Diversity of root nodulating bacteria associated with *Cyclopia* species

by

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I certify that the thesis hereby submitted to the University of Pretoria for the degree PhD (Microbiology) has not previously been submitted by me in respect of a degree at any other university.

Signature: AKock

Date: 26/02/2004

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CONTENTS

DIVERSITY OF ROOT NODULATING BACTERIA ASSOCIATED WITH *CYCLOPIA* SPECIES

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SUMMARY

In recent years, the rhizobial taxonomy changed significantly with the discovery of novel symbiotic associations between legumes and nodulating bacteria. This was aided by the focus shift from studying only agricultural crops to legumes indigenous to certain regions, ultimately to discover new inoculant strains and to uncover the secrets of the rhizobium-legume symbiosis. In previous studies on the diversity of South African rhizobia, it has become clear that our country has a wealth of rhizobia.

Cyclopia is a legume genus, which belongs to the fynbos biome of South Africa. Honeybush tea is a herbal infusion manufactured from the leaves and stems of certain *Cyclopia* spp. Commercial cultivation of this potentially new agricultural crop is now developed to protect the natural *Cyclopia* spp. populations from harvesting and ultimately extinction. Superior inoculant strains are necessary for these commercial seedlings.

The diversity of root-nodulating strains isolated from 14 *Cyclopia* spp. was determined using 16S-23S IGS-RFLP and partial 16S rDNA base sequencing. Based on 16S-23S IGS-RFLP and partial 16S rDNA base sequencing most of the isolates, with the exception of seven strains, were found to belong to the genus *Burkholderia*. More extensive phylogenetic,

symbiotic and phenotypic studies of selected strains were performed using near full-length 16S rDNA base sequencing, *nodA* base sequencing and substrate utilisation analysis. In the genus *Burkholderia*, the isolates belonged to the novel root-nodulating species *Burkholderia tuberum* and several novel, undescribed *Burkholderia* genotypes. However, no new *Burkholderia* species could formally be proposed, since DNA-DNA hybridisation analysis, which is a prerequisite for the description of new species could not be performed in our laboratory