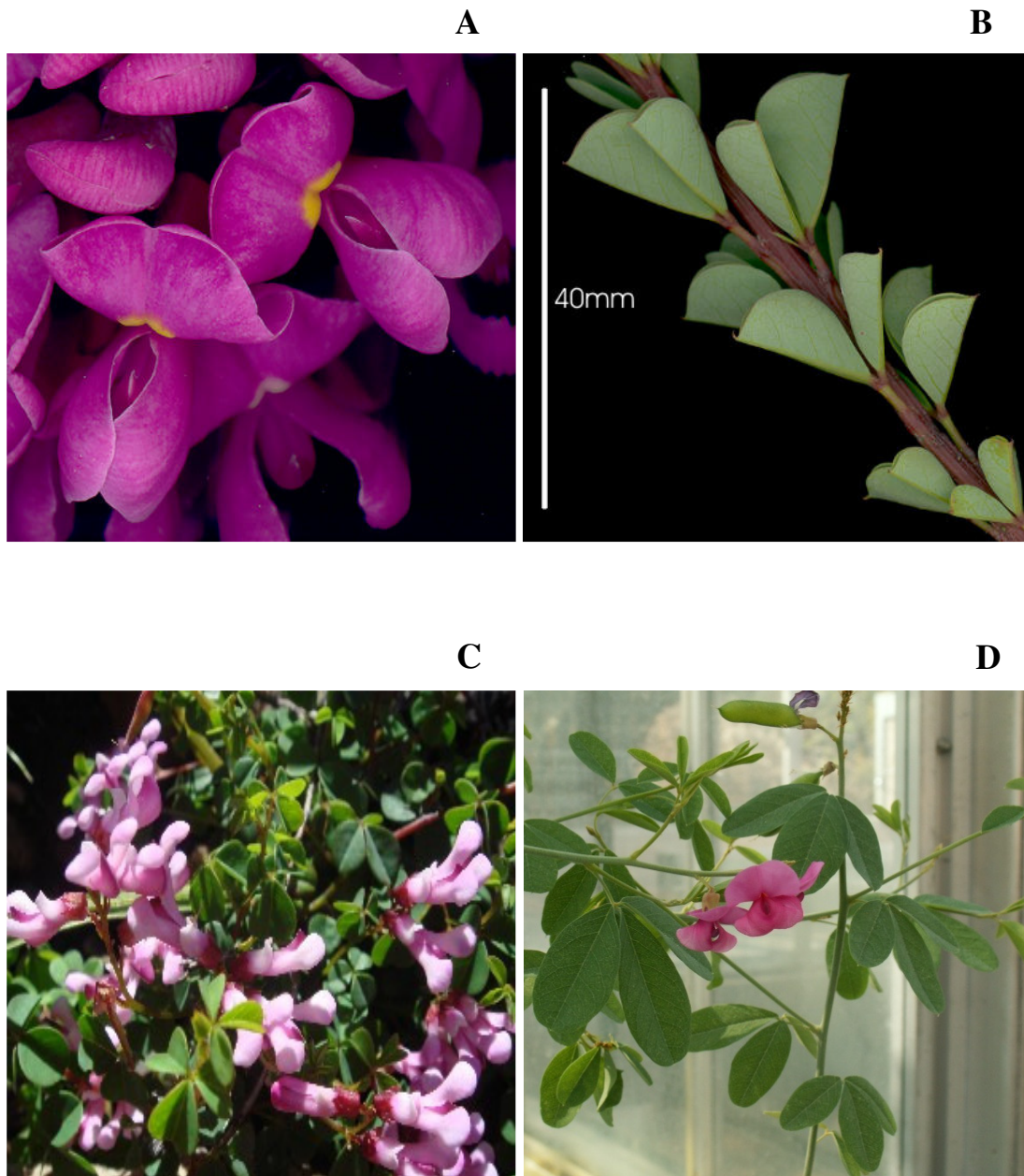
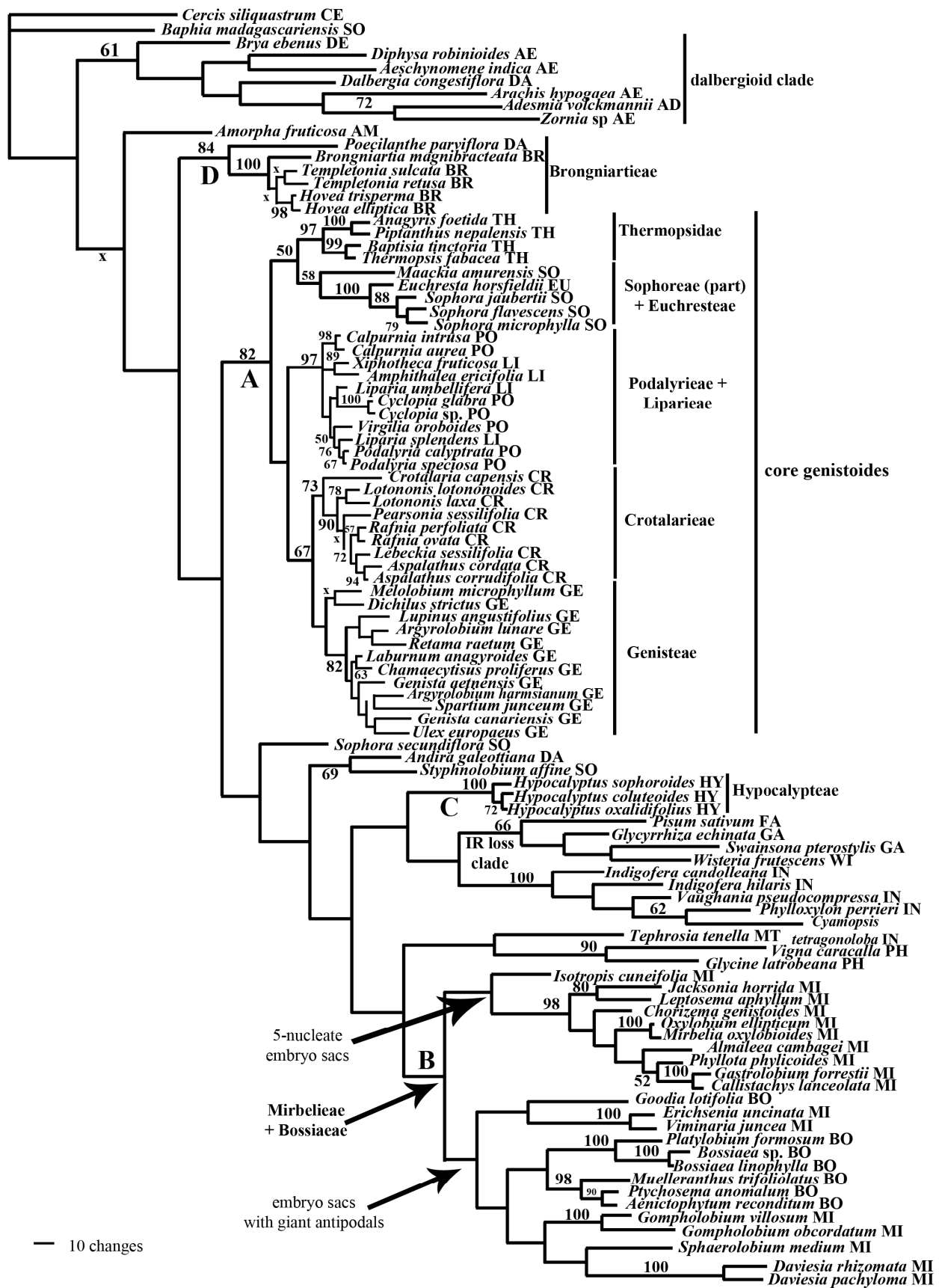


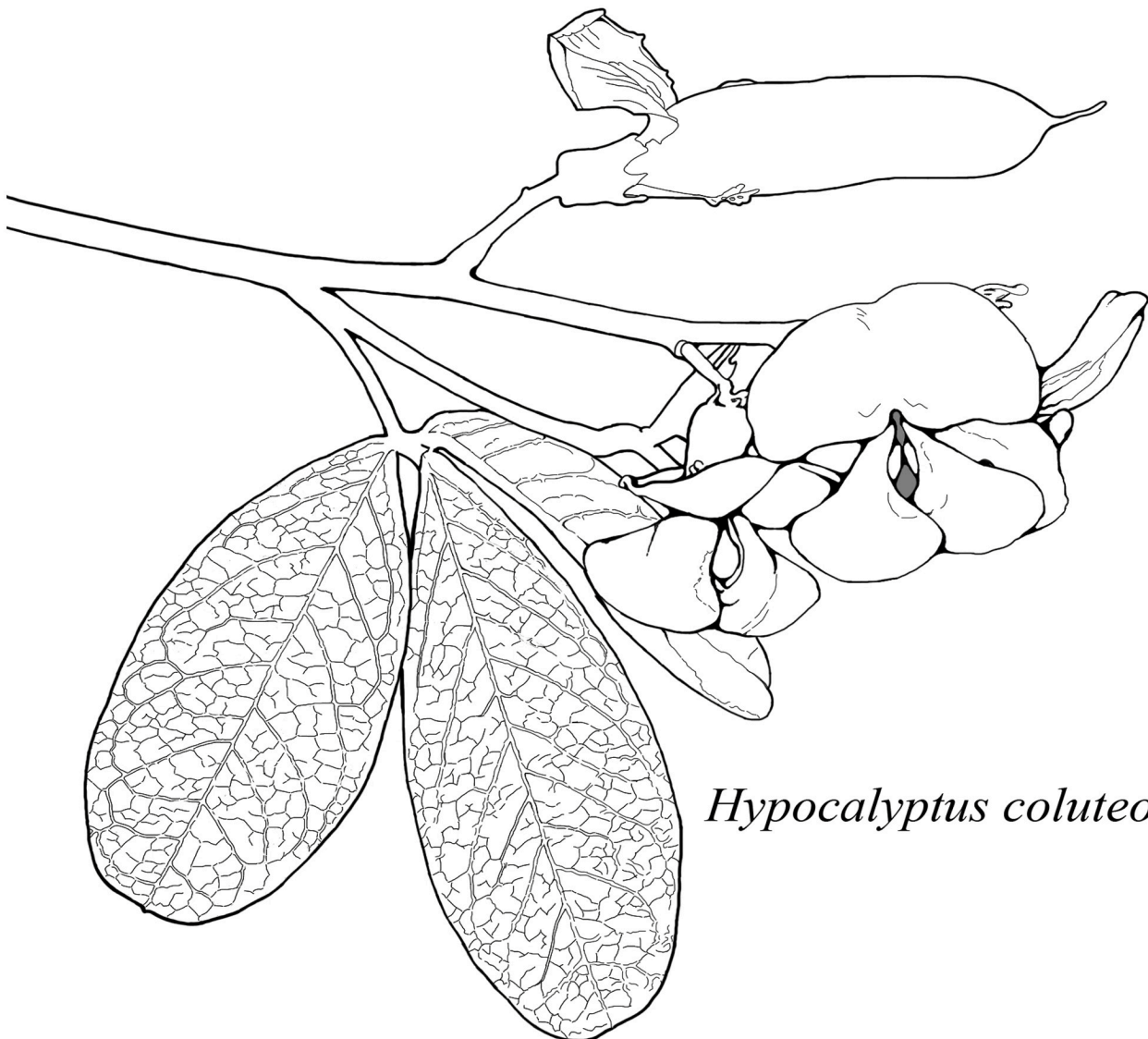
Fig. 1.6 The three species found in the genus *Hypocalyptus*. **A.** *H. sophoroides* flowers (source: <http://www.plantweb.co.za>). **B.** *H. sophoroides* leaf arrangement (source: <http://www.plantweb.co.za>). **C.** *H. oxalidifolius*, flowers on shrub (source: <http://www.fernkloof.com>). **D.** *H. coluteoides* flowers and pod (author's photo).

Fig. 1.7 A most parsimonious tree constructed from legumes ITS data (source: Crisp *et al.*, 2000). Bootstrap values above 50% appear on the branches. Those branches marked by an “x” are those which would collapse when drawing a strict consensus tree. The four clades resulting from this data are indicated by uppercase letters (A – D). The two letters after each species name is the abbreviation for the tribe of that species.



Chapter 2

Diverse beta-rhizobia nodulate legumes in the South African indigenous tribe Hypocalyptae



Hypocalyptus coluteoides

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Abstract

The legume tribe Hypocalypteae (family Leguminosae, subfamily Papilionoideae) is indigenous to the Cape Floristic Region of South Africa. Although one *Hypocalyptus* species was known to be nodulated, the identity of the nitrogen-fixing bacteria in the root nodules of these plants has never been studied before. The aim of this chapter was, therefore, to identify and assess the diversity of the root nodule bacteria of *Hypocalyptus* species and related species in the genera *Podalyria*, *Virgilia* and *Cyclopia*. For this purpose, the rhizobial symbionts of the targeted host species were isolated and their nodulation capabilities confirmed. To identify these bacteria, phylogenetic analyses based upon both 16S rRNA and *recA* gene sequence information was used. The results indicated that the isolates represent so-called beta-rhizobia that form part of the genus *Burkholderia*. This finding was congruent with previous studies in which some of the rhizobial symbionts associated with *Cyclopia* species were also identified as belonging to this genus. The results of this study hints at a wider diversity of indigenous beta-rhizobia found in South Africa than previously anticipated, with *Burkholderia* species likely being a major if not dominant nodulator of fynbos legumes.

Key words: *Hypocalyptus*, *Burkholderia*, Hypocalypteae, beta-rhizobia, nodulation, Cape Floristic Region

2.1 Introduction

The legume genus *Hypocalyptus* Thunberg (Family Leguminosae; Subfamily Papilionoideae) contains three species (*H. sophoroides*, *H. oxalidifolius* and *H. coluteoides*) that are endemic to South Africa (Schutte and van Wyk, 1998a). The members of this genus form part of the sclerophyllous shrubland or ‘fynbos’ vegetation of the Cape Floristic Region (CFR) (Schutte and van Wyk, 1998a), where they are typically found in sandy soils close to waterways and especially on sheer slopes and in kloofs (Dahlgren, 1972). Members of the Leguminosae, including *Hypocalyptus*, constitute roughly 10% of the estimated 6210 endemic plant species within the CFR, thus representing the second largest family (Cocks and Stock, 2001; Goldblatt and Manning, 2002) in this internationally recognised biodiversity ‘hot spot’ (Cowling *et al.*, 2009; Myers *et al.*, 2000).

The three species of *Hypocalyptus* are all characterised by magenta-pink flowers with fused stamens and yellow nectar guides, as well as intrusive calyx bases, trifoliolate leaves and elongated seed pods (Schutte and van Wyk, 1998b; see also Fig. 2.1). Since the establishment of the genus in 1800 by Thunberg (Dahlgren, 1972), the classification of *Hypocalyptus* at tribal level remained unresolved (Polhill, 1976; Schutte and van Wyk, 1998a) since *Hypocalyptus* had been associated with various tribes, including Loteae, Crotalariae, Cytiseae, Genisteae, Lipariae and Podalyrieae (Harborne, 1969; Polhill, 1981; Schutte and van Wyk, 1998a; 1998b). However, *Hypocalyptus* species differ significantly from other genera and tribes, especially in terms of flavonoid and anthocyanin chemistry (De Nysshen *et al.*, 1998; van Wyk *et al.*, 1995) as well as gene sequence evolution (Crisp *et al.*, 2000; Lavin *et al.*, 1990). As a result, the tribe Hypocalypteae (Yakovlev) A.L. Schutte was erected to accommodate this genus and its three species (Schutte and van Wyk, 1998a). Nevertheless, the *Hypocalyptus* species share a number of characters with the members of the tribe Podalyrieae (Schutte and van Wyk, 1998b), which includes the genera *Cyclopia*, *Virgilia*, *Liparia*, *Amphithalea*, *Podalyria*, *Stirtonanthus*, *Xiphotheca*, *Calpurnia* and possibly *Cadia* (Boatwright *et al.*, 2008; Schutte and van Wyk, 1998b). Some of the shared characteristics include tridentate bracts (e.g. in *Hypocalyptus*, *Podalyria*, *Stirtonanthus* and *Virgilia* species), xylem vessels that are arranged in remote radial groups (e.g. in *Hypocalyptus*, *Virgilia* and *Calpurnia* species), unusual chromosome base numbers (e.g. in *Hypocalyptus*, *Cyclopia* and *Virgilia* species) and the lack of any of the major alkaloids (e.g.

in *Hypocalyptus* and *Cyclopia* species) (Schutte and van Wyk, 1998b). They all thus share greater or lesser degrees of relatedness.

Like many other legumes, members of the tribes Hypocalyptea and Podalyrieae are capable of establishing nitrogen-fixing symbioses with rhizobia or root-nodule bacteria (Elliott *et al.*, 2007a; Grobbelaar and Clarke, 1972; Kock, 2003; Sprent, 2001). The characteristic outcome of this symbiosis is the development of external structures known as nodules on the roots of these legumes in which nitrogen-fixation occurs (Liu *et al.*, 2005; Long, 2001; Markmann *et al.*, 2008; Soto *et al.*, 2006). Earlier information regarding the nodulation status of the Hypocalyptea and Podalyrieae is limited. Grobbelaar and Clarke (1972) examined the presence of nodules only on the roots of *H. sophoroides*, while the identity and diversity of the rhizobial symbionts of this plant remained unknown. Among the Podalyrieae, species in the genus *Cyclopia* were shown to be nodulated by diverse rhizobia (Kock, 2003).

Despite the unique nature of the legume-rhizobium symbiosis, the diverse and non-monophyletic nature of rhizobial symbionts is widely recognised (Amadou *et al.*, 2008; Masson-Boivin *et al.*, 2009; Sawada *et al.*, 2003; Willems, 2006). Rhizobial bacteria are currently classified into either the Alpha- or Betaproteobacteria classes (Sawada *et al.*, 2003; Wei *et al.*, 2009), and are correspondingly termed alpha- or beta-rhizobia (Moulin *et al.*, 2001). Recently, rhizobia were confirmed within the Gammaproteobacteria, as the legume *Robinia pseudoacacia* is nodulated by a *Pseudomonas* strain with nodulation and nitrogen-fixing genes (Shiraishi *et al.*, 2010).

In the last decade, the rhizobia associated with indigenous legumes have increasingly received worldwide attention (Sprent *et al.*, 2010). Studies have, for example, focussed on indigenous legumes in Australia (Lafay and Burdon, 2007; Yates *et al.*, 2004), New Zealand (Weir *et al.*, 2004), the Canary Islands (Lorite *et al.*, 2010) and Sicily (Cardinale *et al.*, 2008). In South Africa, several studies have shown that indigenous papilionoid legumes are nodulated by rhizobia from the Betaproteobacteria (Elliott *et al.*, 2007a; Garau *et al.*, 2009; Kock, 2003; Moulin *et al.*, 2001; Spriggs and Dakora, 2007). These included *Aspalathus carnosus*, which is nodulated by *Burkholderia tuberum*, the first described member of the beta-rhizobia (Moulin *et al.*, 2001; Vandamme *et al.*, 2002a). Indigenous South African papilionoid legume hosts have also been shown to be associated with diverse alpha-rhizobia. These include amongst others species of *Methylobacterium*, *Bradyrhizobium*,

Mesorhizobium, *Sinorhizobium* and *Rhizobium* on *Lotononis*, *Vigna*, *Aspalathus*, *Crotalaria*, *Desmodium*, *Alysicarpus* and *Lebeckia* hosts respectively (Ardley *et al.*, 2009; Jaftha, 2006; Phalane, 2008).

In this chapter I sought to identify the rhizobial symbionts of the three species of *Hypocalyptus*. For comparative purposes, root-nodule bacteria from some members of the related tribe Podalyriaceae (*Virgilia*, *Podalyria* and *Cyclopia*; Fig. 2.2) were also included. My aims were three-fold: (i) obtain the rhizobial bacteria from the root-nodules of the respective host plants; (ii) identify the bacteria using phylogenetic analyses based on the genes encoding the 16S subunit of the ribosomal RNA (16S rRNA) and the 38 kilodalton DNA recombinase RecA; (iii) confirm that the isolated bacteria can nodulate their original *Hypocalyptus* species.

2.2 Materials and Methods

2.2.1 Rhizobial cultures, bacterial isolation and maintenance of isolates

A total of 69 rhizobial isolates were included in this study (Table 2.1). Of these, 40 were received from the South African Rhizobium Collection (Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI), Roodeplaat, Pretoria, SA) and were originally isolated from the root nodules of *H. coluteoides*, *H. oxalidifolius*, *H. sophoroides*, *Virgilia oroboides* or *Podalyria calyptata*. The 19 isolates associated with the root nodules of *Cyclopia* species originated from a previous study performed at the University of Pretoria (Kock, 2003) (Table 2.1). All of the above isolates were obtained either directly from root nodules collected in the field or from nodules obtained by means of ‘trapping’ from collected soil (see below; Table 2.1). By making use of the latter approach I obtained an additional ten isolates from the root nodules of either *H. oxalidifolius* or *H. sophoroides* (Table 2.1).

For the ‘trapping’ experiments, *H. sophoroides* and *H. oxalidifolius* seeds were soaked in concentrated sulphuric acid (H₂SO₄; Saarchem, Wadeville, SA) for 45 and 30 min, respectively. Both sets of seeds were then rinsed in five changes of sterile distilled water (sH₂O), followed by a soaking step of 3 to 4 h in sH₂O. The imbibed seeds were then placed onto 15% (w/v) water agar (Biolab, Merck, SA) and incubated at 15 °C in the dark for about

7 days. Afterwards, the germinated seeds were planted in Leonard jars containing nitrogen-free Hoagland growth solution (Somasegaran and Hoben, 1994) and soil collected from the rhizosphere surrounding the roots of the corresponding wild host (Table 2.1). Seedlings were grown in a glasshouse at a 14h-day temperature of 27-28 °C and a 10 h-night temperature of 15 °C. Plants were examined for nodule development after 6-8 weeks.

Nodules were carefully excised from roots and surface sterilised by soaking in 3.5% (m/v) sodium hypochlorite for 10-15 min, before being rinsed five times with sH₂O. Rhizobia were isolated from these nodules by crushing a nodule with sterilised forceps onto Yeast-Mannitol agar (YMA) containing 0.5 g/L Yeast extract (Biolab), 10 g/L Mannitol (Saarchem), 0.5 g/L K₂HPO₄, 0.2 g/L MgSO₄.7H₂O, 0.1 g/L NaCl, 15 g/L bacteriological agar (Biolab) and Congo red (diphenyldiazo-bis- α -naphthylaminesulfonate; 2.5 g/L; Saarchem) (Somasegaran and Hoben, 1994). After incubation at 28 °C for 4 to 5 days, single cream or light pink colonies (approx. 3-10 mm in diameter) were selected and pure cultures prepared by streaking for growth on Tryptone-Yeast agar (TYA) (5 g/L Tryptone; Oxoid, Hampshire, UK; 3 g/L Yeast Extract; Biolab; 15 g/L agar; Biolab) to which is added 2 ml of a separate calcium chloride dihydrate solution (CaCl₂.2H₂O; 440 g/L) (Somasegaran and Hoben, 1994). Use of TY agar reduced copious slime formed by the relatively fast-growing isolates on YM agar. Suspensions of purified isolates were stored at -70 °C using 20% glycerol as a cryoprotectant.

2.2.2 Confirmation of nodulation capabilities

The nodulation capabilities of the 36 *Hypocalyptus* isolates were tested on a *Hypocalyptus* host as well as the promiscuous legumes cowpea (*Vigna unguiculata*) and siratro (*Macroptilium atropurpureum*). However, only *H. sophoroides* and *H. coluteoides* seeds were included in these tests as *H. oxalidifolius* seeds could not be obtained. The rhizobial isolates from this species were instead tested on *H. coluteoides*, for which sufficient seed was available. The nodulation abilities of the isolates obtained from *Virgilia* and *Podalyria* were confirmed on siratro as well as cowpea. Seeds for both cowpea and siratro were obtained from the ARC-PPRI, while seeds for the *Hypocalyptus* species were obtained from Prof. B.-E. van Wyk (Department of Botany, University of Johannesburg, SA) and Silverhill Seeds (Kenilworth, Cape Town, SA).

For the nodulation tests, *Hypocalyptus* seed imbibition and germination was performed as described above. The siratro seeds were pre-treated as described for *Hypocalyptus*, but required 15 min scarification in concentrated sulphuric acid. Cowpea seeds were surface-sterilised by soaking in 3.5% (m/v) sodium hypochlorite for 15 min followed by rinsing with five changes of sH₂O. Germination of the imbibed cowpea and siratro seeds was achieved following 7 days of incubation at 28 °C on water agar in the dark.

The rhizobial inocula used in nodulation tests were prepared from the glycerol stock suspensions. They were first cultured on TYA to ensure purity before a single colony of each isolate was selected and again grown on fresh TYA. From the subsequent bacterial growth, two inoculation loopfuls (of which the size of the loop is 1/200 ml) of culture were added to 4 ml of sH₂O and suspended by vortexing for use as an inoculum. One ml of a specific inoculum was then applied to each of three germinated seeds planted in a Leonard jar containing playpen sand and nitrogen-free Hoagland solution. The cowpea and siratro seedlings were grown for 4 and 6 weeks, respectively, in the glasshouse under conditions described above for the *Hypocalyptus* nodulation tests.

After the appropriate growth period, nodulated seedlings were carefully removed from the Leonard jars and the roots rinsed free of sand with sH₂O. For each isolate that was used as inoculum, three nodules were chosen for further experimentation. In each case, one of these nodules were used to evaluate the effectiveness of nitrogen-fixation by dissecting the nodule and inspecting it for the presence of leghaemoglobin that gives the effective nodule interior a pink colour (Somasegaran and Hoben, 1994). The bacteria were then isolated from the remaining nodules, following surface-sterilisation as described above. In order to verify the identities of the resulting nodule inhabitants and determine possible contamination, the 16S rRNA gene sequences of all these ‘secondary’ isolates were compared to those from the stock cultures used to inoculate (see below).

2.2.3 DNA extraction

DNA was extracted from bacterial cultures grown in TY broth at 28 °C with shaking at *ca.* 150 rpm for 1-5 days. The bacteria in 5 ml of broth were harvested by centrifugation (3 824 *rcf*, 3 min). For genomic DNA extraction, the pelleted cells were resuspended in 250 µl extraction buffer containing 2% sodium dodecyl sulphate (SDS), 100 mM Tris-HCl (pH 8), 10 mM EDTA (pH 8) and 1.2 µl/µg Proteinase K (Roche Diagnostics, Manheim, Germany)

(Sambrook and Russell, 2001). These mixtures were frozen at $-70\text{ }^{\circ}\text{C}$ for 15 min and then incubated at $64\text{ }^{\circ}\text{C}$ for 60 min, after which 0.3 volume of a 5 M NaCl₂ solution and 0.1 volume of a 10% CTAB (N-cetylN, N, N-trimethyl ammonium bromide) (Saarchem) solution was added to the mixtures, followed by mixing and incubation at $65\text{ }^{\circ}\text{C}$ for 10 min. The nucleic acids were then extracted by homogenisation in the presence of 1 volume of a phenol-chloroform-isoamyl alcohol [25:24:1] solution, incubation on ice for 30 min, centrifugation at room temperature (20 817 *rcf*, 15 min) and transfer of the aqueous phase to a fresh tube (Sambrook and Russell, 2001). These phenol-chloroform-isoamyl alcohol extractions were repeated until the aqueous-organic interphase was clear. Residual phenol in the aqueous phase was then removed with a final chloroform extraction. The extracted nucleic acids were precipitated by overnight incubation at $-20\text{ }^{\circ}\text{C}$ in the presence of 0.6 volume isopropanol, and harvested by centrifugation (20 817 *rcf*, 30 min, $4\text{ }^{\circ}\text{C}$) (Sambrook and Russell, 2001). After washing the pelleted nucleic acids with 70% ethanol, the air-dried nucleic acids were dissolved in 50 μl sH₂O. The quality and concentration of the extracted DNA was evaluated by electrophoresis (Sambrook and Russell, 2001) using 1% agarose gels (Whitehead Scientific, Brackenfell, SA), TAE buffer (242 g/L Tris, 57.1 ml/L Acetic acid and 100 ml/L EDTA), staining with 1 mg/ml ethidium bromide and a E-box UV transilluminator (Vilber Lourmat, France).

2.2.4 16S rRNA and *recA* amplification and sequencing

For the rhizobia included in this study (Table 2.1), a portion of their 16S rRNA and the *recA* genes were sequenced. For amplification of a nearly complete 16S rRNA gene, the primers 27F (5' AGA GTT TGA TCC TGG CTC AG 3') (Suau *et al.*, 1999) and 485R (5' TAC CTT GTT ACG ACT TCA CCC CA 3') (Logan *et al.*, 2000) were used. For amplification of a portion of the *recA* gene, the primers BUR1 (5' GAT CGA RAA GCA GTT CGG CAA 3') and BUR2 (5' TTG TCC TTG CCC TGR CCG AT 3') (Payne *et al.*, 2005) were used. All of the 50 μl reaction mixtures contained 50-100 ng template DNA, 10 $\mu\text{M}/\mu\text{l}$ of the respective primers, 25 mM MgCl₂, 2.5 mM of each dNTP and 0.1 U/ μl Super-Therm *Taq* DNA polymerase and reaction buffer (Southern Cross Biotechnology, Cape Town, SA). The PCR cycling conditions included an initial denaturation at $94\text{ }^{\circ}\text{C}$ for 2 min, and 30 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ for 1 min, annealing for 1 min (at $55\text{ }^{\circ}\text{C}$ for 16S rRNA and $62\text{ }^{\circ}\text{C}$ for *recA*) and elongation at $72\text{ }^{\circ}\text{C}$ for 1 min, followed by a final elongation step of 7 min at $72\text{ }^{\circ}\text{C}$. All PCR reactions were performed on an iCycler (BioRad, Hercules, California, USA) or

GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, California, USA) or an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany).

Amplified PCR fragments were purified and concentrated by means of polyethylene glycol precipitation (Steenkamp *et al.*, 2006). All PCR products were sequenced in both directions using the original PCR primers and the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems) on the ABI3100 Automated Capillary DNA Sequencer. For the *recA* products, however, another forward primer, BUR-1F-short (5' CGA RAA GCA GTT CGG C 3') was used. The resulting raw sequences were inspected and corrected, where necessary, with Chromas Lite version 2.01 (Technelysium, Tewantin, Queensland, Australia) and BioEdit version 7.0.5.3 (Hall, 1999).

2.2.5 Sequence and phylogenetic analyses

All 16S rRNA and *recA* sequences were compared to those in the GenBank database (National Centre for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>) by making use of *blastn* (Altschul *et al.*, 1990; Benson *et al.*, 2004). Based on the results of these comparisons, all the available 16S rRNA and *recA* sequences for the *Burkholderia* type strains were downloaded from GenBank by using Euzéby's (1997) continuously updated list of bacterial names and type strain information or by making use of the accession numbers provided in the original species descriptions. There was one exception, however, where the type strain sequence could not be obtained (Euzéby, 1997). Instead, the 16S rRNA sequence of a closely related strain was used to represent the species *B. anthina* (Vandamme *et al.*, 2002b). Sequences were aligned online with MAFFT (Multiple Alignment using Fast Fourier Transformation; <http://align.bmr.kyushu-u.ac.jp/mafft/online/server/>) (Kato *et al.*, 2002). For the *recA* dataset, BioEdit was used to ensure that the nucleotide alignment corresponded with the inferred amino acid alignment. The 16S rRNA alignment included sequences for the rhizobia associated with hosts indigenous to South Africa and those for the majority of described *Burkholderia* type strains, as well as a broader spectrum of nodulating *Burkholderia* strains not yet formally described (Barrett and Parker, 2006; Chen *et al.*, 2005a; Chen *et al.*, 2005b; Garau *et al.*, 2009; Parker *et al.*, 2007).

For each dataset, phylogenies were inferred based on maximum likelihood (ML) and Bayesian inference (BI) by making use of PHYML version 3 (Guindon and Gascuel, 2003)

and MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), respectively. These analyses utilised the best-fit substitution models as indicated by jModelTest version 0.1.1 (Felsenstein, 2005; Guindon and Gascuel, 2003; Posada, 2008). ML analysis of the *recA* dataset utilised the General Time Reversible evolutionary model (GTR) (Tavaré, 1986), while the 16S rRNA dataset required the “transitional model” (TIM2) (Posada, 2008). Analysis of both datasets utilised gamma correction for among-site rate variation and a proportion of invariable sites. Bootstrap analyses (Felsenstein, 1985) based on 100 pseudoreplicates were performed using the same parameter settings. For the BI analyses, the Metropolis-coupled Markov chain Monte Carlo search algorithm as implemented in MrBayes, was used. The *recA* analysis consisted of 2 500 000 generations and the 16S rRNA analysis of 5 000 000 generations running one cold and three heated chains, and in both instances sampling every 100th tree. After discarding a burnin of 1 200 in the *recA* analysis and 5 000 in the 16S rRNA analysis, posterior probabilities (BIpp) were calculated based on the remaining trees.

To determine whether or not these two datasets were congruent and could be combined, the incongruence length difference (ILD) test (Farris *et al.*, 1995) was performed in PAUP* version 4b10 (Swofford, 2003). In order to perform the test, 1000 repartitions of the datasets were created which were then submitted to heuristic searches by means of 100 cycles of simple sequence additions and tree bisection reconnection branch swapping.

2.3 Results

2.3.1 Bacterial isolates and their nodulation capabilities

Tests were carried out to confirm the nodulation capabilities of the 50 isolates obtained from *Hypocalyptus*, *Virgilia* and *Podalyria* species (Table 2.1). A major hindrance was establishment of *Hypocalyptus* seedlings, which proved exceedingly difficult in the artificial glasshouse environment away from the natural conditions of the CFR. Of those rhizobia originally isolated from *H. coluteoides* and *H. oxalidifolius*, only five out of eleven and two out of five isolates, respectively, formed effective nodules on their corresponding host approximately eight weeks after inoculation (Table 2.1; Fig. 2.3). Pink, fingerlike to fan-shaped indeterminate nodules were formed on these plants, as shown for *H. coluteoides* in Figure 2.3. Such nodules were observed on all three *Hypocalyptus* species in the glasshouse and under field conditions (I. Law personal communication). Nodulation by the isolates was

more successful on the promiscuous nodulators siratro and cowpea (Table 2.1). Of the 11 *H. coluteoides* isolates, nine formed nodules on siratro and seven nodulated cowpea, for the five *H. coluteoides* isolates, four nodulated both siratro and cowpea, while of the 20 *H. sophoroides* isolates 13 formed nodules on siratro and ten nodulated cowpea. The ability to nodulate either *H. coluteoides*, siratro or cowpea could not be confirmed for two *H. coluteoides*, one *H. oxalidifolius* and three *H. sophoroides* isolates (Table 2.1). Of the six *P. calyptrata* isolates, three nodulated only siratro, but nodulation for the last three isolates could not be confirmed (Table 2.1). Of the eight *V. oroboides* isolates, seven nodulated siratro, three nodulated cowpea and one did not nodulate either legume (Table 2.1). The 41 isolates from *Hypocalyptus*, *Virgilia* and *Podalyria* that nodulated either siratro or cowpea, formed effective determinate nodules on these legumes (Fig. 2.3). In cases where the nodulation tests were successful, the 16S rRNA gene sequences of isolates recovered from the induced nodules were all similar to those of the original isolates (data not shown). The nodulation capabilities of the isolates from the root nodules of the *Cyclopia* species were confirmed in a previous study and thus were not repeated (Kock, 2003). All of the isolates from *Virgilia oroboides* (eight isolates) and *Podalyria calyptrata* (six isolates) had previously been proven capable of nodulating their original hosts in glasshouse tests at the ARC-PPRI (J.F. Bloem personal communication).

2.3.2 16S rRNA gene sequence analyses

The 16S rRNA sequences for the isolates examined in this study were $\geq 98\%$ similar to those of known *Burkholderia* strains in the GenBank database (Fig. 2.4). The sequences of many of these isolates were also highly similar to those of the rhizobia previously reported from *Cyclopia* species (Kock, 2003). This indicated that the *Cyclopia* and *Hypocalyptus* spp., as well as, *V. oroboides* and *P. calyptrata* are all nodulated by bacteria from the genus *Burkholderia* in the class Betaproteobacteria (Fig. 2.4).

The dataset used for phylogenetic analysis of the 16S rRNA sequences included the sequences determined in this study for all the isolates and those for the type strains of described *Burkholderia* species. The corresponding published sequences for a number of undescribed *Mimosa*-nodulating rhizobia and two isolates from the indigenous South African legume *Rhynchosia ferulifolia* (Garau *et al.*, 2009) were also included (Fig. 2.4). The aligned dataset was 1321 bases in length and proved to be very uniform; overall the

alignment only included nine sites with alignment gaps, of which none were longer than three bases (data not shown).

ML analyses of this dataset separated the taxa into a number of well-supported clades or groups (Fig. 2.4). The dataset was divided into two large clusters, A and B (Fig. 2.4), which each contained a number of smaller clades. Most of the beta-rhizobia were contained in Cluster A, with 73% ML bootstrap support (MLbs). Cluster A thus included all of the isolates from *Hypocalyptus*, *Virgilia*, *Podalyria*, *Cyclopia*, *Mimosa* and *Rhynchosia ferulifolia*, as well as all the described nodulating species of *Burkholderia*: *B. caribensis* (Achouak *et al.*, 1999; Vandamme *et al.*, 2002a), *B. nodosa* (Chen *et al.*, 2007), *B. sabiae* (Chen *et al.*, 2008), *B. mimosarum* (Chen *et al.*, 2006), *B. phymatum* (Vandamme *et al.*, 2002a) and *B. tuberum* (Vandamme *et al.*, 2002a). Cluster A also included several non-nodulating species of *Burkholderia* with nitrogen-fixing capabilities, i.e., *B. xenovorans* (Goris *et al.*, 2004), *B. silvatlantica* (Perin *et al.*, 2006), *B. unamae* (Caballero-Mellado *et al.*, 2004), *B. tropica* (Reis *et al.*, 2004), *B. kururiensis* (Estrada-de los Santos *et al.*, 2001) and *B. terrae* (Yang *et al.*, 2006). A number of species that are not known to possess nitrogen-fixing or nodulation abilities were also included in this cluster. They were *B. caledonica* (Coenye *et al.*, 2001), *B. bryophila* (Vandamme *et al.*, 2007), *B. phenazineum* (Viallard *et al.*, 1998), *B. phytofirmans* (Sessitsch *et al.*, 2005), *B. sartisoli* (Vanlaere *et al.*, 2008), *B. sedimicola* (Lim *et al.*, 2008), *B. megapolitana* (Vandamme *et al.*, 2007), *B. fungorum* (Coenye *et al.*, 2001), *B. terricola* (Goris *et al.*, 2002), *B. graminis* (Viallard *et al.*, 1998), *B. phenoliruptrix* (Coenye *et al.*, 2004), *B. ginsengisoli* (Kim *et al.*, 2006), *B. heleia* (Aizawa *et al.*, 2009), *B. ferrariae* (Valverde *et al.*, 2006), *B. sacchari* (Brämer *et al.*, 2001) and lastly *B. hospita* (Goris *et al.*, 2002).

The only beta-rhizobial species not included in Cluster A was *B. dolosa* (Fig. 2.4). This species, found within Cluster B, was reported to nodulate *Alysicarpus glumaceus* (Vandamme *et al.*, 2002a), but is now believed to have lost its nodulation capability (Coenye and Vandamme, 2003). The only species with known nitrogen-fixing capability included in Cluster B is *B. vietnamiensis* (Chiarini *et al.*, 2006; Gillis *et al.*, 1995). The two above mentioned species form part of the medically important *B. cepacia* complex (Chiarini *et al.*, 2006; Coenye and Vandamme, 2003), which consists of another seven species (*B. ambifaria*, *B. anthina*, *B. cenocepacia*, *B. cepacia*, *B. multivorans*, *B. pyrrocinia* and *B. stabilis*; Chiarini *et al.*, 2006), which are all found within Cluster B. There are also another 22

Burkholderia species grouped into Cluster B, none of which as far as I am aware are capable of either nitrogen-fixation or nodulation (Fig. 2.4).

Within Cluster A (Fig. 2.4), the beta-rhizobia used in this study separated into 25 distinct lineages (referred to as SA1-SA25). Fourteen of these (i.e., SA3-SA5, SA7-SA11, SA13, SA14, SA16, SA21, SA22 and SA24) were represented by single isolates. The remaining 11 lineages (SA1, SA2, SA6, SA12, SA15, SA17-SA20, SA23 and SA25) were supported by the results of either or both phylogenetic methods of analyses, with values of MLbs $\geq 70\%$ and BIpp ≥ 0.7 . Of the 25 lineages, only five (SA1, SA6, SA12, SA15 and SA23) consisted of groups of isolates associated with a single host legume. Among these, the lineage SA12 isolates were characterised by 16S rRNA sequences identical to that of the type strain of *B. caledonica*. Still, the majority of the indigenous groups (i.e., SA1-SA8, SA11, SA15-SA17, SA19, SA23 and SA24) did not group with known *Burkholderia* species. However, lineage SA18 grouped closely with the diazotrophic *B. xenovorans* and lineage SA25 was closely related to the nitrogen-fixing *B. terrae*. Of the beta-rhizobia isolated in this study, only lineages SA20-SA22 grouped with a described nodulating species, *B. tuberum*. Furthermore, some of the beta-rhizobia isolates included appeared to be associated with species that have no described nodulating or even nitrogen-fixing capabilities. Examples of these are SA13 (represented by isolate CS2 from *C. subternata*) and SA14 (represented by isolate UCT71 from *C. glabra*) closely related to the type strains of *B. phenazinium* and *B. sediminicola*, respectively, as well as the large single host isolate lineage, SA12, for which the sequences appear identical to that of *B. caledonica* (Fig. 2.4).

Figure 2.4 shows that the isolates included in this study for the most part did not group closely with the *Mimosa*-nodulating isolates reported previously (Barrett and Parker, 2006; Chen *et al.*, 2005a; Chen *et al.*, 2005b; Parker *et al.*, 2007). The only exception was the association of isolate B. Mcas 7.1, obtained from a *Mimosa* species in Panama (Parker *et al.*, 2007), with lineages SA20-SA22. Nevertheless, the indigenous beta-rhizobia formed part of at least three larger clusters that grouped at less stringent levels than the lineages and contained indigenous South African isolates, as well as those isolated from the root nodules of *Mimosa* species (Fig. 2.4). One of these larger clusters (93% MLbs; 0.71 BIpp) included lineage SA25, several as yet undescribed strains from *Mimosa* (Barret and Parker, 2006; Chen *et al.*, 2005a), the type strains for nodulating species *B. sabiae*, *B. phymatum* and *B. caribensis*, all three of which are associated with *Mimosa* hosts (Chen *et al.*, 2008; Elliott *et*

al., 2007b; Vandamme *et al.*, 2002a), as well as the diazotrophic *B. terrae* and the soil-inhabiting *B. hospita* (Goris *et al.*, 2002). Lineages SA20-SA24 formed part of a second large well-supported cluster (70% MLbs) that included various undescribed strains isolated from the root nodules of *Mimosa* species from Australia, Costa Rica, Brazil, Venezuela and Taiwan (Barrett and Parker, 2006; Chen *et al.*, 2005a; Chen *et al.*, 2005b; Parker *et al.*, 2007), as well as some of the type strains for the described diazotrophic species *B. silvatlantica*, *B. unamae*, *B. tropica* and *B. kururiensis*, and nodulating species *B. mimosarum*, *B. nodosa* and *B. tuberum*. The third larger assemblage of South African rhizobia from indigenous legumes and *Mimosa* species included the remaining lineages SA1-SA19, but this grouping lacked statistical support in both ML and BI analyses (Fig. 2.4). Together with lineages SA1-SA19, this larger assemblage included strains that were isolated from the root nodules of *Mimosa* species in Australia and Brazil (Chen *et al.*, 2005a; Parker *et al.*, 2007), two strains associated with the indigenous South African papilionoid species *Rhynchosia ferulifolia* (Garau *et al.*, 2009), as well as the type strains for the diazotrophic species *B. xenovorans* and various other *Burkholderia* species, none of which are known to nodulate legumes (Fig. 2.4).

2.3.3 *recA* gene sequence analyses

The results of the *blastn* analyses of the *recA* sequences corroborated those of the 16S rRNA sequences. The *recA* sequences for all of the isolates examined in this study, showed 94-99% sequence similarity to those of *Burkholderia* species in the GenBank database. The limited availability of *recA* sequences in databases for previously characterised *Burkholderia* strains and species explains why there were fewer taxa in the *recA* dataset (Fig. 2.5) than in the 16S rRNA dataset (Fig. 2.4). The aligned dataset was 816 bases in length and contained no insertions or deletions. Most of the variation in this dataset was due to synonymous substitutions (i.e., nucleotide substitutions that do not change the inferred amino acid coded by a specific codon), while only a small number of non-synonymous substitutions (i.e., nucleotide substitutions that change the inferred amino acid coded by a specific codon) were detected (Fig. 2.6).

To confirm the phylogenetic groupings obtained with the 16S rRNA data, I also analysed data for a portion of the *recA* gene. ML and BI analyses of the *recA* dataset recovered a well-supported Cluster A from among the *Burkholderia* sequences included (92% MLbs; 0.99

BIpp) (Fig. 2.5). However, the *recA*-based Cluster A did not include the diazotrophic *B. kururiensis*, the two nodulating species *B. nodosa* and *B. mimosarum* nor *B. sacchari*, which is capable of accumulating polyhydroxyalkanoic acids (Brämer *et al.*, 2001). Within the *recA*-based Cluster A, many of the lineages identified in the 16S rRNA phylogeny were also recovered. Seven of the 25 16S rRNA-based lineages (SA1, SA6, SA12, SA15, SA18, SA19 and SA23) were well supported in the *recA* phylogeny (Fig. 2.5), and showed better resolution than what was obtained for the 16S rRNA phylogeny (Fig. 2.4). For example, SA12 which only had 100% MLbs support in the 16S rRNA phylogeny increased to show support for both ML and BI analyses in the *recA* phylogeny (94% MLbs; 1.00 BIpp). Other examples are SA1, which group together in the 16S rRNA phylogeny with support of 88% MLbs and 0.96 BIpp but shows an overall increase to 94% MLbs and 0.93 BIpp for the *recA* phylogeny; and SA6, which has only 72% MLbs support in the 16S rRNA phylogeny but increases to 98% MLbs and 0.96 BIpp in the *recA* phylogeny. Whereas 14 single taxon lineages were obtained in the 16S rRNA phylogeny (Fig. 2.4), the *recA* data recovered only five such lineages, SA5, SA9, SA11, SA13 and SA14 (Fig. 2.5). Other major differences between the respective 16S rRNA- and *recA*-based phylogenies of Figures 2.4 and 2.5 were as follows. According to the *recA* data, the assemblage containing 16S rRNA lineage SA20 also includes isolates from lineages SA21-SA24 as well as one SA25 isolate (HC1.1ba), whereas the remaining three SA25 isolates form a separate group closely related to *B. hospita* as in the 16S rRNA phylogeny. Furthermore, according to the *recA* data, SA17 represents an assemblage that includes lineage SA17 isolates, as well as isolates from lineages SA15 and SA16. Yet another difference between the phylogenies is the grouping within lineage SA2 consisting of UCT34, UCT43 and UCT56, which groups together with support based upon the 16S rRNA phylogeny (98% MLbs; 0.84 BIpp). In contrast the *recA* phylogeny shows a tighter association between UCT34 and UCT43 (99% MLbs; 1.00 BIpp support) with UCT56 still remaining their closest relative although now with no support. Of note was the phylogenetic placement of four isolates (Kb2, UCT31, RAU2i and HC1.1ba), which was not consistent and varied quite substantially between the two phylogenies (Figs. 2.4 and 2.5).

The information on the *recA* alignment (Fig. 2.6) was used to construct a rough grouping in which it is possible to see the underlying amino acid differences conceivably responsible for the associations encountered in the *recA* phylogeny (Fig. 2.5). For example, the movement of the isolates UCT31 and HC1.1ba to different lineages between the two phylogenies can be

explained as follows: In the *recA* phylogeny, isolate HC1.1ba moved from lineage SA25, in which the isolates all contain glutamine (Q) at position 85 in the sequence alignment followed by an aspartic acid (D) residue at position 90 and serine (S) at position 92, to a cluster containing the lineages SA20-SA24, with which it more closely aligns as it together with the lineages SA20-SA24 substitute glycine (G) in place of glutamine at position 85 and asparagine (N) in place of aspartic acid at position 90 and lastly alanine (A) in place of serine at position 92. Similarly, isolate UCT31 moved from lineage SA20 in the 16S rRNA phylogeny (Fig. 2.4) to a larger cluster containing lineages SA1 to SA8 and SA10 in the *recA* phylogeny (Fig. 2.5). The possible reason for this move is apparent in Figure 2.6 as UCT31 does not contain an alanine residue at position 92 as did the rest of the isolates from lineage SA20, but rather an asparagine residue, and instead of an aspartic acid residue at position 93, it has a glutamic acid (E) residue, the same as for isolates from lineages SA1 to SA8, SA10 and SA11.

The other two isolates that showed movement between the two phylogenies, Kb2 and RAU2i, appeared of less importance in this respect. RAU2i was a single isolate lineage (SA10) in the *recA* phylogeny (Fig. 2.5), and the same grouping was encountered when considering similarities in the protein alignment (Fig. 2.6). Similarly, isolate Kb2 was the sole representative of lineage SA16 in the 16S rRNA phylogeny, but it grouped very closely with a lineage SA17 isolate in the *recA* phylogeny, this being corroborated by protein alignment similarities between Kb2 and isolates from lineages SA12, SA15 and SA17. Some of the type strains do have non-synonymous changes in common with isolates from the indigenous sample group, for instance the isolate CS2 (from single strain lineage SA13) shares four such changes with *B. fungorum* (Fig. 2.6).

There are also a couple of instances where such similarities found on the protein alignment (Fig. 2.6) are reflected in the *recA* phylogeny (Fig. 2.5), for example the placement of *B. hospita* with isolates from lineage SA25 in the phylogeny corresponds to its placement in the protein alignment where it shares six non-synonymous changes with those same isolates (Fig. 2.6). The majority of type strains however did not show significant similarities (with regards to non-synonymous changes) to the indigenous isolates, except between the type strains themselves with groupings such as *B. sabiae*, *B. phymatum* and *B. Br3405* and separately the grouping of *B. latens*, *B. cenocepacia*, *B. ambifaria*, *B. arboris*, *B. dolosa*, *B.*

diffusa, *B. ubonensis* and *B. vietnamiensis* (Fig. 2.6). As far as I am aware there are no underlying features which connect these type strains and which could cause these groupings.

2.4 Discussion

All 69 isolates of rhizobia from the legumes *Hypocalyptus*, *Podalyria*, *Virgilia* and *Cyclopia* examined in this study were identified as *Burkholderia* using DNA sequence information for the genes encoding the 16S rRNA subunit and *recA* (Figs. 2.4 and 2.5). Amongst these isolates, 25 distinct lineages of *Burkholderia* were identified based upon phylogenetic relatedness (Figs. 2.4 and 2.5). Amongst the lineages containing more than one isolate, only five were represented by isolates from a single host species (Fig. 2.4), and divergence was evident even between clades containing isolates from the same hosts, notably SA1 and SA6, both of which contained *H. coluteoides* isolates, while SA12, SA15 and SA25 all contained *H. sophoroides* isolates. Distribution of isolates amongst the remaining lineages was often not related to host plant origin, some of the rhizobial lineages being associated with multiple plant hosts. For example, SA17 included isolates from all three species of *Hypocalyptus* as well as *V. oroboides*, a similar assortment was seen for SA20 which included a range of *Cyclopia* isolates as well as one isolate each of *H. oxalidifolius* and *H. sophoroides* (Fig. 2.4).

Despite the fact that strong host-based groups were not detected in this study, nodulation studies indicated that some isolates appeared to be specific to certain hosts. For example, none of the isolates obtained from *H. sophoroides* were capable of nodulating the other two species of *Hypocalyptus* (Table 2.1). The structure of the rhizobial Nod factor (NF) is the major determinant of nodulation and host range (Cooper, 2007; Gage and Margolin, 2000; Perret *et al.*, 2000). The NF backbone is encoded by the common *nod* genes, *nodABC*, while modifications to this backbone by the host-specificity-determining *nod* genes enable the recognition of this molecule by a particular set of legume hosts (Broughton *et al.*, 2000; Fujishige *et al.*, 2008; Spaink, 2000). An overall lack of host-associated groupings among the majority of isolates in my study suggests that these diverse *Burkholderia* rhizobia harbour symbiotic plasmids encoding similar sets of nodulation genes enabling them to nodulate the same host species (Chen *et al.*, 2003). In contrast, if a relatively specific NF is required for successful nodulation of *H. coluteoides* or *H. oxalidifolius*, the NF of the *H. sophoroides* rhizobia may have modifications that prevent its recognition by the other two

species. This could explain why inoculation tests performed on *H. coluteoides* plants (the only host for which enough seeds were available) with the rhizobial isolates from all *Hypocalyptus* spp., only led to successful nodulation in a few cases, every one of which occurred with isolates originating from *H. coluteoides* and *H. oxalidifolius*, but not *H. sophoroides* (Table 2.1). In contrast, most of the isolates were, able to induce nodules on the promiscuous nodulators siratro and cowpea. The observation that ability to nodulate siratro and cowpea often differed amongst individual *Burkholderia* isolates from *Hypocalyptus*, *Podalyria* and *Virgilia*, while several could not be induced to nodulate either siratro or cowpea (Table 2.1), resembles the varying degrees of specificity previously observed when bradyrhizobia were inoculated on these and other so-called promiscuous legumes (Thies *et al.*, 1991). More exhaustive cross-inoculation tests will be required to investigate both nodulation ability of all the *Burkholderia* isolates that tested negative in this study, as well as the apparent specificity noted above amongst *Hypocalyptus* symbionts. Of further interest will be to determine the presently unknown host boundaries that might exist between the four legume tribal groups. This, together with phylogenetic analyses of nodulation genes such as the common *nod* gene, *nodA*, would enable more concrete conclusions regarding nodulation specificity within this set of rhizobia, and might help explain why the *H. sophoroides* isolates do not group more closely with isolates from the other two *Hypocalyptus* hosts with regard to both cross-inoculation specificity and lineage differentiation (Figs. 2.4 and 2.5).

I had hoped to avoid some of the caveats associated with reliance on single genes or the 16S rRNA gene specifically, by including the ubiquitous *recA* gene, which is thought to occur as a single copy in the bacterial genome. Although apparently more variable than the 16S rRNA (Payne *et al.*, 2005), it is sufficiently conserved to allow its amplification with universal primers (Eisen, 1995; Martens *et al.*, 2007). Some of the issues surrounding single gene phylogenies are phenomena such as low resolution, unusual evolutionary rates (Gontcharov *et al.*, 2004), horizontal transfer, homologous recombination and gene conversion associated with a specific gene region which could all potentially obscure or obliterate the true phylogenetic relationships among taxa (Jaspers and Overmann, 2004; Martens *et al.*, 2007; van Berkum *et al.*, 2003; Vinuesa *et al.*, 2005). Indeed, a previous study investigating the genome structure of the majority of diazotrophic *Burkholderia* strains (*B. vietnamiensis*, *B. kururiensis*, *B. tropica*, *B. unamae*, *B. xenovorans* and *B. silvatlantica*), found that the 16S rRNA gene was present in multiple copies (up to four copies in both *B.*

unamae and *B. silvatlantica*) whereas the *recA* gene was found only as a single copy in all of the strains (Martínez-Aguilar *et al.*, 2008).

Inconsistencies between the *recA* and 16S rRNA phylogenies, notably the placement of four isolates (Kb2, UCT31, RAU2i and HC1.1ba) were, however, evident in this study as noted above (Section 2.3.3; Figs. 2.4 to 2.6). With the available data it was not possible to ascertain which of the two housekeeping gene phylogenies gave a more reliable picture of the phylogenetic groupings among my indigenous isolates as well as the *Burkholderia* type strains. Although both of the phylogenies were taken into consideration when the 25 lineages were assigned, it was decided to use the 16S rRNA phylogeny for comparative purposes. Inconsistencies consequent among use of single gene phylogenies have been attributed to factors such as horizontal gene transfer and incomplete lineage sorting. In terms of the former, inconsistent positions occupied by a taxon between single gene phylogenies is attributed to the presence of non-orthologous copies of the respective genes in the genome of the specific taxon (Lorenz and Wackernagel, 1994; Omelchenko *et al.*, 2003; Thomas and Nielsen, 2005). Based on our current understanding of the dynamic architecture of the bacterial genome (Amadou *et al.*, 2008; Lindström *et al.*, 2010; Young *et al.*, 2006), such horizontal gene transfer events are often used to explain the incongruent placement of rhizobia in single gene phylogenies (Martens *et al.*, 2008; Rivas *et al.*, 2009; Turner and Young, 2000). In comparison, incomplete lineage sorting or deep coalescence (Maddison, 1997; Maddison and Knowles, 2006) is not commonly invoked to explain incongruence among single gene trees of bacteria. In our dataset, the effects of this phenomenon can, however, not be discounted as it is often associated with short internal branches and early divergence as observed for the indigenous dataset in the phylogenies constructed in this chapter (Maddison, 1997; Maddison and Knowles, 2006).

As the isolates examined in this study generally originated from hosts in single localities, the possible wider geographic distribution of lineages SA1 to SA25 is still unclear. Nevertheless, it is striking that diverse isolates were obtained from all host nodules collected at single localities (Table 2.1). For example, *H. sophoroides* plants collected in the Old du Toit's Kloof pass near Paarl in the Western Cape Province of South Africa were nodulated by seven of the 25 distinct lineages of *Burkholderia* (SA12, SA15, SA17, SA20, SA21, SA24 and SA25). This diversity of rhizobial symbionts at single sites was particularly pronounced for the five lineages (SA5, SA9, SA16, SA17 and SA20) isolated from *V.*

oroboides, as these bacteria were all isolated from nodules collected from a single tree at the Kirstenbosch Botanical Gardens (J.F. Bloem personal communication). The same diversity can be seen when taking the *H. coluteoides* isolates into consideration as these grouped into five different lineages but were all isolates from the Storms River Bridge area (SA1, SA6, SA10, SA17 and SA19).

The shape of a root nodule is typically determined by the region in which cell division occurs (Crespi and Gálvez, 2000; Foucher and Kondorosi, 2000). The nodules induced on *H. coluteoides* were indeterminate in shape (Fig. 2.3), which generally resembled the elongated, finger-like shapes of the ‘caesalpinoid’ nodules or in some cases ‘astragaloid’ nodules with their fused lobes and fan-shape appearance (Doyle, 1998). Although the root-nodule structure of the *Cyclopia*, *Virgilia* and *Podalyria* included here (J.F. Bloem personal communication; Kock, 2003) have not yet been studied, legume hosts in the tribe Podalyrieae have been shown to have nodules that are ‘astragaloid’ indeterminate in shape (Doyle, 1998). This data is also consistent with what is known for the predominantly Australian tribes Bossiaeeae and Mirbelieae that together represent a sister group of the Hypocalypeteae (Lewis *et al.*, 2005; Wojciechowski *et al.*, 2004). Nodules from these two tribes are also indeterminate (Sprent, 2007) and a ‘caesalpinoid’ shape according to Doyle (1998), while Sprent (2009) finds them to be frequently branched. On the basis of the shape assumed by the nodule on the original host we could make assumptions regarding characteristics of the associated rhizobia. For example, in the indeterminate nodules of *Hypocalyptus*, the *Burkholderia* species within the nodules may have undergone endoreduplication during their differentiation into bacteroids and are thus unable to re-assume growth although this development (and subsequent changes in the plant cells) leads to more efficient nitrogen-fixation (Mergaert *et al.*, 2006; Vinardell *et al.*, 2003). In contrast, differentiated bacteroids from determinate nodules do not undergo this process and are able to re-assume growth similar to that experienced as rhizospheric bacteria (Mergaert *et al.*, 2006).

The results of previous studies have shown that the distribution of rhizobial *Burkholderia* species is determined by environmental factors rather than the presence of a certain host (Bontemps *et al.*, 2010; Garau *et al.*, 2009). These environmental factors include altitude and its effect on rainfall and temperature, as well as geology and soil type. It has been noted that nodulating *Burkholderia* symbionts are encountered more frequently in acid and infertile

soils than any of the alpha-rhizobia (Bontemps *et al.*, 2010; Garau *et al.*, 2009). In South Africa, nodulating *Burkholderia* strains have similarly been found associated with legumes growing in soils with low fertility and pH, including *Burkholderia* spp. WSM3937 and WSM 3930 nodulating *Rhynchosia ferulifolia* (Garau *et al.*, 2009), and *B. tuberum* that nodulates *Aspalathus carnosa*, as well as a range of *Cyclopia* species (Elliott *et al.*, 2007a). Examples from other parts of the world include *Burkholderia* sp. strains Br3405 and Br3407 that nodulate *Mimosa caesalpiniaefolia* in Brazil (Chen *et al.*, 2005a). The finding that the rhizobia obtained from the fynbos legumes included in this study all represent *Burkholderia* species, is therefore not unexpected as the CFR is characterised by nutrient poor, acidic soils (Cocks and Stock, 2001; Elliott *et al.*, 2007a). It must be emphasised, however, that soil rhizobia associated with legumes in the CFR are not restricted to beta-rhizobia such as *Burkholderia* (Dagutat, 1995). Diverse members of the alpha-rhizobia (*Sinorhizobium*, *Bradyrhizobium* and *Mesorhizobium*) are associated with species of *Lebeckia*, while *Aspalathus linearis* used for rooibos tea is nodulated by slow-growing bradyrhizobia (Dagutat, 1995; le Roux, 2003; Phalane, 2008).

Consistent with previous studies, our *recA* and 16S rRNA phylogenies (Figs. 2.4 and 2.5) divide the species of *Burkholderia* into two large clusters (Bontemps *et al.*, 2010; Caballero-Mellado *et al.*, 2007; Suárez-Moreno *et al.*, 2008), which appears to be associated with the lifestyle of these bacteria. Cluster B (Figs. 2.4 and 2.5) includes species that interact with plants, animals and/or humans mainly as pathogens (Bontemps *et al.*, 2010; Chiarini *et al.*, 2006; Suárez-Moreno *et al.*, 2008). Known exceptions are *B. ambifaria* that has plant growth promoting properties, and *B. vietnamiensis*, the first nitrogen-fixing *Burkholderia* species to be described (Chiarini *et al.*, 2006; Gillis *et al.*, 1995; Suárez-Moreno *et al.*, 2008). Species in the second cluster (Cluster A; Figs. 2.4 and 2.5) are often beneficial to their environment and appear to be mostly associated with plants (Suárez-Moreno *et al.*, 2008). A large number of the species in this cluster are capable of nitrogen-fixation in association with non-legumes such as sugarcane, maize and coffee, e.g. *B. silvatlantica* and *B. unamae* (Caballero-Mellado *et al.*, 2004; Perin *et al.*, 2006), while many others are capable of nodulating legumes (Chen *et al.*, 2006; Chen *et al.*, 2007; Chen *et al.*, 2008; Vandamme *et al.*, 2002a). The latter group can be extended to include all of the isolates obtained from the root nodules of *H. coluteoides*, *H. oxalidifolius*, *H. sophoroides*, *V. oroboides* and *P. calyptrata* used in my study. Presumably, the predisposition for establishing the nitrogen-fixing symbiosis of *Burkholderia* with legumes evolved in the ancestor of Cluster A.

Most of the *Burkholderia* isolates examined in this study appear to be novel. More than half of the 25 identified taxa (i.e., lineages SA1-SA25) are represented by single isolates that do not group with any known species of *Burkholderia* (Fig. 2.4). Even though the remaining taxa represented well-supported phylogenetic groups that included multiple isolates, they showed no conspecificity with described members of the genus at species level (Fig. 2.4). The only exception is clade SA20 that was found to be closely related to *B. tuberum* (Fig. 2.4), which was originally isolated from *Aspalathus carnososa*, another native to the CFR, like *Hypocalyptus* (Moulin *et al.*, 2001; Vandamme *et al.*, 2002a). *Burkholderia tuberum* also effectively nodulates a range of *Cyclopia* spp. found in the CFR fynbos (Elliott *et al.*, 2007a). If *B. tuberum* is indeed conspecific with SA20, its host range could extend to include *H. oxalidifolius*, *H. sophoroides*, and *V. oroboides*, i.e. the various host species for the isolates grouped in lineage SA20 (Fig. 2.4). Unambiguous identification of lineage SA20 isolates as *B. tuberum* will require their phenotypic characterisation coupled with DNA-based approaches such as multilocus sequence analysis (MLSA) (Gevers *et al.*, 2005). As for the remainder of the new taxa tentatively identified here (Figs. 2.4 and 2.5), future research should similarly focus on their polyphasic characterisation leading to possible formal description (Tindall *et al.*, 2010). Another lineage which warrants closer inspection is that of SA12, that associates closely with the type strain *B. caledonica* which is not known for its nitrogen-fixing or nodulation capabilities and was isolated from a rhizosphere soil sample taken in Scotland.

The wide use of DNA-based methods for identifying and recognizing rhizobia (Moulin *et al.*, 2001; Sawada *et al.*, 2003; Willems, 2006) has in the last decade stimulated a growing appreciation for the diversity associated with the legume-rhizobium symbiosis in indigenous environments (Cardinale *et al.*, 2008; Kock, 2003; Lafay and Burdon, 2007; Lorite *et al.*, 2010; Phalane, 2008). By making use of DNA sequence information and nodulation tests, I demonstrated for the first time that *Hypocalyptus* spp. in the enigmatic tribe Hypocalypeteae establish nitrogen-fixing symbioses with diverse members of the genus *Burkholderia*. I further provided evidence that bacteria in this genus nodulate *Podalyria calyptrata* and *Virgilia oroboides* in the related tribe Podalyrieae, and that they are closely related to the *Burkholderia* strains isolated from associated *Cyclopia* species (Kock, 2003). This complements the observation that nodulation of legumes by *Burkholderia* is fairly widespread (Sprent, 2007). Protein profile studies by Dagut (1995) provided the first

indication of the wide rhizobial diversity likely to be associated with indigenous South African legumes, as did similar later studies of rhizobia associated with a range of endemic legumes and more specifically indigenous *Lotononis* species (Kock, 1999; le Roux, 2003). Several of these legume species are used commercially in the production of herbal teas e.g., *Cyclopia* is used to prepare honeybush tea and *Aspalathus linearis* is used to prepare rooibos tea (Boone *et al.*, 1999; Elliott *et al.*, 2007a; Joubert *et al.*, 2008; Kock, 2003; Sprent, 2009), while others such as *Lotononis bainesii* have agricultural potential as forage plants in pastures (Ardley *et al.*, 2009). My study further advances our knowledge of the root-nodule bacteria associated with the indigenous legume flora of South Africa. Considering the unexpected diversity of *Burkholderia* rhizobia associated with the four taxonomically related plant tribes examined here (Figs. 2.4 and 2.5), it is not unrealistic to surmise that the diversity of other CFR legumes will extend to their respective rhizobial symbionts. My results hint at a wider diversity of indigenous beta-rhizobia found in South Africa than previously anticipated, with *Burkholderia* species likely to be a major if not dominant nodulator of fynbos legumes. Further study of the distribution and host associations of these bacteria is required, entailing sampling strategies targeting as many hosts as possible across a wide geographic region.

2.5 References

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2.6 Tables

Table 2.1 Rhizobial strains associated with indigenous Hypocalypteae and members of the Podalyrieae used in this study.

Legume Host ^a	Isolate ^b	Geographic Origin	GenBank Accession No.	16S rRNA Lineage
<i>H. coluteoides</i>	RAU2b (S, C, HC)	Storms River Bridge		SA17
<i>H. coluteoides</i>	RAU2c (S)	Storms River Bridge		SA6
<i>H. coluteoides</i>	RAU2d	Storms River Bridge		SA6
<i>H. coluteoides</i>	RAU2d2 (S, HC)	Storms River Bridge		SA17
<i>H. coluteoides</i>	RAU2f (S, C)	Storms River Bridge		SA1
<i>H. coluteoides</i>	RAU2g (S, HC)	Storms River Bridge		SA6
<i>H. coluteoides</i>	RAU2h	Storms River Bridge		SA1
<i>H. coluteoides</i>	RAU2i (S, C, HC)	Storms River Bridge		SA10
<i>H. coluteoides</i>	RAU2j (S, C)	Storms River Bridge		SA19
<i>H. coluteoides</i>	RAU2k (S, C)	Storms River Bridge		SA1
<i>H. coluteoides</i>	RAU2l (S, C, HC)	Storms River Bridge		SA1
<i>H. oxalidifolius</i>	RAU6.4a (S, C)	Fernkloof Nature Reserve		SA22
<i>H. oxalidifolius</i>	RAU6.4b (S, C, HC)	Fernkloof Nature Reserve		SA19
<i>H. oxalidifolius</i>	RAU6.4d (S, C, HC)	Fernkloof Nature Reserve		SA17
<i>H. oxalidifolius</i>	RAU6.4f (S, C)	Fernkloof Nature Reserve		SA17
<i>H. oxalidifolius</i>	HC6.4b ^c	Fernkloof Nature Reserve		SA20
<i>H. sophoroides</i>	WK1.1a (S, C)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1c (C)	Old du Toit's Kloof Pass		SA25
<i>H. sophoroides</i>	WK1.1d (S)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1e (S, C)	Old du Toit's Kloof Pass		SA20
<i>H. sophoroides</i>	WK1.1f (S)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1g (S)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1h (S, C)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1i (S, C)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1j (S, C)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1k (S, C)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	WK1.1m (C)	Old du Toit's Kloof Pass		SA12
<i>H. sophoroides</i>	HC1.1a(1) ^c	Old du Toit's Kloof Pass		SA24
<i>H. sophoroides</i>	HC1.1a(2) ^c (C)	Old du Toit's Kloof Pass		SA15
<i>H. sophoroides</i>	HC1.1a(3) ^c (S)	Old du Toit's Kloof Pass		SA25
<i>H. sophoroides</i>	HC1.1b(a) ^c (S)	Old du Toit's Kloof Pass		SA25

Legume Host ^a	Isolate ^b	Geographic Origin	GenBank Accession No.	16S rRNA Lineage
<i>H. sophoroides</i>	HC1.1b(b) ^c (S)	Old du Toit's Kloof Pass		SA25
<i>H. sophoroides</i>	HC1.1b(c) ^c	Old du Toit's Kloof Pass		SA17
<i>H. sophoroides</i>	HC1.1b(d) ^c	Old du Toit's Kloof Pass		SA21
<i>H. sophoroides</i>	HC1.1b(e) ^c (S)	Old du Toit's Kloof Pass		SA15
<i>H. sophoroides</i>	HC1.1b(h) ^c (C)	Old du Toit's Kloof Pass		SA12
<i>P. calytrata</i>	WC7.3a	Paarl Rock Nature Reserve		SA11
<i>P. calytrata</i>	WC7.3b	Paarl Rock Nature Reserve		SA23
<i>P. calytrata</i>	WC7.3c (S)	Paarl Rock Nature Reserve		SA18
<i>P. calytrata</i>	WC7.3d (S)	Paarl Rock Nature Reserve		SA23
<i>P. calytrata</i>	WC7.3f (S)	Paarl Rock Nature Reserve		SA18
<i>P. calytrata</i>	WC7.3g	Paarl Rock Nature Reserve		SA23
<i>V. oroboides</i>	Kb1A (S)	Kirstenbosch Botanical Gardens		SA20
<i>V. oroboides</i>	Kb2 (S)	Kirstenbosch Botanical Gardens		SA16
<i>V. oroboides</i>	Kb6	Kirstenbosch Botanical Gardens		SA9
<i>V. oroboides</i>	Kb12 (S)	Kirstenbosch Botanical Gardens		SA5
<i>V. oroboides</i>	Kb13 (S, C)	Kirstenbosch Botanical Gardens		SA17
<i>V. oroboides</i>	Kb14 (S)	Kirstenbosch Botanical Gardens		SA17
<i>V. oroboides</i>	Kb15 (S, C)	Kirstenbosch Botanical Gardens		SA17
<i>V. oroboides</i>	Kb16 (S, C)	Kirstenbosch Botanical Gardens		SA17
<i>C. buxifolia</i>	CB2	Helderberg, Somerset-West	AY178059	SA3
<i>C. genistoides</i>	UCT2	Rein's Farms	AY178073	SA20
<i>C. genistoides</i>	UCT15	Constantiaberg	AY178068	SA20
<i>C. glabra</i>	UCT34	Matroosberg, Ceres	AY178056	SA2
<i>C. glabra</i>	UCT71	Unknown	AY178064	SA14
<i>C. intermedia</i>	CI1	Dennehoek, Joubertina	AY178060	SA8
<i>C. intermedia</i>	CI2	Dennehoek, Joubertina	AY178069	SA20
<i>C. intermedia</i>	CI3	Dennehoek, Joubertina	AY178072	SA20
<i>C. longifolia</i>	Clong1	Thornhill, Humansdorp	AY178061	SA7
<i>C. longifolia</i>	Clong3	Thornhill, Humansdorp	AY178070	SA20
<i>C. maculata</i>	CM1	Paarlberg, Paarl	AY178058	SA4
<i>C. maculata</i>	UCT70	Jonkershoek	AY178075	SA20
<i>C. meyeriana</i>	UCT43	Hottentots Holland mountains	AY178055	SA2
<i>C. meyeriana</i>	UCT56	Hottentots Holland mountains	AY178054	SA2
<i>C. pubescens</i>	Cpub6	Next to N1, Port Elizabeth	AY178071	SA20
<i>C. sessiliflora</i>	Cses4	Plattekloof, Heidelberg	AY178063	SA18
<i>C. sessiliflora</i>	UCT30	Callie's Farm, Heidelberg	AY178067	SA19
<i>C. sessiliflora</i>	UCT31	Grootvadersbosch	AY178074	SA20

Legume Host ^a	Isolate ^b	Geographic Origin	GenBank Accession No.	16S rRNA Lineage
<i>C. subternata</i>	CS2	Dennehoek, Joubertina	AY178065	SA13

^a The legume hosts are all listed with an abbreviated genus name followed by the species name i.e., *Hypocalyptus coluteoides* = *H. coluteoides*.

^b Letters in brackets indicate the host plants on which nodulation occurred. S = siratro (*Macroptilium atropurpureum*; Tribe Phaseoleae), C = cowpea (*Vigna unguiculata*; Tribe Phaseoleae), HC = *Hypocalyptus coluteoides*. No brackets indicate nodulation not confirmed by the author.

^c Isolated by the author using trapping techniques.

2.7 Figures

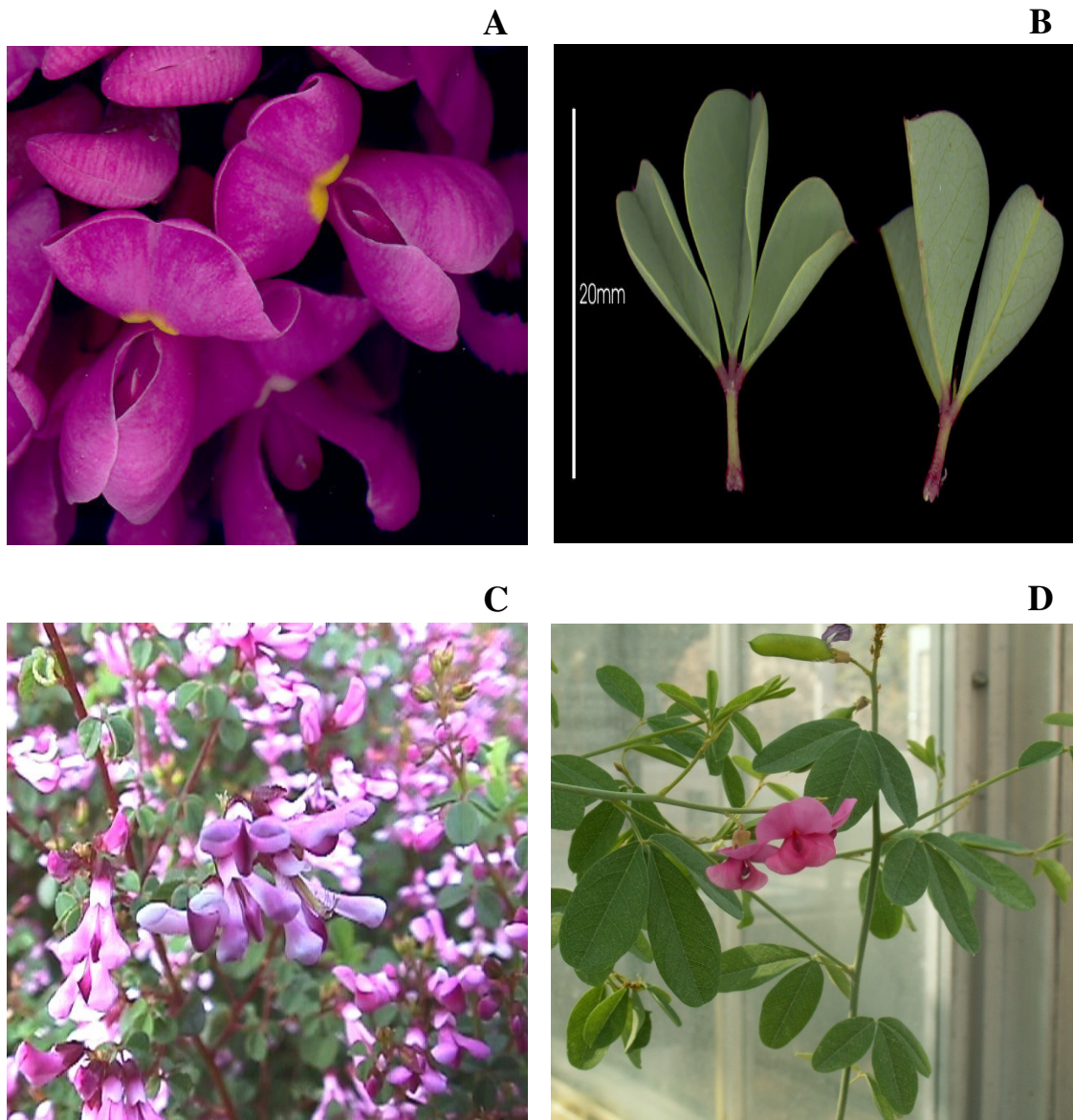


Fig. 2.1 Photographic representation of the three *Hypocalyptus* species and examples of their characteristic traits such as flowers and seed pods. **A** and **B**. *H. sophoroides* (source: <http://www.plantweb.co.za>). **C**. *H. oxalidifolius* (source: <http://www.fernkloof.com>) and **D**. *H. coluteoides* (personal photograph).

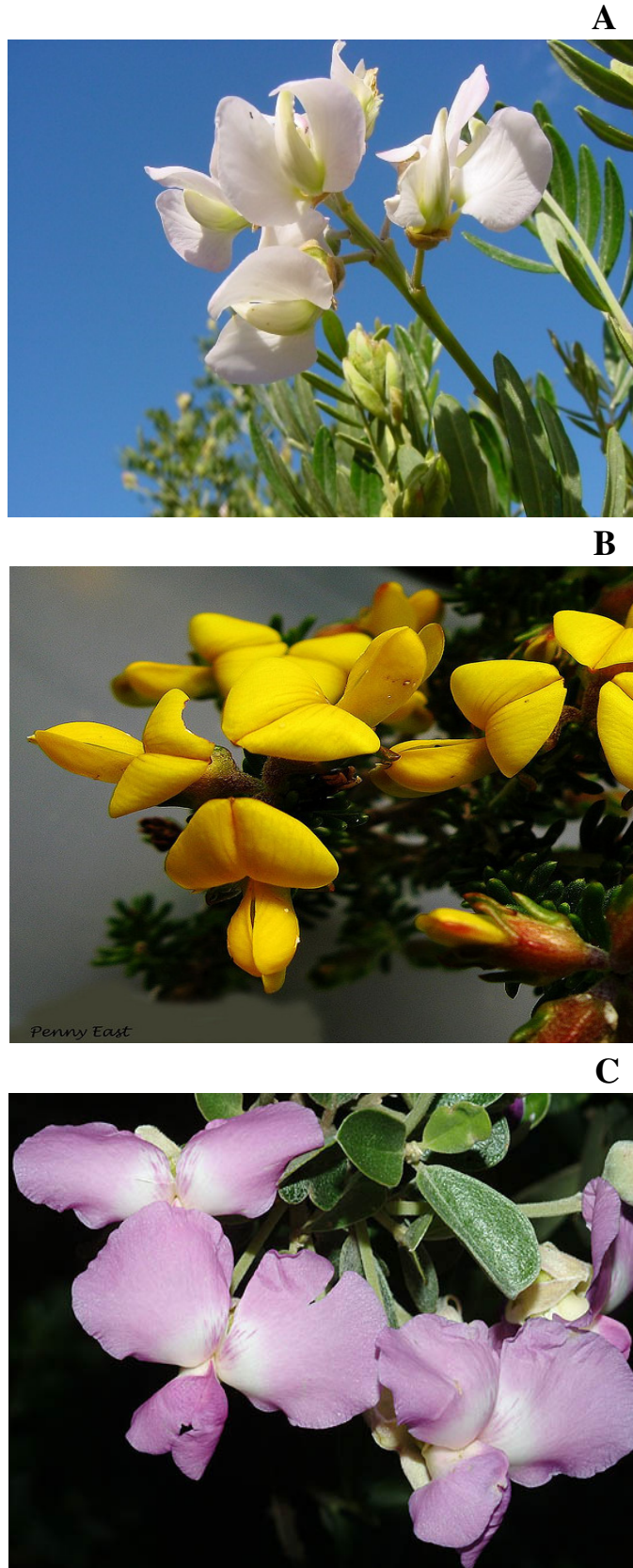


Fig. 2.2 Photographic examples of some of the Podalyrieae relatives of *Hypocalyptus* which form part of this study. **A.** *Virgilia oroboides* (source: www.fernkloof.com) **B.** *Cyclopia genistoides* (source: www.flickr.com) **C.** *Podalyria calyprata* (source: www.tropicamente.it).

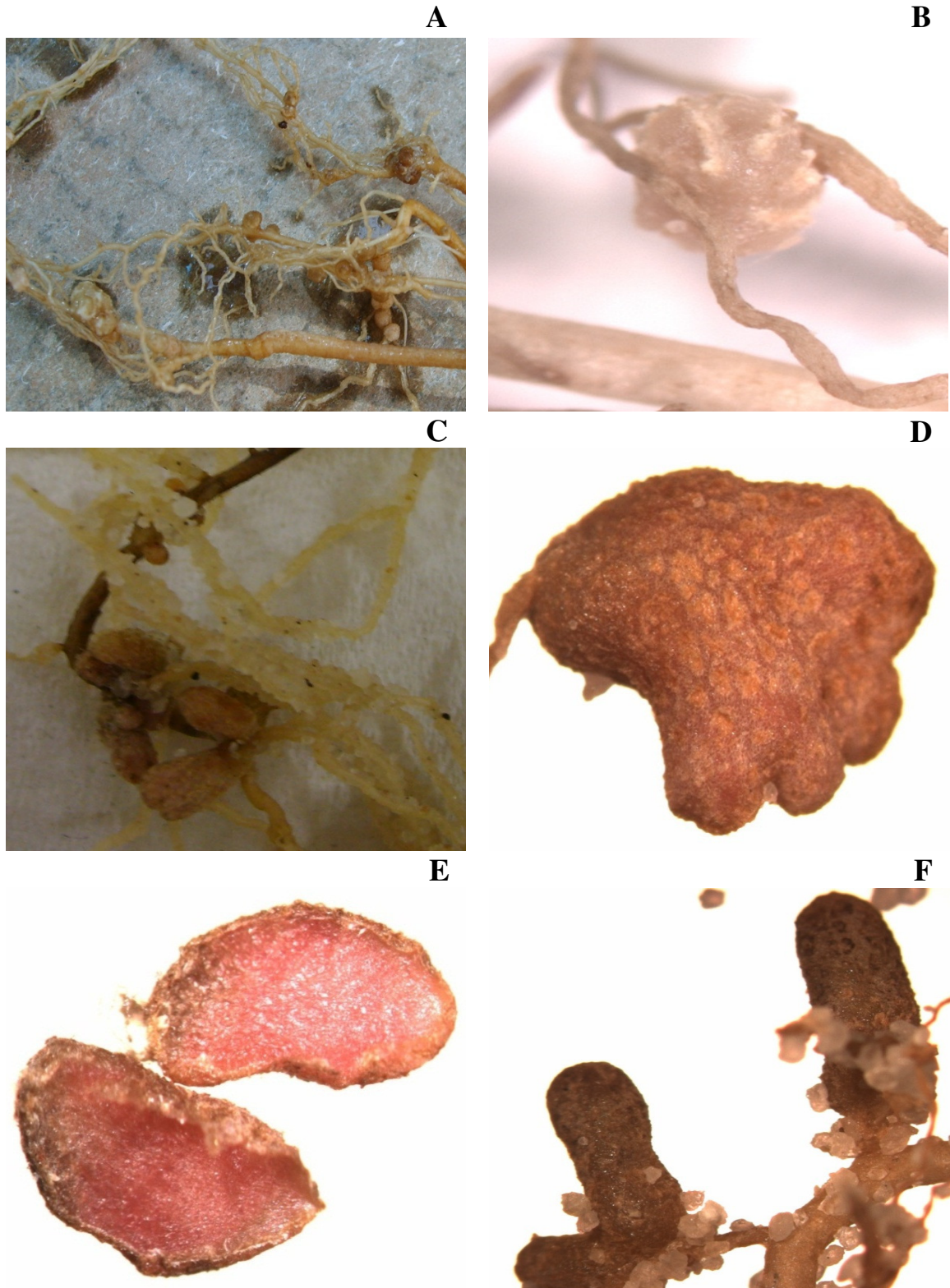


Fig. 2.3 Examples of nodulation test results observed in this study. **A.** Nodules formed after inoculation of siratro with isolate RAU6.4a obtained from *Hypocalyptus oxalidifolius*. **B.** A determinate nodule typical of those induced on cowpea roots by isolates obtained from *Hypocalyptus*. **C.** Indeterminate nodules induced by isolate RAU2i from *H. coluteoides* on the roots of *H. coluteoides*. **D.** An example of the fan-shaped nodule occasionally found on the roots of *H. coluteoides*. **E.** Dissection showing the pink interior of an effective nodule excised from the roots of *H. coluteoides* following inoculation with isolate RAU6.4b. **F.** Indeterminate nodules found abundantly on the roots of *H. coluteoides* following inoculation with a beta-rhizobial isolate of *Burkholderia*.

Fig. 2.4 A 16S rRNA maximum likelihood phylogeny for the genus *Burkholderia*. Known nodulating type strains appear in green and nitrogen-fixing type strains appear in orange. Shaded areas indicate the smallest well-supported (maximum likelihood bootstrap support $\geq 60\%$; Bayesian posterior probabilities ≥ 0.60) groups of isolates examined from *Hypocalyptus*, *Virgilia*, *Podalyria* and *Cyclopia* species. For each of these, specific lineage designations are indicated with SA1-SA25. Following the name of the undescribed nodulating *Burkholderia* strains are the names of their original host plant. Culture collection numbers and GenBank accession numbers for each type strain are listed in brackets at the back of each taxon. The *B. anthina* strain included in this analysis is not the type strain but it is the only *B. anthina* strain which has a 16S rRNA sequence available. This tree is rooted by both *Pandorea apista* and *P. norimbergensis*. Maximum likelihood support values as well as Bayesian posterior probabilities are indicated in the order BI/ML.

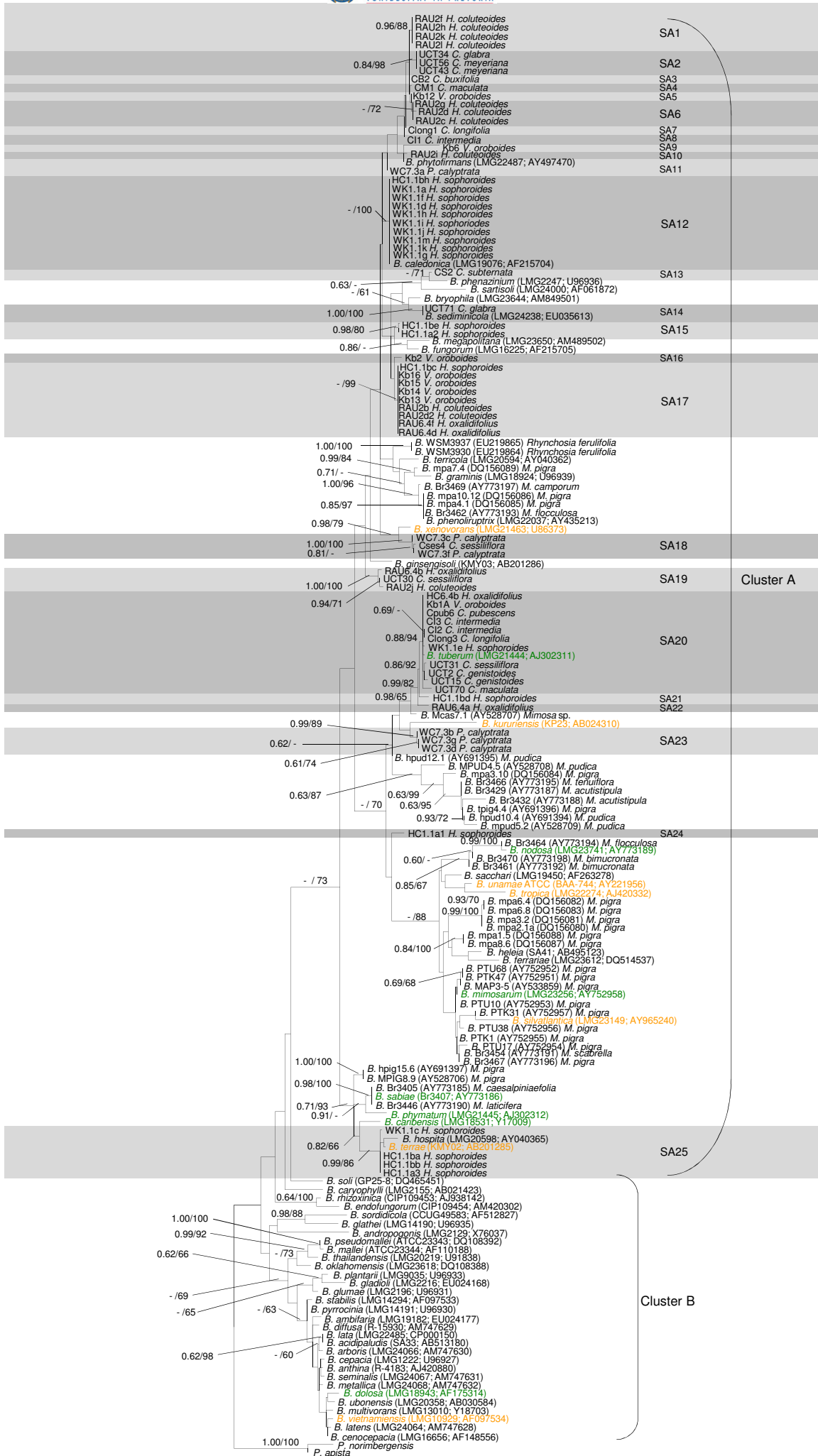
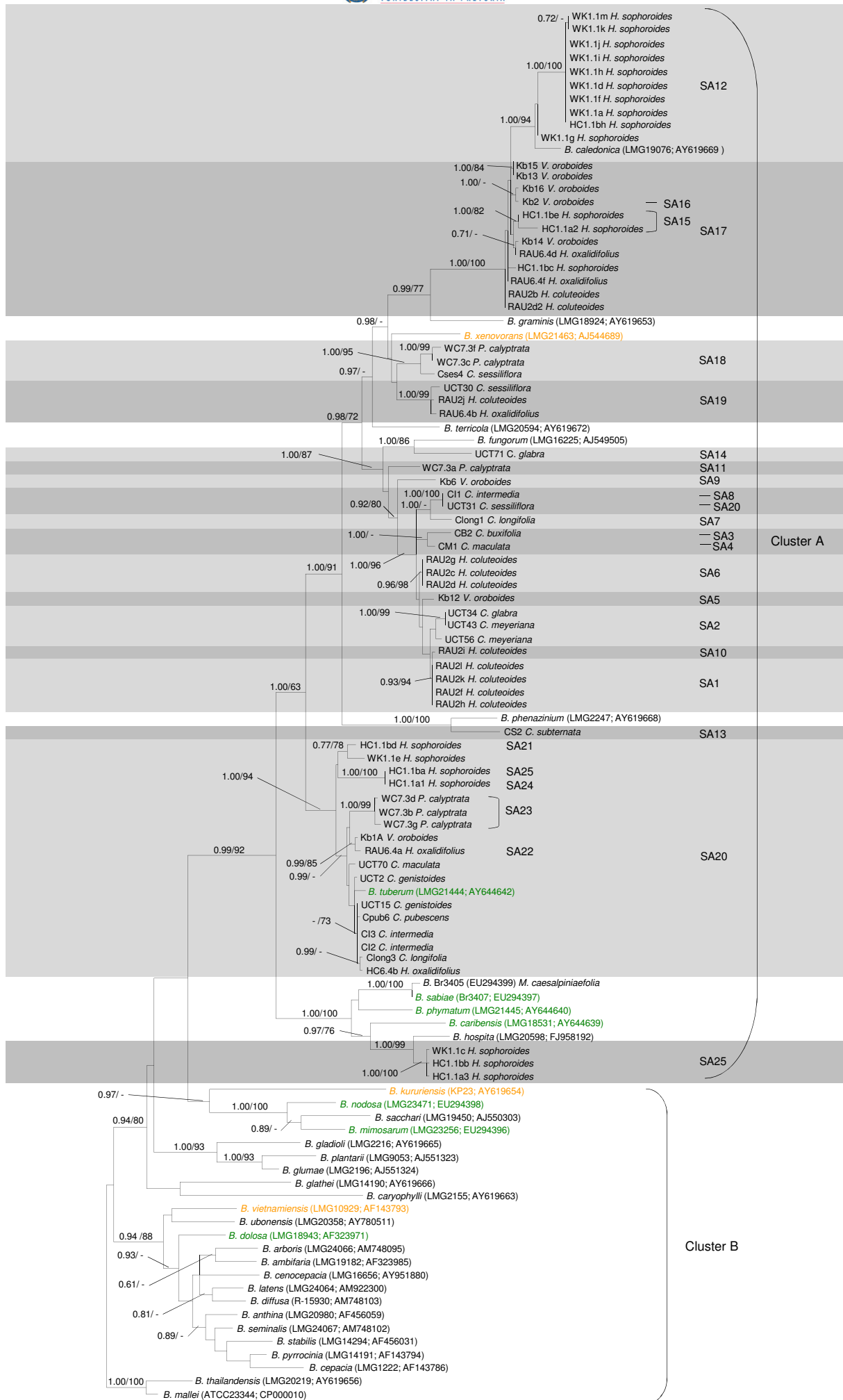


Fig. 2.5 A *recA* maximum likelihood phylogeny of the genus *Burkholderia*. Known nodulating type strains appear in green and nitrogen-fixing type strains appear in orange. Shaded areas indicates the smallest well-supported groups (maximum likelihood bootstrap support $\geq 60\%$; Bayesian posterior probabilities ≥ 0.60) of isolates examined from *Hypocalyptus*, *Virgilia*, *Podalyria* and *Cyclopia* species. For each of these, specific lineage designations based on both the 16S rRNA and *recA* data are indicated with SA1-SA25. The original host plants for the nodulating strains are also indicated, while type strain numbers as well as GenBank accession numbers are provided in brackets. This tree is rooted with *Burkholderia mallei* and *B. thailandensis*. Maximum likelihood support values as well as Bayesian posterior probabilities are indicated in the order BI/ML.



	1111 111111 11111 222 222222	
	666889999999 0001 234566 67799022 2333345	
	036150123678 5898 202114 80689201 6258974	
RAU6.4d SA17	IMLIGNVNDVVSQATAIVVSMTRLIRYARNEVSFAIGV	
RAU6.4f SA17	}
RAU2d2 SA17	
RAU2b SA17	
Kb13 SA17	
Kb14 SA17	
Kb15 SA17	
Kb16 SA17	
HC1.1bc SA17P.....V.....	
WK1.1g SA12	
WK1.1d SA12	
WK1.1f SA12	
WK1.1k SA12	
WK1.1m SA12	
WK1.1j SA12	
WK1.1i SA12	
WK1.1h SA12	
HC1.1bh SA12	
WK1.1a SA12	
<i>B. caledonica</i> SA12	
HC1.1be SA15	
HC1.1a2 SA15	
Kb2 SA16N.....	
RAU6.4b SA19	..I.....I	}
RAU2j SA19	..I.....I	
UCT30 SA19	..I.....K.....I	
WC7.3c SA18	..I.....IA.....I	}
WC7.3f SA18	..I.....G.....IA.....I	
Cses4 SA18	..I.....IA.....I	
<i>B. graminis</i>	..I.....I	
<i>B. terricola</i>	..I.....A...??	
Kb6 SA9	V.....A.....	
UCT71 SA14	V.....A.....	
WK1.1c SA25	V...QD.S.....A...I	}
HC1.1a3 SA25	V...QD.S.....AS...I	
HC1.1bb SA25	V...QD.S.....V.....A...I	
<i>B. hospita</i> SA25	V...QD.S.....T.....A.....I	
RAU6.4a SA22	V.....A.....A...I	}
WC7.3b SA23	V.....A.....A...I	
WC7.3d SA23	V.....A.....C.....A...I	
WC7.3g SA23	V.....A.....A...I	
Kb1A SA20	V.....A.....A...I	
HC1.1a1 SA24	V.....A.....A...I	
HC1.1ba SA25	V.....A.....A...I	
HC1.1bd SA21	V.....A.....A...I	
WK1.1e SA20	V.....A.....A...I	
HC6.4b SA20	V.....A.....A...I	
Clong3 SA20	V.....A.....A...I	
CI2 SA20	V.....A.....A...I	
UCT15 SA20	V.....A.....A...I	
UCT70 SA20	V.....A.....A...I	
Cpub6 SA20	V.....A.....A...I	
UCT2 SA20	V.....A.....A...I	
CI3 SA20	V.....A.....A...I	
<i>B. tuberum</i> SA20	V.....A.....A...I	
RAU2i SA10	V.....E.....A...I	}
RAU2l SA1	V.....E.....A...I	
RAU2f SA1	V.....E.....A...I	
RAU2h SA1	V.....E.....A...I	
RAU2k SA1	V.....E.....A...I	
RAU2g SA6	V.....E.....A...I	
RAU2d SA6	V.....E.....A...I	
RAU2c SA6	V.....E.....A...I	
WC7.3a SA11	V.....E.....A...I	
Kb12 SA5	V.....E.....A...I	
UCT31 SA20	V.....E.....A...I	

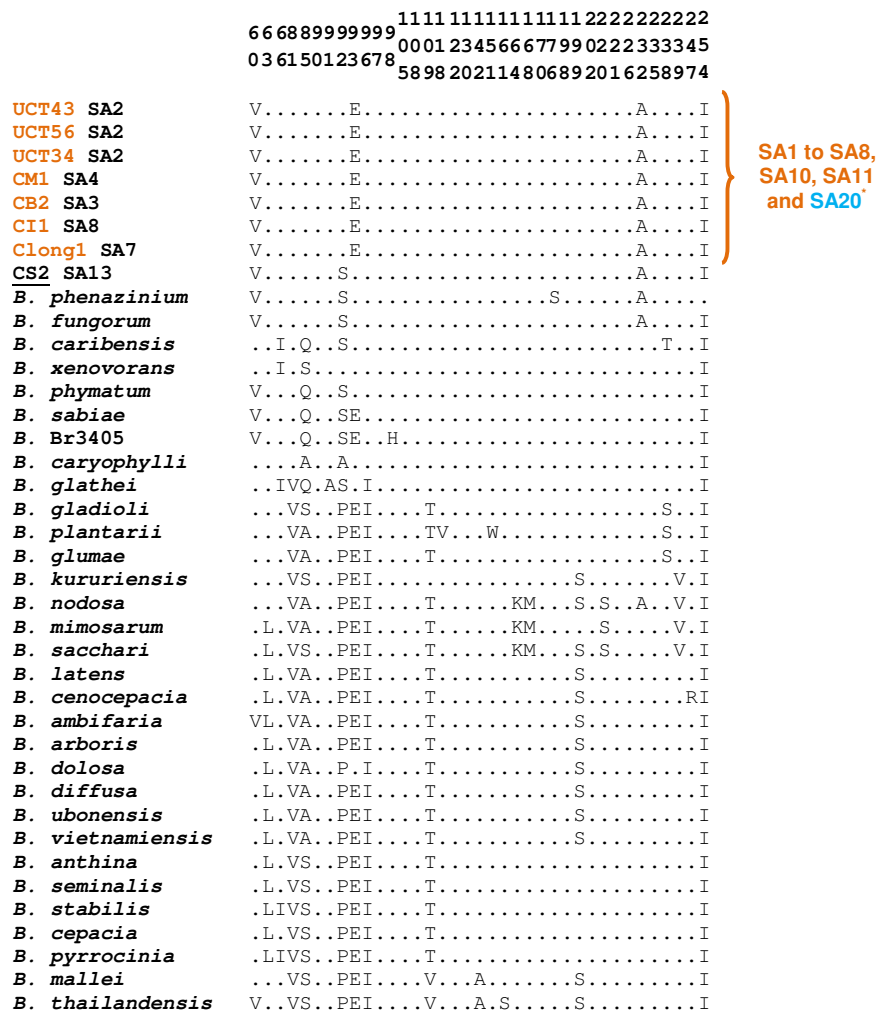
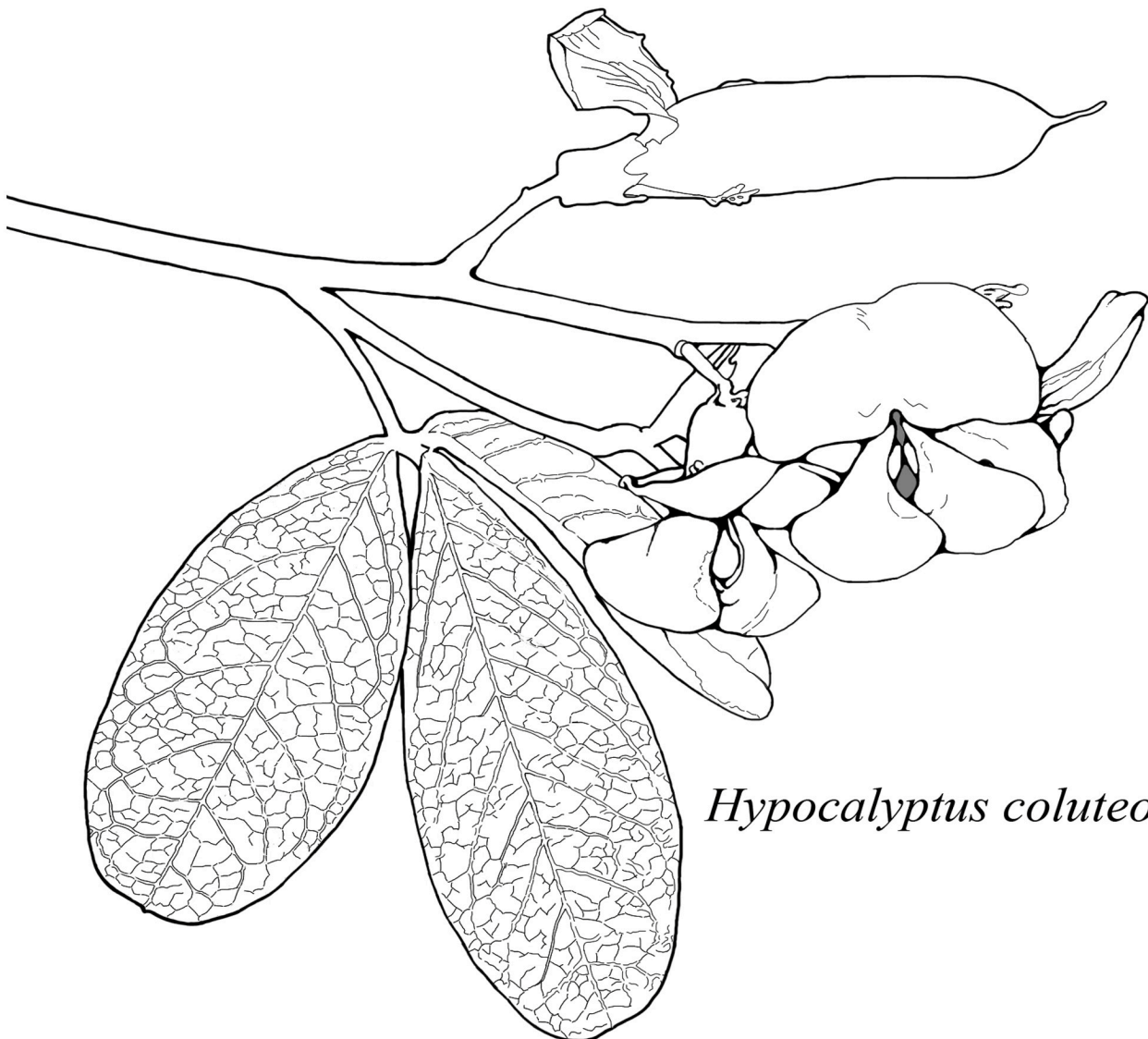


Fig. 2.6 Polymorphic amino acid residues in the alignment inferred from the *recA* sequences for the *Burkholderia* isolates included in this study. Isolate RAU6.4d was used as the reference. Groups containing similar sequences among the isolates from *Hypocalyptus*, *Virgilia*, *Podalyria* and *Cyclopia* are indicated with different colors. Isolate numbers are followed by lineage designations inferred from the 16S rRNA phylogeny presented in Fig. 2.4. The type strains are in black, as well as the three isolates Kb6, UCT71 and CS2 that correspond to single strain lineages SA9, SA14 and SA13, respectively. The numbers at the top of each column in the alignment are read vertically and correspond to the sequence position of that amino acid in the original alignment, e.g. the first polymorphism appeared at position 60 of the original DNA sequence alignment.

Chapter 3

African origins for fynbos associated beta-rhizobia



Hypocalyptus coluteoides

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Abstract

The discovery of beta-rhizobia in 2001 broadened the field of rhizobiology considerably. One of the first beta-rhizobia to be described, *Burkholderia tuberum*, was isolated from the indigenous South African fynbos legume *Aspalathus carnosa*. This species has since proven to be quite unique with regards to phylogenetic placement as determined by symbiotic loci. My study focused on a wide range of beta-rhizobia associated with the indigenous fynbos tribe Hypocalypeteae and related papilionoid species of the tribe Podalyrieae. Segments of the symbiotic genes, *nifH* and *nodA* for these indigenous isolates were amplified and phylogenetically analysed in order to determine if their symbiotic gene groupings were related to their tentative species identities, host plant origins, or geographic locations, or whether horizontal gene transfer was of importance. The origins of the *nodA* and *nifH* genes for these isolates were also investigated by comparing their respective placement amongst the broader spectrum of beta-rhizobial species. No clear correlation between the symbiotic groups of the isolates and their species identities based upon housekeeping gene lineages, host origin or geographic location could be uncovered. That there was only one group common to the two symbiotic gene phylogenies suggested that both vertical and horizontal gene transfer played a role in the evolution of both loci in this array of fynbos isolates. The broader analyses including beta-rhizobia showed that the fynbos isolates grouped closely with one another and with the described indigenous species *B. tuberum*. Interestingly, *nifH* analyses placed the free-living diazotrophic/nitrogen-fixing *Burkholderia* strains nearer to the indigenous isolates than to the nodulating *Burkholderia* strains, such as *B. phymatum*, *B. sabbiae* and *B. nodosa*. The same analyses further found that free-living diazotrophs from genera not associated with nodulation were more closely related to the isolates than alpha-rhizobia included in the dataset. The *nodA* analyses of the isolates further confirmed the close phylogenetic link between nodulating South African *Burkholderia* and the alpha-rhizobia *Methylobacterium nodulans* and strains of *Bradyrhizobium*. This was even closer than the link between the nodulating South African *Burkholderia* and the rest of the beta-rhizobia.

Key words: *Burkholderia*, *nodA*, *nifH*, *Hypocalyptus*, beta-rhizobia

3.1 Introduction

Biological nitrogen-fixation (the conversion of atmospheric nitrogen to ammonium) is facilitated by the nitrogenase enzyme produced by various diazotrophic bacteria and archaea (Dixon and Kahn, 2004; Hirsch *et al.*, 2001; Menna and Hungria, 2011). Among these, rhizobia (diazotrophic Gram-negative soil bacteria), together with their legume (Family Leguminosae) symbionts, have the largest quantitative effect on the nitrogen cycle (Menna and Hungria, 2011; Zahran, 1999). The most prominent feature of this symbiosis is the development of nodules on plant roots or stems in which the nitrogen is fixed. Establishment of this interaction is dependent on the exchange of molecular signals between the two symbionts (Angus and Hirsch, 2010), during which the rhizobial Nod factor (NF) initiates morphological changes in the legume resulting in the formation of nodules (Amadou *et al.*, 2008; Banba *et al.*, 2008; Cooper, 2007; Geurts *et al.*, 2005). Within the low-oxygen environment of these nodules, differentiated bacteroid-forms of the rhizobia perform the nitrogen-fixation process (Angus and Hirsch, 2010; Liu *et al.*, 2005; Wei *et al.*, 2009).

In rhizobia the genetic determinants of nitrogen-fixation and nodulation are carried on plasmids or symbiotic genomic islands (Flores *et al.*, 2005; MacLean *et al.*, 2007; Stępkowski and Legocki, 2001). For nodulation, these determinants include the genes involved in formation of the NF backbone (i.e., *nodABC*, the common *nod* genes) (Debellé *et al.*, 2001; Spaink, 2000), as well as the host-specificity nod (*hsn*) genes that are responsible for modifying this backbone for recognition by a subset of specific legumes (Debellé *et al.*, 2001; Downie, 2010; Stępkowski and Legocki, 2001). For nitrogen-fixation, the genetic determinants include the *nif* genes *nifHDK*, where *nifH* encodes dinitrogenase reductase necessary for the formation of a functional nitrogenase enzyme from the products of *nifD* and *nifK* (Franche *et al.*, 2009). In contrast with the *nif* genes that appear to be common among diazotrophs, the nodulation genes appear to be restricted to rhizobial taxa (Bontemps *et al.*, 2010; Giraud *et al.*, 2007; Moulin *et al.*, 2001).

The *nod* and *nif* genes form part of the so-called accessory genome (Harrison *et al.*, 2010; Lindström *et al.*, 2010; Young *et al.*, 2006) as they are positioned on mobile genetic elements such as plasmids and symbiotic islands. The determinants of nodulation and nitrogen-fixation are thus prone to horizontal gene transfer (HGT) (MacLean *et al.*, 2007; Pérez-Mendoza *et al.*, 2004; Sullivan and Ronson, 1998), a process that is thought to have

shaped the evolution of rhizobia (González *et al.*, 2003; MacLean *et al.*, 2007). It has also been shown that these symbiotic loci are transferable between genera (Barcellos *et al.*, 2007). The effects of HGT on these bacteria are perhaps most strikingly revealed when *nif* and *nod* gene phylogenies are compared with those inferred from housekeeping genes located on their so-called core genomes (Bailly *et al.*, 2007; Haukka *et al.*, 1998; Ibáñez *et al.*, 2010; Rivas *et al.*, 2007).

Initially, the legume-rhizobium symbiosis was thought to be a trait limited to diverse members of the bacterial class Alphaproteobacteria (Sawada *et al.*, 2003; Willems, 2006), but rhizobia are now known to also occur in the class Betaproteobacteria (Chen *et al.*, 2003; Moulin *et al.*, 2001). To distinguish between the rhizobia in these two classes, the terms alpha-rhizobia and beta-rhizobia have been introduced (Moulin *et al.*, 2001). Currently the alpha-rhizobia spans 12 genera found across seven families (see Table 1.1 of this dissertation). The beta-rhizobia described so far include species in the genera *Burkholderia* (Moulin *et al.*, 2001), *Cupriavidus* (Chen *et al.*, 2001; Vandamme and Coenye, 2004), *Herbaspirillum* (Valverde *et al.*, 2003) and *Achromobacter* (Benata *et al.*, 2008). However, the majority of known beta-rhizobia belong to *Burkholderia*, a large genus of diverse species (Coenye and Vandamme, 2003; Compant *et al.*, 2008) that include medically important species, as well as species with antifungal properties and plant growth promotion traits (Chiarini *et al.*, 2006; Coenye and Vandamme, 2003; Vandamme *et al.*, 2007). The genus *Burkholderia* currently include six rhizobial taxa (*B. tuberum*, *B. phymatum*, *B. caribensis*, *B. mimosarum*, *B. nodosa* and *B. sabiae*) (Chen *et al.*, 2006; Chen *et al.*, 2007; Chen *et al.*, 2008; Vandamme *et al.*, 2002) and seven non-rhizobial diazotrophic species (*B. silvatlantica*, *B. tropica*, *B. xenovorans*, *B. unamae*, *B. terrae*, *B. kururiensis* and *B. vietnamiensis*) (Caballero-Mellado *et al.*, 2004; Estrada-de los Santos *et al.*, 2001; Gillis *et al.*, 1995; Goris *et al.*, 2004; Perin *et al.*, 2006; Reis *et al.*, 2004; Yang *et al.*, 2006).

Current data on the symbiotic loci of the beta-rhizobia is sparse and heavily skewed towards those *Burkholderia* species and *Cupriavidus taiwanensis* strains associated with the genus *Mimosa* (Leguminosae, subfamily Mimosoideae). It has, however, been established that *nifH* generally represents a single copy gene in the free-living diazotrophic *Burkholderia* (Martínez-Aguilar *et al.*, 2008) and that it is found on the same 0.5 Mb plasmid as the *nod* genes of both *Burkholderia phymatum* and *Cupriavidus taiwanensis* (Chen *et al.*, 2003). Also, in contrast to effects suggested for other rhizobia (Galibert *et al.*, 2001; Suominen *et*

al., 2001), recent HGT appears to have had a limited impact on the evolution of the symbiotic loci of these symbionts of *Mimosa* species as very little incongruence could be detected among the *nif*, *nod* and housekeeping loci (Bontemps *et al.*, 2010). In addition, the *Mimosa* symbionts apparently group according to environmental factors (Bontemps *et al.*, 2010) rather than host or geography as suggested for rhizobia such as *Bradyrhizobium* species (Steenkamp *et al.*, 2008; Vinuesa *et al.*, 2005).

This research chapter describes a phylogenetic analyses of two symbiotic loci (*nodA* and *nifH*) for a group of indigenous South African *Burkholderia* associated with Papilionoideae legume species in the fynbos tribes Hypocalypteae (i.e., *Hypocalyptus sophoroides*, *H. coluteoides*, *H. oxalidifolius*) and Podalyrieae (i.e., *Podalyria calyptrata*, *Virgilia oroboides*, *Cyclopia buxifolia*, *C. genistoides*, *C. glabra*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. meyeriana*, *C. pubescens*, *C. sessiliflora* and *C. subternata*). The aim was to answer three questions: Is there evidence of HGT with regards to the respective symbiotic loci? Is there any indication of host-specific associations? What are the evolutionary origins of their nodulation and diazotrophic properties?

3.2 Materials and Methods

3.2.1 Rhizobial cultures and DNA extraction

A study set of 69 *Burkholderia* isolates was used, representing 25 distinct lineages (SA1-25) that are potentially separate species as previously characterised in Chapter 2 of this dissertation. Table 3.1 captures the information for the host legumes and geographic origins of these isolates. DNA was extracted from all 69 isolates following the procedure described in Chapter 2 of this dissertation.

3.2.2 *nodA* and *nifH* amplification and sequencing

In order to amplify a portion (*ca.* 500 bp) of the *nodA* gene region, primers NodAunivF145u (5' TGG GCS GGN GCN AGR CCB GA 3') and NodARbrad (5' TCA CAR CTC KGG CCC GTT CCG3') (Kock, 2003; Moulin *et al.*, 2001) were used. The PCR cycling conditions were as follows: an initial denaturation step of 15 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 63.5 °C and elongation for 1 min at 72 °C, with a final elongation step of 5 min at 72 °C. For the amplification of a

fragment of the *nifH* region (ca. 800 bp), primers NifHF (5' GCG AAT CTA CGG NAA RGG NGG 3') and NifH2R (5' CTC CAT CGT DAT NGG NGT NGG 3') were designed and used in PCR. The cycling conditions for the *nifH* PCR was as follows: initial denaturation for 15 min at 95 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 64 °C, and lastly 1 min of elongation at 72 °C, finishing with a longer elongation step of 10 min also at 72 °C. The total volume of the *nifH* and *nodA* amplification reaction mixtures were 50 µl, which contained 50-100 ng DNA, 50 µM/µl of each of the respective primers, 25 mM MgCl₂, 2.5 mM of each dNTP and 5U/µl FastStart Taq DNA polymerase and reaction buffer (Roche Diagnostics, Mannheim, Germany). All PCRs were performed on any one of the following machines an iCycler (BioRad, Hercules, California, USA) or GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, California, USA) or an Eppgradient Mastercycler (Eppendorf, Hamburg, Germany). PCR fragments were visualised using agarose gel (Whitehead Scientific, Brackenfell, SA) electrophoresis (Sambrook and Russell, 2001), during which the gels were stained with 1mg/ml ethidium bromide.

Following purification with a PCR Purification Kit (Qiagen, Hilden, Germany), amplicons were cloned using the pGEM®-T Easy Vector System (Promega Corp., Madison, WI, USA) and Library Efficiency® DH5α™ competent cells (Invitrogen, Paisley, UK), according to the manufacturers' protocols. Cloned inserts were amplified directly from the transformed bacteria using vector-specific primers T7 and SP6 (Steenkamp *et al.*, 2006) as described above, except that 10 µM/µl of each primers, and 1U/µl Super-Therm Taq DNA polymerase and PCR buffer (Southern Cross Biotechnology, Cape Town, SA) were used. PCR cycling conditions included an initial denaturation for 2 min at 94 °C, followed by 30 cycles containing the following steps: denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C and lastly elongation for 2 min at 72 °C before a final elongation step for 7 min at 72 °C. All colony PCR products were purified and concentrated by means of a polyethylene glycol precipitation protocol (Steenkamp *et al.*, 2006). The clean products were then sequenced using primers T7 and SP6, the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and the ABI3100 Automated Capillary DNA Sequencer (Applied Biosystems).

3.2.3 DNA sequence alignment and analysis

ABI trace files were analysed using Chromas Lite version 2.01 (Technelysium, Tewantin, Queensland, Australia) and BioEdit version 7.0.5.3 (Hall, 1999) to remove vector-specific sequences. The completed sequences were then compared to *nifH* and *nodA* sequences in the GenBank database (National Centre for Biotechnology Information <http://www.ncbi.nlm.nih.gov/>) using *blastn* (Altschul *et al.*, 1990; Benson *et al.*, 2004). These *blastn* analyses were also used to select known sequences for the type strains of *Burkholderia* species to be used, together with those for the examined 69 isolates, in multiple sequence alignments. All multiple alignments were generated manually in BioEdit 7.0.5.3, by making use of the inferred amino acids for each sequence set. This software was also used for routine visualisation of all alignments.

Four different datasets were subjected to phylogenetic analyses. The first two datasets included information for the *nifH* and *nodA* regions of the 69 *Burkholderia* isolates included in this study. The third dataset included *nifH* sequences for a representative isolate from each of the 25 lineages (where possible) identified using housekeeping loci (Chapter 2 of this dissertation), as well as the type strains for nodulating and non-nodulating diazotrophic *Burkholderia* species and known, but as yet undescribed, isolates of nodulating *Burkholderia*. This dataset also included representatives of free-living diazotrophs from the Betaproteobacteria (*Leptothrix cholodnii*), Alphaproteobacteria (*Xanthobacter autotrophicus*) and the Gammaproteobacteria (*Methylococcus capsulatus*), as well as the alpha-rhizobia (*Rhizobium alkalisoli* and *Ensifer fredii*). The fourth dataset included *nodA* sequences for representatives of the 25 lineages previously identified (Chapter 2 of this dissertation) and a range of alpha-rhizobia (i.e., *Methylobacterium nodulans*, several *Bradyrhizobium* species, two *Rhizobium* species and three *Ensifer* species). The fourth dataset also contained the *nodA* sequences known for all other *Burkholderia* species and the wider beta-rhizobia.

3.2.4 Phylogenetic analyses

All datasets were subjected to maximum likelihood (ML) phylogenetic analyses using PHYML version 3 (Guindon and Gascuel, 2003). These analyses utilised the best-fit evolutionary models as indicated by jModelTest version 0.1.1 (Felsenstein, 2005; Guindon and Gascuel, 2003; Posada, 2008). The *nifH* dataset with the 69 *Burkholderia* sequences

used the “transitional model” (TIM3) (Posada, 2008), while the broader *nifH* dataset used the “transversional model” (TVM) (Posada, 2003). The *nodA* dataset with the 69 *Burkholderia* sequences used the Hasegawa-Kishino-Yano (HKY) model (Hasegawa *et al.*, 1985), while the broader *nodA* dataset used the TVM model. All analyses also included a proportion of invariable sites and gamma correction for among site rate variation, except for the first *nifH* analysis for which the proportion of invariable sites was negligible. Branch support was estimated with bootstrap (MLbs) analyses (Felsenstein, 1985) based on 100 pseudoreplicates under the same best-fit model parameters.

All dataset were further subjected to Bayesian inference (BI) analyses with MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). As indicated by MrModeltest version 2.3 (Nylander, 2004), these analyses all used the General Time Reversible (GTR) model (Tavaré, 1986) with a proportion of invariable sites and gamma correction for between site rate variation. The only exception was the *nodA* dataset with the 69 *Burkholderia* isolates, which used the HKY model. All four BI phylogenies were determined by means of the Metropolis-coupled Markov chain Monte Carlo search algorithm. The analyses of the *nifH* and *nodA* datasets for the 69 *Burkholderia* isolates were based on 1 500 000 and 2 000 000 generations respectively, and burnin values of, respectively, 40 and 50 to calculate posterior probability (BIpp) values from the remaining trees. The analyses for the more broadly sampled *nifH* and *nodA* datasets included 5 000 000 and 2 000 000 generations, and burnin values of respectively 100 and 50 for calculation of BIpp values from the remaining trees.

3.2.5 Detection of congruence

In order to determine whether or not the indigenous *nifH* and *nodA* phylogenies were congruent, the incongruence length difference (ILD) (Farris *et al.*, 1995) test was performed in PAUP* version 4b10 (Swofford, 2003). During this test 1000 repartitions of each dataset was generated and then submitted to heuristic searches based upon 1000 cycles of simple sequence additions and tree bisection reconnection branch swapping. A visual inspection of the similarity between trees generated from the two datasets was also performed.

3.3 Results

3.3.1 DNA sequence alignment and analysis

Despite several attempts, the *nifH* primers used here was not successful in amplifying the corresponding region for two isolates (RAU2h from *H. coluteoides* and CM1 from *C. maculata*). Also, the *nifH* region sequenced in isolate Kb12 from *V. oroboides* apparently harboured a frameshift mutation. With respect to the *nodA* sequences, those of isolate WK1.1e from *H. sophoroides* and isolate UCT15 from *C. genistoides* were unusual and not included in subsequent analyses.

The *blastn* searches against the NCBI database indicated that all 67 of the new *nifH* sequences examined were most similar to that of the *Burkholderia tuberum* type strain STM678 isolated from *Aspalathus carnosus* (Tribe Crotonarieae). The maximum identity with this database sequence was 96-99%, followed by various diazotrophic *Burkholderia* species (e.g., *B. xenovorans*, *B. tropica*, *B. vietnamiensis* or *B. unamae*) with identity values of 88-92%. In all cases, the new *nifH* sequences were also highly similar to those of one or both of the strains (WSM3937 and WSM3930) previously isolated from the root nodules of *Rhynchosia ferulifolia* (Tribe Crotonarieae) in Darling, South Africa (Garau *et al.*, 2009).

For the 747-base pair (bp) *nifH* sequences, the corresponding 249 amino acid residues were inferred. However, the nucleotide sequences contained relatively few substitutions, with only a small proportion being non-synonymous and causing amino acid changes. Among the 66 sequences examined, nucleotide substitutions were observed at 94 sites, of which only 39 were non-synonymous (Fig. 3.1). Because of this overall uniformity, no obvious pattern of grouping could be observed from the deduced amino acid data.

The *blastn* searches against the NCBI database indicated that the 67 *nodA* sequences generated here were all highly similar (> 95%) to those of other *Burkholderia* isolates. The top hit for all was any one of *B. tuberum* strain DUS833, isolate WSM3937 or a range of isolates obtained from *Cyclopia* species (Kock, 2003). These sequences were also closer to the *nodA* sequences of known alpha-rhizobia such as *Methylobacterium nodulans* or the *Bradyrhizobium* species than any of the other *Burkholderia* and beta-rhizobial species.

A *nodA* amino acid alignment consisting of 145 residues were deduced for the 435 bp region examined in the 64 *Burkholderia* isolates. Overall this dataset contained 59 nucleotide substitutions of which 25 gave rise to amino acid substitutions (Fig. 3.2). In contrast to the *nifH* amino acid data, 17 groups of isolates could be identified in the *nodA* amino acid alignment, especially when the *nodA* phylogenetic tree was taken into consideration (see below). Of these, only one was represented by isolates originating from a single legume species (*H. sophoroides*), while the remainder of the groups each included isolates originating from various legume species.

3.3.2 Phylogenetic analyses of the *nifH* sequences

ML and BI analyses of the *nifH* sequences generated in this study separated the *Burkholderia* species into a number of supported ($\geq 60\%$ MLbs and ≥ 0.60 BIpp) groups (i.e., *nifH* groups 1, 4, 5, 6, 17, 18, 19, 20 and 21; Fig. 3.3). These mostly included several different *Burkholderia* lineages, each of which possibly represents species (see Chapter 2 of this dissertation). There were, however, some similarities between groupings observed here and those found previously in the phylogeny of the housekeeping gene *recA* (Chapter 2 of this dissertation, Fig. 2.5). For example, lineage SA16 represented by isolate Kb2 grouped with lineage SA17 isolates, while lineage SA10 represented by isolate RAU2i grouped with lineage SA1 isolates. Additionally, the *nifH* groups mostly included isolates that originated from more than one host species (Fig. 3.3). In only four instances (i.e., *nifH* groups 6, 17, 19 and 21), were isolates from the same host included within one group. The isolates in *nifH* groups 6 and 17 all originated from *P. calytrata*, and the isolates in *nifH* groups 19 and 21 all originated from *H. sophoroides*. Therefore, the isolate groups observed in the *nifH* phylogeny generally did not match those previously observed for these isolates in the housekeeping phylogenies (e.g., compare Fig. 3.3 with Fig. 2.4 in Chapter 2 of this dissertation), or their known host associations. There was also no clear correlation to geographic origin, as for example none of the *P. calytrata* isolates were placed in one group but instead occupied four separate groups (5, 6, 17 and 18; Fig. 3.3) although they were all isolated from the Paarl Rock Nature Reserve (Table 3.1).

The evolutionary origin(s) of the *nifH* sequences generated in this study used a dataset containing sequences for a representative of each of 23 of the 25 lineages identified from housekeeping gene sequences (i.e., SA1-25 excepting SA3 and SA5; see Chapter 2 of this

dissertation), as well as a broader set of diazotrophic species (Fig. 3.4). Phylogenetic analyses of this dataset placed the *nifH* sequences of South African origin in a single clade (100% MLbs) (Fig. 3.4). In addition to the isolates from fynbos hosts of the present study, this clade also included the type strain for *B. tuberum*, and strain WSM3937 isolated from the root nodules of *R. ferulifolia*, both being from the fynbos region in South Africa (Fig. 3.4). The sister taxon (90% MLbs; 1.00 BIpp) to the South African isolates clade was the diazotrophic, but apparently non-nodulating species, *Burkholderia xenovorans*, isolated from a landfill site in the USA (Goris *et al.*, 2004). Within the South African clade, two isolates from *V. oroboides* (Kb14 and Kb2) and two from *H. sophoroides* (HC1.1a2 and WK1.1h) grouped with isolate WSM3937 (82% MLbs; 1.00 BIpp). *Burkholderia tuberum* was placed in a larger group (0.64 BIpp) with six isolates predominantly originating from *Hypocalyptus* (HC1.1bd; HC1.1bb; HC1.1a1; HC6.4b) as well as *P. calytrata* (WC7.3g) and *C. subternata* (CS2) (Fig. 3.4).

In the *nifH* phylogeny (Fig. 3.4), the sister clade (86% MLbs; 1.00 BIpp) of the South African assemblage of isolates and *B. xenovorans* included all the other species and isolates of *Burkholderia* (nodulating and non-nodulating) and the beta-rhizobium *Cupriavidus taiwanensis* (Chen *et al.*, 2001). Within the latter clade, the beta-rhizobia formed a distinct group (98% MLbs; 1.00 BIpp), with the various non-nodulating diazotrophs (*B. silvatlantica*, *B. unamae*, *B. tropica* and *B. vietnamiensis*) representing lineages basal to it. This clade, together with the South African isolates and *B. xenovorans* clade clustered together (83% MLbs; 1.00 BIpp) with the basal lineages being represented by, respectively, the Betaproteobacterial species *Leptothrix cholodnii*, and the Alpha- and Gammaproteobacterial sequences of *Xanthobacter autotrophicus* and *Methylococcus capsulatus* (Fig. 3.4).

3.3.3 Phylogenetic analyses of the *nodA* sequences

ML and BI analyses of the *nodA* sequences generated in this study separated the *Burkholderia* species into 17 groups (i.e., *nodA* groups 1-17) (Fig. 3.5) that broadly corresponded to the 17 groups identified according to the polymorphisms observed in the *nodA* deduced amino acid sequences (Fig. 3.2). The majority of these groups were supported in both the ML and BI analyses ($\geq 60\%$ MLbs and ≥ 0.60 BIpp), but support was lacking for five of the groups (1, 2, 3, 4, and 12). As with the *nifH* data, the *nodA* sequences did not

allow grouping of the isolates according to housekeeping gene lineage designations (SA1-25; Chapter 2 of this dissertation). For example, isolates from lineage SA20 occur in six of the *nodA* groups (3, 4, 5, 6, 8 and 9). Also, the data generally did not group the isolates according to their respective hosts. For example, *nodA* group 10 included isolates that originated from *H. coluteoides*, *C. longifolia*, *C. glabra* and *C. intermedia*. Notable exceptions were *nodA* groups 5 and 17 that both associated with only one host each, respectively, *C. intermedia* and *H. sophoroides* (Fig. 3.5). Neither did the groupings show any real specificity towards geographic location as the *P. calytrata* isolates (all isolated from the Paarl Rock Nature Reserve; Table 3.1) was found in five separate groups (1, 2, 4, 13 and 14; Fig. 3.5).

To investigate the evolutionary origins of the *nodA* sequences obtained for the isolates examined in this study, a dataset containing representatives of the lineages SA1-25 (again excepting SA3 and SA5), as well as the known *nodA* sequences for various beta- and alpha-rhizobia were analysed. For both of the ML and BI analyses, the isolates originating from the fynbos taxa clustered together with high support (100% MLbs; 1.00 BIpp; Fig. 3.6). This monophyletic group also included the *nodA* sequence determined previously for isolate STM687 of *B. tuberum* (Moulin *et al.*, 2001) as well as the indigenous isolate WSM3937 from *R. ferulifolia* (Garau *et al.*, 2009). According to the *nodA* phylogeny, the closest relative to this assemblage of South African isolates was the alpha-rhizobial species *Methylobacterium nodulans* from Senegal (68% MLbs). The *nodA* sequences from the other beta-rhizobial species (*B. phymatum*, *B. mimosarum* and *C. taiwanensis*) clustered together (100% MLbs; 100 BIpp), but were entirely separate from the clade containing the fynbos isolates (Fig. 3.6).

3.3.4 Detection of congruence

Visual inspection of the *nodA* and *nifH* phylogenies containing only the isolates from fynbos hosts (Figs. 3.3 and 3.5), revealed that only one group was consistently recovered from both datasets. This corresponded to *nifH* group 21 (100% MLbs and 1.00 BIpp) and *nodA* group 17 (97% MLbs and 1.00 BIpp), which both contained isolates originating from root nodules of *H. sophoroides* and represented lineages SA12 and SA15 (Chapter 2 of this dissertation). Lack of obvious correlation between the two datasets was reflected in results of the ILD test

(P value of 0.001), which showed that the two datasets do not represent homogeneous partitions.

3.4 Discussion

This study clearly showed that the genetic determinants of nitrogen-fixation and nodulation of *Burkholderia* isolates originating from the fynbos legumes examined are different from those known for root-nodule bacteria collected elsewhere. In both, the sequences generated for the isolates from *Hypocalyptus*, *Virgilia*, *Podalyria* and *Cyclopia* formed phylogenetic clusters that were distinct and separate from those for *Burkholderia* sequences obtained from isolates growing in Taiwan, French Guiana and Brazil (*B. mimosarum*, *B. phymatum*, *B. sabiae* and *B. nodosa*). These groupings notably also included two strains that were previously isolated in the Western Cape Province (South Africa) from the root nodules of *Aspalathus carnosa* (Moulin *et al.*, 2001) and *Rhynchosia ferulifolia* (Garau *et al.*, 2009), both of which are in the Tribe Crotalarieae (Figs. 3.4 and 3.6). The *nodA* and *nifH* gene uniqueness of these root-nodule bacteria reflected the fact that they apparently represent species distinct from those known in other part of the world (see Chapter 2 of this dissertation). Therefore, nodulation and nitrogen-fixation in the Cape Floristic Region legumes from the papilionoid tribes Hypocalypeteae, Podalyrieae and Crotalarieae appear facilitated, not only by unique *Burkholderia* species, but also distinct forms of the *nif* and *nod* loci.

Comparisons of the *nifH* and *nodA* phylogenies deduced in this study, revealed that the two datasets were strongly incongruent (Section 3.3.4). A similar finding of different phylogenetic histories for these two gene regions was encountered in *B. phymatum* and *C. taiwanensis* (Chen *et al.*, 2003) and also the *Burkholderia* isolates that nodulate *Mimosa* species in South America (Chen *et al.*, 2005a). These incongruencies were further emphasised when the nitrogen-fixation and nodulation gene phylogenies of the fynbos isolates (Figs. 3.3 and 3.5), were compared to their respective lineages inferred from 16S ribosomal RNA (rRNA) and *recA* data (Figs 2.4 and 2.5 in Chapter 2 of this dissertation). The only group of isolates that consistently grouped together (*nifH* group 21 and *nodA* group 17) were those representing the closely related lineages SA12 and SA15, and were all obtained from the root nodules of *H. sophoroides*. Therefore, except for this grouping, the nodulation, nitrogen-fixation and housekeeping genetic loci (*recA* and 16S rRNA) all

apparently have independent evolutionary histories, which is one of the major characteristics of the effects of HGT. As documented for the genus *Bradyrhizobium* (Moulin *et al.*, 2004), the evolution of the symbiotic loci of the *Burkholderia* from the fynbos taxa thus appears influenced by the effects of both HGT and vertical descent.

The placement of symbiotic genes on mobile genetic elements such as plasmids or genomic islands facilitates the transfer (Flores *et al.*, 2005; MacLean *et al.*, 2007) of nodulation and nitrogen-fixation properties among strains (Bailly *et al.*, 2007; Laguerre *et al.*, 2001; Sullivan and Ronson, 1998). For *Burkholderia* species it has been speculated that the symbiotic loci are carried on plasmids (Chen *et al.*, 2003). In the case of *B. phymatum* these loci occur on a 0.5 Mb plasmid, although their location in *B. tuberum* is not yet resolved (Chen *et al.*, 2003). In terms of the nodulation genes, the two alpha-rhizobial taxa (i.e., *Bradyrhizobium* and *Methylobacterium*) that group with the *Burkholderia* sequences examined (Fig. 3.6); encode their nodulation genes on genomic islands (Renier *et al.*, 2008; Wang *et al.*, 1999). In other alpha-rhizobia such as *Mesorhizobium*, the majority of species carry their symbiotic loci on islands, while some carry these loci on plasmids (e.g., *M. amorphae* and *M. huakuii*) (Wang *et al.*, 1999; Xu and Murooka, 1995). In addition, all of the genes involved in symbiosis are not necessarily located together. For example, in the common *nod* genes of *B. tuberum* and *C. taiwanensis*, the functional *nodC* forms part of a separate locus in *B. tuberum* (Moulin *et al.*, 2001), whereas the functional *nodA* gene occurs at a separate locus in *C. taiwanensis* (Chen *et al.*, 2003). Further research is thus needed to determine the genomic locations of the genes determining nodulation and nitrogen-fixation in the South African *Burkholderia* isolates studied here.

Phylogenetic analyses of the *nifH* and *nodA* genes generally did not group isolates according to their geographic origins and/or host legumes (Figs. 3.3 and 3.5). Although geographic grouping has been reported for *Bradyrhizobium* species (Steenkamp *et al.*, 2008; Vinuesa *et al.*, 2005), this was not generally observed with the fynbos isolates, as there were instances where one host could be nodulated by different strains with diverse *nifH* and *nodA* sequences as summarised in Table 3.1. Notably, the isolates from *V. oroboides* obtained from a single tree in Kirstenbosch, contained five distinct lineages (SA5, SA9, SA16, SA17, SA19), three *nifH* groups (5, 12, 20) and four *nodA* groups (4, 7, 11, 16) (see Table 3.1). In terms of geographic origins of the isolates, no firm conclusions can be drawn at present as the different isolates from a specific host were often obtained only from a single site.

Considering that diverse isolates spanning several phylogenetic groups, based on housekeeping and symbiotic locus information, were collected from individual sites, the results indicate that the *Burkholderia* diversity in the various Western Cape regions is probably very high.

The evolutionary origins of nitrogen-fixation in the South African root-nodule *Burkholderia* species appear distinct from those of the species that nodulate legumes in South America and Taiwan. Overall, nitrogen-fixation is thought to be a more ancient trait than nodulation and that rhizobial *Burkholderia* species acquired this trait from free-living diazotrophic *Burkholderia* or closely related Betaproteobacteria (Chen *et al.*, 2003; Hirsch *et al.*, 2001). Consistent with this idea, all the *nifH* sequences for the Betaproteobacteria included in the study, grouped together and were separate from those for Alpha- and Gammaproteobacteria (Fig. 3.4). In further agreement with this hypothesis, all of the *nifH* sequences for the South African *Burkholderia* species and isolates grouped more closely with the diazotrophic, non-nodulating species, *Burkholderia xenovorans*, than to other beta-rhizobia. Previous studies have also noted this association, where the South African species *B. tuberum* grouped with *B. xenovorans* (Andam *et al.*, 2007; Chen *et al.*, 2005a; Chen *et al.*, 2005b). Similarly in the present study, the root-nodule bacteria obtained from legumes in Taiwan and South America were more closely related to species such as *B. vietnamiensis* and *B. tropica* (Fig. 3.4). This suggests that they too acquired their nitrogen-fixation abilities from diazotrophic non-nodulators. However, the overall lack of polymorphism in the *nifH* among the isolates examined (Figs. 3.1 and 3.3) suggests that this acquisition of nitrogen-fixation abilities probably happened relatively recently during the evolution of these beta-rhizobia, which is in direct contrast to the hypothesis put forth by Garau *et al.* (2009), that this event occurred a long time ago.

Consistent with previous hypotheses, the *Burkholderia* root-nodule bacteria examined in this study, likely acquired their nodulation abilities from alpha-rhizobia (Fig. 3.6; Bontemps *et al.*, 2010). Based on *nodA* phylogenetic analyses, the South African species including *B. tuberum* grouped with the alpha-rhizobium *Methylobacterium nodulans* (Fig. 3.6; Chen *et al.*, 2003), while the South American species including *B. phymatum* has a separate grouping and origin (Fig. 3.6; Andam *et al.*, 2007; Chen *et al.*, 2003; Chen *et al.*, 2005a). Also, the *nodA* gene of *B. tuberum* has been shown to be 13 amino-acids longer at the N terminus, which is identical to the situation in the alpha-rhizobia genus *Bradyrhizobium* (Chen *et al.*,

2003; Moulin *et al.*, 2004). In contrast, this insertion is not found among the remainder of the beta-rhizobia and the alpha-rhizobial genera *Mesorhizobium*, *Sinorhizobium*, *Rhizobium* and *Azorhizobium* (Chen *et al.*, 2003). It is thus likely that the South African isolates examined here also encode longer *nodA* genes, which is an issue that will be addressed in future research. Nevertheless, the high *nodA* similarity between the beta-rhizobial genera *Cupriavidus* and *Burkholderia* and the phylogenetic placement of this species (Fig. 3.6) supports the idea that the rhizobia in this genus probably acquired their nodulation genes from other beta-rhizobia and most probably from a ‘South American’ *Burkholderia* isolate (Andam *et al.*, 2007; Chen *et al.*, 2003).

The lack of an apparently monophyletic origin for the *Burkholderia nodA* sequences directly contrasts that proposed for the members of the genus *Bradyrhizobium* (Menna and Hungria, 2011). Also, for *Bradyrhizobium*, irrespective of species identities, various well-supported clades of *nodA* sequences have been reported (Stępkowski *et al.*, 2007), which appear to reflect the biogeography of the strains harbouring these sequences. For example, all of the isolates in *Bradyrhizobium nodA* clade II occur in association with Genisteeae legumes (Stępkowski *et al.*, 2005), while most *nodA* sequences for *Bradyrhizobium* isolates originating from legumes native to Africa form part of clade III that also include diverse sequences with tropical and sub-tropical origins (Steenkamp *et al.*, 2008). Therefore, despite lacking a single origin for all *nodA* sequences, the phylogenetic patterns observed for *Burkholderia* resemble the situation in *Bradyrhizobium*. All isolates of South African origin group together, while those from South America group together. In fact, the South African isolates are most closely related to another African species *Methylobacterium nodulans*, isolated from *Crotalaria podocarpa* in Senegal, thus emphasising the likely common African ancestry of their nodulation abilities. Undoubtedly, a clearer picture of the phylgeography of the beta-rhizobia will emerge once more isolates, legume hosts and geographic areas are considered.

In conclusion, the findings presented in this study unequivocally showed that the *Burkholderia* symbionts of various fynbos legumes have evolutionary origins distinct from those of other beta-rhizobia. In this regard, the fynbos root-nodule bacteria most probably acquired both their nodulation and nitrogen-fixation properties from other South African rhizobial and/or diazotrophic taxa. Once acquired, HGT appears to have played a fundamental role in the evolution of the loci determining these symbiotic properties. It will

be interesting to see whether this pattern of distribution of the *nif* and *nod* loci among *Burkholderia* species remains, as a broader range of fynbos and other South African legumes are evaluated. An extended sampling strategy will elucidate whether soil characteristics inherent to the Cape Floristic Region help determine the identity and genomic diversity of rhizobia that nodulate legumes in this region, as suggested by Garau *et al.* (2009).

3.5 References

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3.6 Tables

Table 3.1 Beta-rhizobia associated with indigenous fynbos legumes featuring in this study.

Legume Host ^a	Isolate ^b	Geographic Origin	16S Lineage ^c	<i>nifH</i> Group ^d	<i>nodA</i> Group ^e
<i>H. coluteoides</i>	RAU2b	Storms River Bridge	SA17	1	11
<i>H. coluteoides</i>	RAU2c	Storms River Bridge	SA6	1	10
<i>H. coluteoides</i>	RAU2d	Storms River Bridge	SA6	1	10
<i>H. coluteoides</i>	RAU2d2	Storms River Bridge	SA17	1	11
<i>H. coluteoides</i>	RAU2f	Storms River Bridge	SA1	5	14
<i>H. coluteoides</i>	RAU2g	Storms River Bridge	SA6	1	10
<i>H. coluteoides</i>	RAU2h	Storms River Bridge	SA1	-	-
<i>H. coluteoides</i>	RAU2i	Storms River Bridge	SA10	5	14
<i>H. coluteoides</i>	RAU2j	Storms River Bridge	SA19	4	7
<i>H. coluteoides</i>	RAU2k	Storms River Bridge	SA1	5	14
<i>H. coluteoides</i>	RAU2l	Storms River Bridge	SA1	5	14
<i>H. oxalidifolius</i>	RAU6.4a	Fernkloof Nature Reserve, Hermanus	SA22	13	9
<i>H. oxalidifolius</i>	RAU6.4b	Fernkloof Nature Reserve, Hermanus	SA19	4	7
<i>H. oxalidifolius</i>	RAU6.4d	Fernkloof Nature Reserve, Hermanus	SA17	20	15
<i>H. oxalidifolius</i>	RAU6.4f	Fernkloof Nature Reserve, Hermanus	SA17	1	7
<i>H. oxalidifolius</i>	HC6.4b	Fernkloof Nature Reserve, Hermanus	SA20	18	6
<i>H. sophoroides</i>	WK1.1a	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1c	Old du Toit's Kloof Pass	SA25	19	1
<i>H. sophoroides</i>	WK1.1d	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1e	Old du Toit's Kloof Pass	SA20	-	-
<i>H. sophoroides</i>	WK1.1f	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1g	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1h	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1i	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1j	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1k	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	WK1.1m	Old du Toit's Kloof Pass	SA12	21	17
<i>H. sophoroides</i>	HC1.1a1	Old du Toit's Kloof Pass	SA24	19	1
<i>H. sophoroides</i>	HC1.1a2	Old du Toit's Kloof Pass	SA15	21	17
<i>H. sophoroides</i>	HC1.1a3	Old du Toit's Kloof Pass	SA25	19	1
<i>H. sophoroides</i>	HC1.1ba	Old du Toit's Kloof Pass	SA25	19	1
<i>H. sophoroides</i>	HC1.1bb	Old du Toit's Kloof Pass	SA25	19	1
<i>H. sophoroides</i>	HC1.1bc	Old du Toit's Kloof Pass	SA17	19	1
<i>H. sophoroides</i>	HC1.1bd	Old du Toit's Kloof Pass	SA21	16	8
<i>H. sophoroides</i>	HC1.1be	Old du Toit's Kloof Pass	SA15	21	17
<i>H. sophoroides</i>	HC1.1bh	Old du Toit's Kloof Pass	SA12	21	17
<i>P. calyptrata</i>	WC7.3a	Paarl Rock Nature Reserve	SA11	5	14
<i>P. calyptrata</i>	WC7.3b	Paarl Rock Nature Reserve	SA23	17	2

Legume Host ^a	Isolate ^b	Geographic Origin	16S Lineage ^c	<i>nifH</i> Group ^d	<i>nodA</i> Group ^e
<i>P. calyptрата</i>	WC7.3c	Paarl Rock Nature Reserve	SA18	6	13
<i>P. calyptрата</i>	WC7.3d	Paarl Rock Nature Reserve	SA23	18	1
<i>P. calyptрата</i>	WC7.3f	Paarl Rock Nature Reserve	SA18	6	13
<i>P. calyptрата</i>	WC7.3g	Paarl Rock Nature Reserve	SA23	17	4
<i>V. oroboides</i>	Kb1A	Kirstenbosch Botanical Gardens	SA20	12	4
<i>V. oroboides</i>	Kb2	Kirstenbosch Botanical Gardens	SA16	20	11
<i>V. oroboides</i>	Kb6	Kirstenbosch Botanical Gardens	SA9	5	16
<i>V. oroboides</i>	Kb12	Kirstenbosch Botanical Gardens	SA5	-	-
<i>V. oroboides</i>	Kb13	Kirstenbosch Botanical Gardens	SA17	20	7
<i>V. oroboides</i>	Kb14	Kirstenbosch Botanical Gardens	SA17	20	7
<i>V. oroboides</i>	Kb15	Kirstenbosch Botanical Gardens	SA17	20	7
<i>V. oroboides</i>	Kb16	Kirstenbosch Botanical Gardens	SA17	20	7
<i>C. buxifolia</i>	CB2	Helderberg, Somerset-West	SA3	5	14
<i>C. genistoides</i>	UCT2	Rein's Farms	SA20	7	3
<i>C. genistoides</i>	UCT15	Constantiaberg	SA20	-	-
<i>C. glabra</i>	UCT34	Matroosberg, Ceres	SA2	1	9
<i>C. glabra</i>	UCT71	Unknown	SA14	1	10
<i>C. intermedia</i>	CI1	Dennehoek, Joubertina	SA8	1	10
<i>C. intermedia</i>	CI2	Dennehoek, Joubertina	SA20	10	5
<i>C. intermedia</i>	CI3	Dennehoek, Joubertina	SA20	2	5
<i>C. longifolia</i>	Clong1	Thornhill, Humansdorp	SA7	1	10
<i>C. longifolia</i>	Clong3	Thornhill, Humansdorp	SA20	11	6
<i>C. maculata</i>	CM1	Paarlberg, Paarl	SA4	-	-
<i>C. maculata</i>	UCT70	Jonkershoek	SA20	15	8
<i>C. meyeriana</i>	UCT43	Hottentots Holland mountains	SA2	1	9
<i>C. meyeriana</i>	UCT56	Hottentots Holland mountains	SA2	1	9
<i>C. pubescens</i>	Cpub6	Next to N1, Port Elizabeth	SA20	14	9
<i>C. sessiliflora</i>	Cses4	Plattekloof, Heidelberg	SA18	9	13
<i>C. sessiliflora</i>	UCT30	Callie's Farm, Heidelberg	SA19	3	13
<i>C. sessiliflora</i>	UCT31	Grootvadersbosch	SA20	8	6
<i>C. subternata</i>	CS2	Dennehoek, Joubertina	SA13	18	12

^a Legume hosts are indicated by the abbreviated genus name, followed by their respective species name i.e., *Hypocalyptus coluteoides* = *H. coluteoides*.

^b These isolates all form part of Chapter 2 of this dissertation.

^c Lineages are based on 16S and recA phylogenies (refer to Chapter 2 of this dissertation; see Fig. 2.4).

^d See Fig. 3.3. Sequences not included for a specific isolate indicated with -.

^e See Fig. 3.4. Sequences not included for a specific isolate indicated with -.

3.7 Figures

Fig. 3.1 Polymorphisms observed among the deduced NifH amino acid sequences for the *Burkholderia* isolates obtained from the root-nodules of *Hypocalyptus*, *Cyclopia*, *Virgilia* and *Podalyria* hosts. Positions similar to those of the first sequence, RAU6.4a, are indicated with dots. The numbers at the top of each column in the alignment are read vertically and correspond to the sequence position of that amino acid in the original alignment. Shorter sequences contain positions marked with 'X', which indicates positions at which there is no amino acid.


```

111111111111222222222
157 1123333446679 122234566771122334444
5610139041614 617802025486901490189
RAU6.4a GTQELLSRKAIDGRNVFENAMIGIQEFESKHNGTME
RAU6.4b .....D...T...
RAU6.4d .....
RAU6.4f .....A..
RAU2b .....
RAU2c .....
RAU2d .....H.....
RAU2d2 .....
RAU2f .....T...R
RAU2g .....
RAU2i .....S...T...
RAU2j .....D...T...
RAU2k .....T...
RAU2l .....V.....T...
HC1.1a1 .....
HC1.1a2 .....
HC1.1a3 .....V.....S...
HC1.1ba .....
HC1.1bb .....
HC1.1bc .....
HC1.1bd .....
HC1.1be R.....D.....
HC1.1bh .....
HC6.4b .S....P.....G.....
WK1.1a .....
WK1.1c .....
WK1.1d .....
WK1.1f .....
WK1.1g .....
WK1.1h .....
WK1.1i .....
WK1.1j .....
WK1.1k .....
WK1.1m .....
Kb1A .....CR.....
Kb2 .....C.....IT
Kb6 .P.....L...T...
Kb13 .....N.....I...T...
Kb14 .....
Kb15 .....I.....
Kb16 .....S...T...XXXXXXXXXX
WC7.3a .....T...
WC7.3b .....S...
WC7.3c .....
WC7.3d .....
WC7.3f .....P...R.....
WC7.3g .R...R...M.....
Cpub6 .....
CI1 .....
CI2 .....V.....NG
CI3 .K.....V.....
Clong1 .....
Clong3 .....S...
Cses4 .....
UCT2 .....
UCT31 .....
UCT34 .....
UCT70 .....N.....
UCT71 .....
CB2 .....T...
CS2 .....K...R...
UCT30 .....
UCT43 .....
UCT56 .....

```

Figure 3.2 Polymorphisms observed among the deduced NodA amino acid sequences for the *Burkholderia* isolates obtained from the root-nodules of *Hypocalyptus*, *Cyclopia*, *Virgilia* and *Podalyria* hosts. Positions similar to those of the first sequence, RAU2b, are indicated with dots and only those at which at least two isolates were polymorphic are shown. The 17 *nodA* groups (see Figure 3.5) are indicated to the right of the sequences. The numbers at the top of each column in the alignment are read vertically and correspond to the sequence position of that amino acid in the original alignment. Shorter sequences contain positions marked with ‘X’, these are positions at which that sequence does not have an amino acid.

	1111111 11 22335 677889 0122334 1234568 0624567901023 0505262	<i>nodA</i> Group
RAU2b	RGNSIPVARIADLHLGVHLHTKI IRDL	
RAU2d2	LAGAR.....S.....	
Kb2	WAGAR.....S.....	11
CI1	L.GRET...V.....ST.....	
Clong1	WAGAR...V.....ST.....	
RAU2c	WAGAR...V.....ST.....	
RAU2d	DLGAR...V.....ST.....	
RAU2g	WAGAR...V.....ST.....	
UCT71	XXXXXXXXXXXX.....ST.....	10
WC7.3a	WAGAR.....S.....I	
RAU2i	L.GGR.....S.....I	
RAU2k	LAGAR.....S.....I	
RAU2l	LAGAR.....S.....I	
CB2	WAGAR.....S.....I	
RAU2f	WAGAR.....YS.....I	14
WK1.1a	WAGAR...V.....M.SV.VQEI	
WK1.1d	WAGAR...V.....M.SV.VQEI	
WK1.1f	WAGAR...V.....M.SV.VQEI	
WK1.1g	WAGAR...V.....M.SV.VQEI	
WK1.1h	WAGAR...V.....M.SV.VQEI	
WK1.1i	WAGAR...V.....M.SV.VQEI	
WK1.1j	L.GAR...V.....M.SV.VQEI	
WK1.1k	WAGAR...V.....M.SV.VQEI	
WK1.1m	WAGAR...V.....M.SV.VQEI	
HC1.1bh	WAGAR...V.....M.SV.VQEI	
HC1.1be	WAGAR...V..V.....M.SV.VQEI	
HC1.1a2	WAGAR...V..V.....M.SV.VQEI	17
Kb6	WAGAR...VE.....M.ST.V.EI	16
RAU6.4d	L.GGR.....ST.V.EI	15
HC6.4b	WAGAR.....Q.D.L.PSR...I	
Clong3	WAGAR...V...Q.D.L.PSR...I	
UCT31	L.GAR...V...Q.D.L.PSR...I	6
WK1.1c	WAGAR.F.....Q.....PST...I	
HC1.1a1	WAGAR.F.....Q.....PST...I	
HC1.1a3	WAGAR.F.....Q.....PST...I	
HC1.1ba	WAGAR.F.....Q.....PST...I	
HC1.1bb	WAGAR.F.....Q...R.PST...I	
HC1.1bc	WAGAR.F.....Q.....PST...I	
WC7.3d	WAGAR.F.....Q.....PST...I	1
WC7.3b	WAGAR.F.....Q.....PST...I	2
UCT2	WAGAR.F.....L.....PST...I	3
Kb1A	L.GGR.F.....Q.....PST...I	
WC7.3g	L.GGR.....PST...I	4
CI2	L.GGETF.....Q.....PPT...I	
CI3	XXXXXXX.....Q.....PPT...I	5
HC1.1bd	WAGAR..V.....A..PSTT...I	
UCT70	L.GAR..V.....A..PSTT...I	8
RAU6.4f	WAGAR.....D.....PST...I	
Kb14	WAGAR.F.....D.....PST...I	
Kb15	WAGAR.F.....D.....PST...I	
Kb13	WAGAR.F.....D.....PST...I	
Kb16	L.GGR.F.....D.....PST...I	
RAU2j	L.GGR.....G..D.....PST...I	
RAU6.4b	L.GAR.....D.....PST...I	7
WC7.3c	WAGAR...H.....F.....S.....I	
WC7.3f	WAGAR...H.....F.....S.....I	
Cses4	LAGAR.....F.....S.....I	
UCT30	WAGAR.....F.....S.....I	13
CS2	WAGAR...V.....RS.....I	12
RAU6.4a	WAGAR...V.....D.....ST...I	
Cpub6	WAGAR...V.....D.....ST...I	
UCT34	WAGAR...V.....D.....ST...I	
UCT43	XXXXXXXXXXXX.....D.....ST...I	
UCT56	WAGAR...V.....D.....ST...I	9

Fig. 3.3 A midpoint-rooted *nifH* maximum likelihood phylogeny of 64 of the *Burkholderia* isolates obtained from the root-nodules of *Hypocalyptus*, *Cyclopia*, *Virgilia* and *Podalyria* hosts. Bootstrap support of $\geq 60\%$ for maximum likelihood and Bayesian posterior probabilities ≥ 0.60 are shown, in the order BI/ML. Original legume hosts and lineage designations based on housekeeping gene phylogenies (Chapter 2 of this dissertation) are indicated after the isolate numbers.

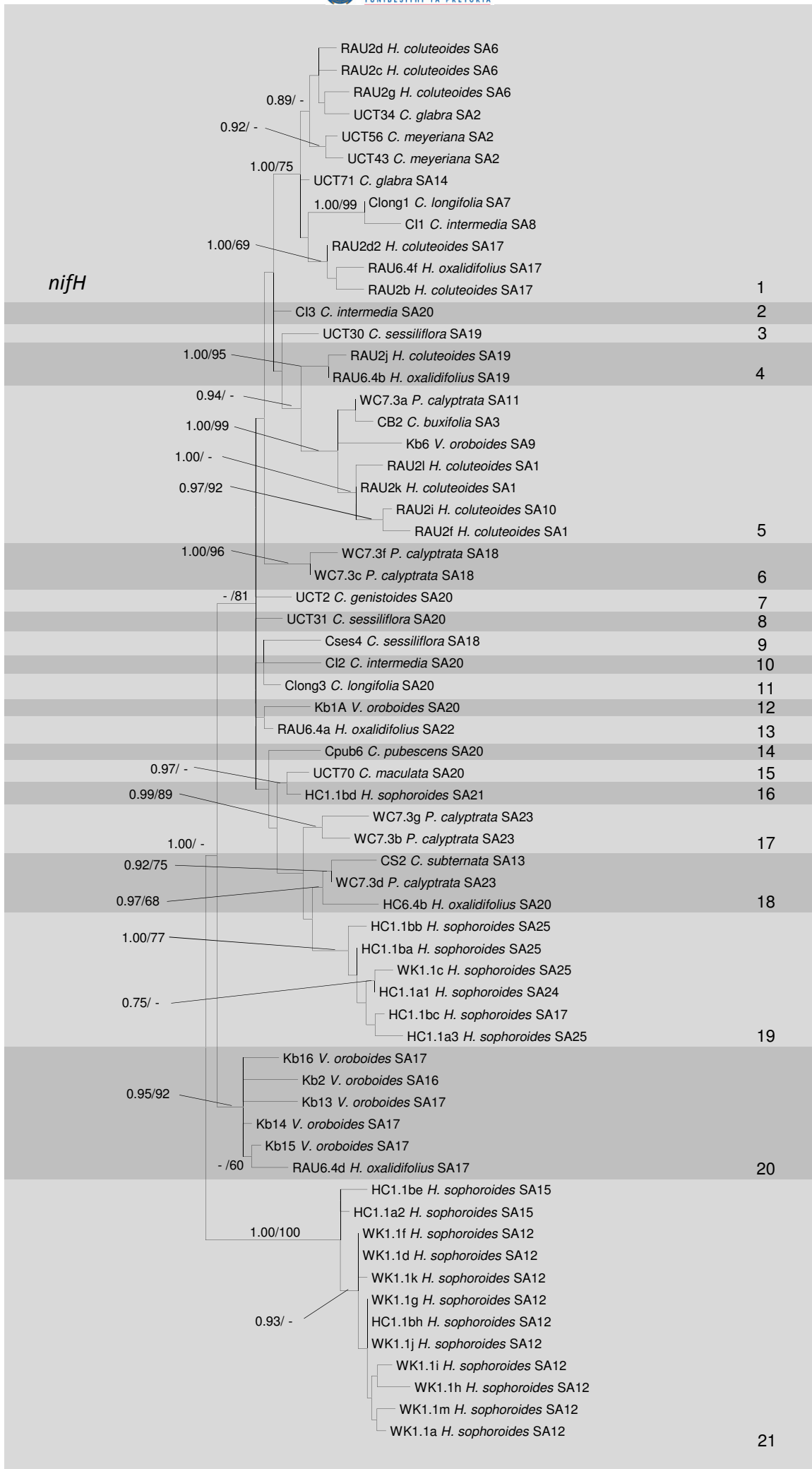


Fig. 3.4 A *nifH* maximum likelihood phylogeny that includes sequences for representatives of 23 of the 25 *Burkholderia* lineages examined in this study, as well as known free-living and nodulating *Burkholderia* strains. The phylogeny also includes sequences for the diazotrophs (all indicated in red) *Leptothrix cholodnii*, *Xanthobacter autotrophicus* and *Methylococcus capsulatus* and the alpha-rhizobia *Rhizobium alkanisoli* and *Ensifer fredii*, which were used for outgroup purposes. Original legume hosts and lineage designations based on housekeeping gene phylogenies (Chapter 2 of this dissertation) are indicated after the isolate numbers. For the known strains, isolate names are followed by the source, while isolate number and GenBank accessions are indicated in parentheses. Bootstrap support of $\geq 60\%$ for maximum likelihood and Bayesian posterior probabilities ≥ 0.60 are shown, in the order BI/ML. The non-nodulating diazotrophic *Burkholderia* strains are indicated in orange, while those in green are nodulating strains mostly associating with *Mimosa* spp.

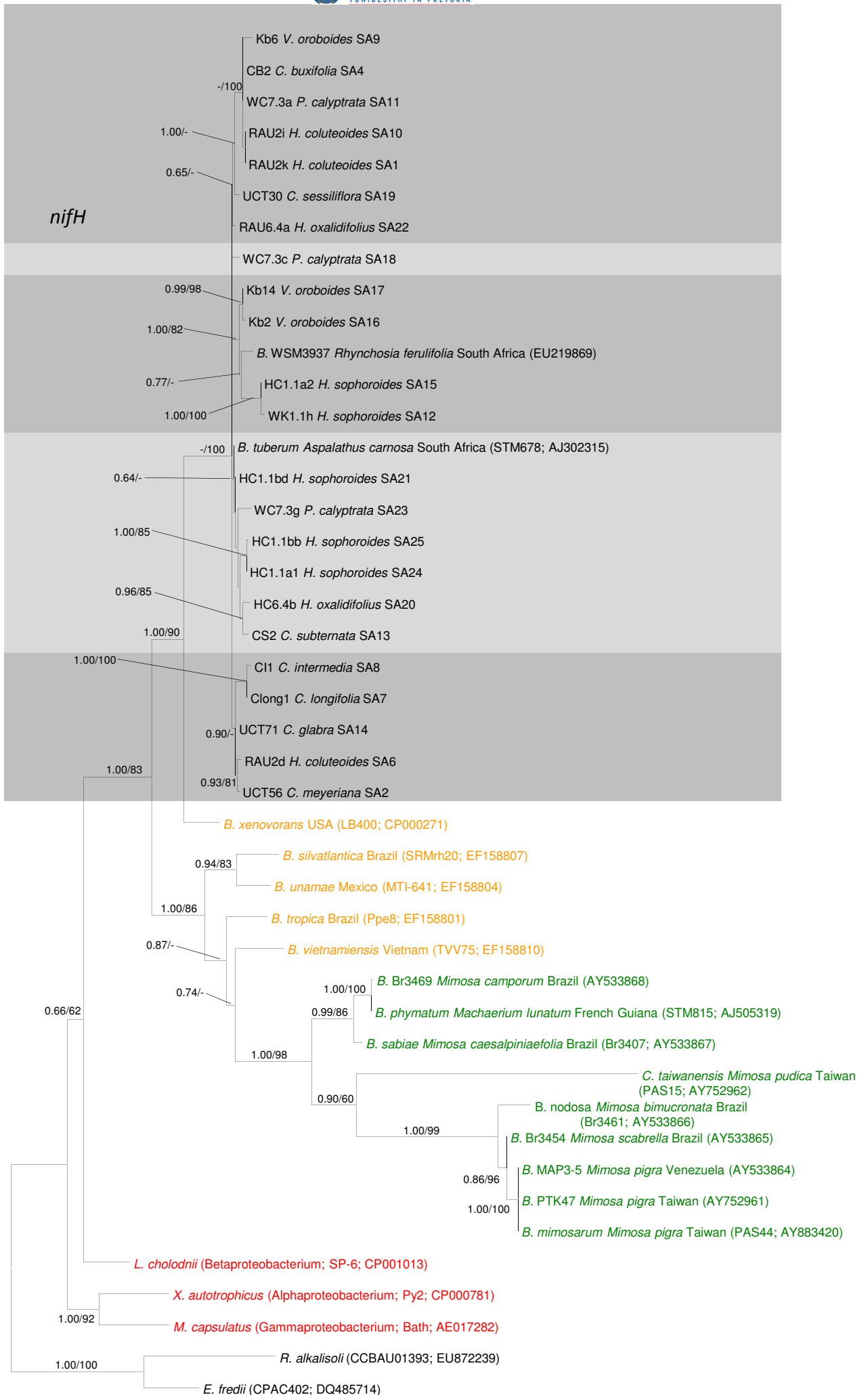


Fig. 3.5 A midpoint-rooted *nodA* maximum likelihood phylogeny of 64 of the *Burkholderia* isolates obtained from the root-nodules of *Hypocalyptus*, *Cyclopia*, *Virgilia* and *Podalyria* hosts. Bootstrap support of $\geq 60\%$ for maximum likelihood and Bayesian posterior probabilities ≥ 0.60 are shown, in the order BI/ML. Original legume hosts and lineage designations based on housekeeping gene phylogenies (Chapter 2 of this dissertation) are indicated after the isolate numbers.

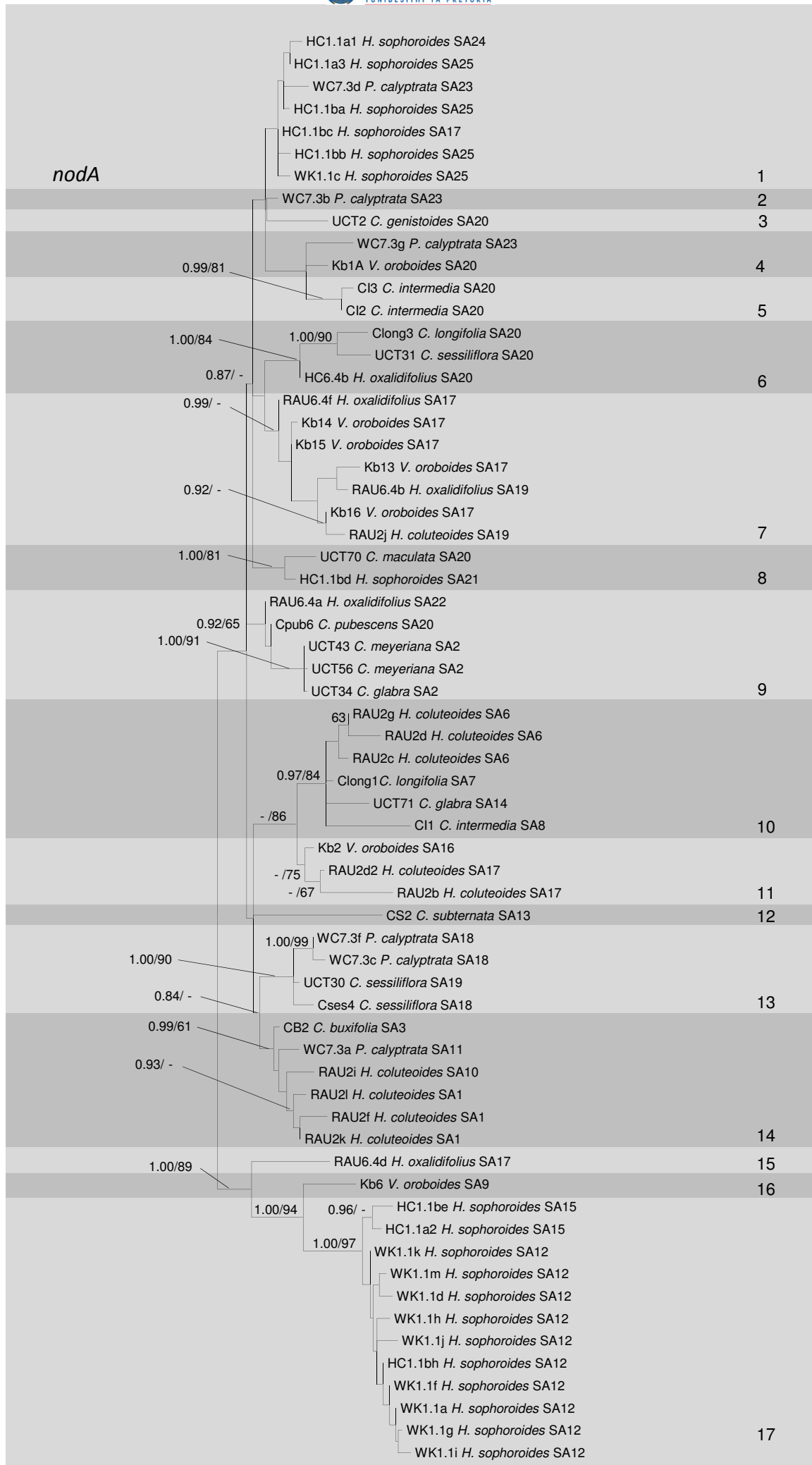
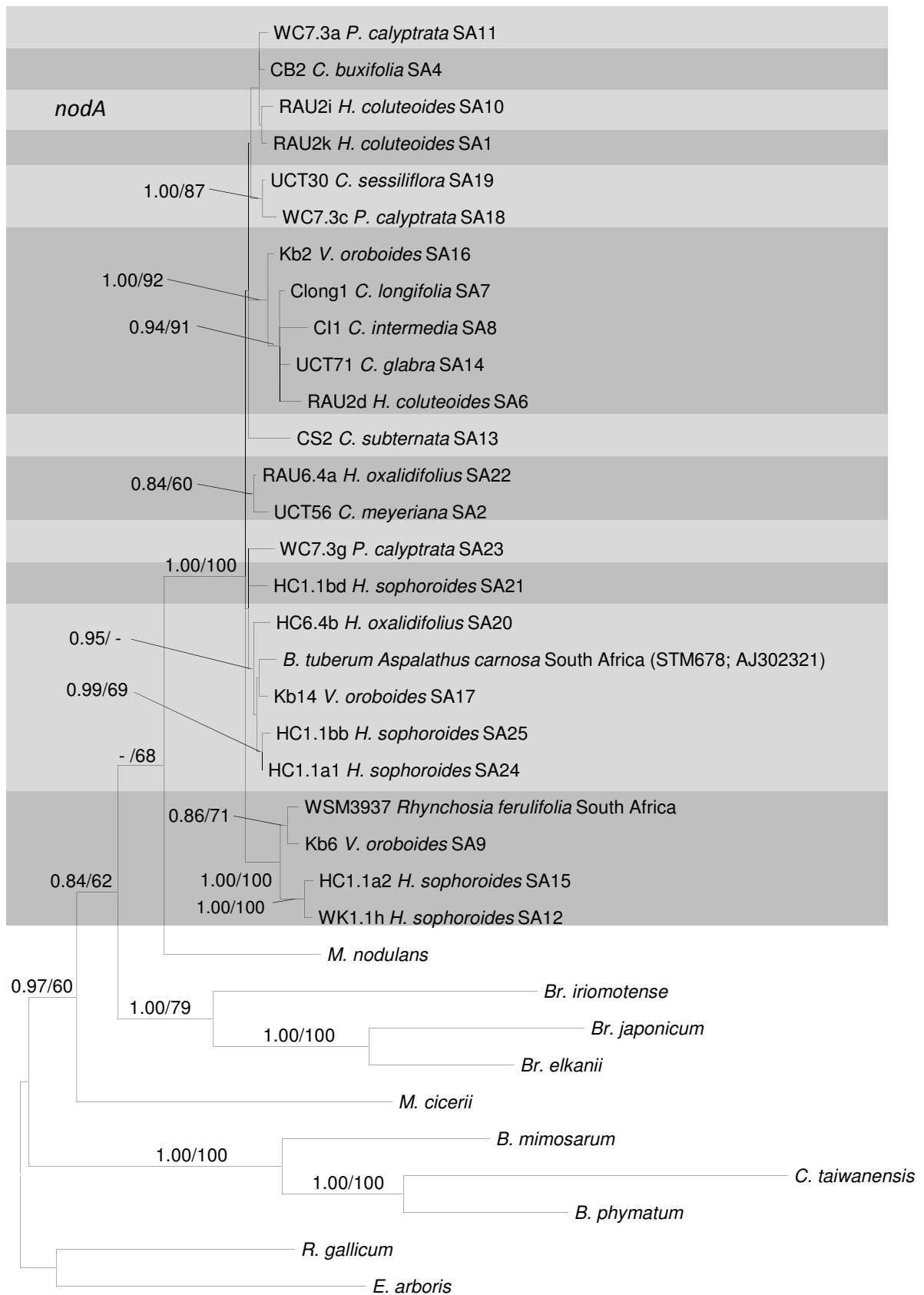


Fig. 3.6 A *nodA* maximum likelihood phylogeny that includes sequences for representatives of 23 of the 25 *Burkholderia* lineages examined in this study, as well as a range of beta-rhizobia and the alpha-rhizobia *Rhizobium gallicum* and *Ensifer arboris*, which were used for outgroup purposes. Original legume hosts and lineage designations based on housekeeping gene phylogenies (Chapter 2 of this dissertation) are indicated after the isolate numbers. For the known strains, isolate names are followed by the source, while isolate number and GenBank accessions are indicated in parentheses. Bootstrap support of $\geq 60\%$ for maximum likelihood and Bayesian posterior probabilities ≥ 0.60 are shown, in the order BI/ML. The bacterial species, legume host, country of origin, isolate number and GenBank accession number for the reference strains are consecutively: *Methylobacterium nodulans*: *Crotalaria podocarpa*, Senegal, ORS2060, AM712915; *Bradyrhizobium iriomotense*: *Entada koshunensis*, Japan, EK05, AB300999; *Bradyrhizobium japonicum*: *Glycine max*, Japan, USDA 6, AM117545; *Bradyrhizobium elkanii*: *Glycine max*, USA, USDA76, AM117554; *Mesorhizobium cicerii*: *Cicer arietinum*, Spain, UPM-Ca7, AJ300247; *Burkholderia mimosarum*: *Mimosa pigra*, Taiwan, PAS44, EU434822; *Cupriavidus taiwanensis*: *Mimosa pudica*, Taiwan, AY752964; *Burkholderia phymatum*: *Machaerium lunatum*, French Guiana, STM815, AJ505318; *Rhizobium gallicum*: *Phaseolus vulgaris*, France, R602sp, AJ300236; *Ensifer arboris*: *Prosopis chilensis*, Sudan, HABI1552, Z95234.



Until recently, most of the legumes that have been studied in South Africa were known to be nodulated by diverse alpha-rhizobia in the class Alphaproteobacteria. Our knowledge regarding the occurrence of so-called beta-rhizobia were limited and restricted to *Aspalathus* and *Cyclopia* species. The aim of this study was, therefore, to explore the diversity and evolution of the root-nodule bacteria of various papilionoid legumes indigenous to southern Africa. By making use of housekeeping gene sequence information, the research presented here showed that all 69 of the bacteria isolated from the root-nodules of species in the genera *Hypocalyptus*, *Virgilia*, *Podalyria* and *Cyclopia* represented beta-rhizobia in the genus *Burkholderia* (class Betaproteobacteria). Based on these DNA sequences, the isolates could be assigned to 25 independent lineages that most probably represent distinct species. With the exception of one group that tended to associate with *B. tuberum* in my phylogenies, the majority of these lineages or species appeared to be new to science as they did not group with any of the known diazotrophic and/or nodulating species. Phylogenetic analyses of the *nifH* and *nodA* gene sequences also separated the isolates into a number of groups, but surprisingly the groups recovered with these two gene regions did not match, nor did they match with those inferred using housekeeping gene sequences. In general, there was only one exception where the same group of isolates were recovered from phylogenies inferred for the various loci. These findings thus suggested a significant impact of horizontal gene transfer on the evolutionary histories of the determinants of nodulation and nitrogen-fixation in these bacteria. The phylogenetic groups recovered from the various sequences also did not match those expected based on the host or geographic origin of the isolates. However, isolates from the South African legumes generally appeared to group separate from those isolated in other parts of the world. The distinctness of the South African isolates was most pronounced in the *nifH* and *nodA* gene trees, where they formed a well-supported cluster separate from all of isolates associated with *Mimosa* species elsewhere, which suggest a unique and possibly African origin for the root-nodule bacteria examined in this study. The findings presented in this dissertation thus present an important contribution to our understanding of the diversity and evolution of these bacteria from both a Southern African and a global perspective.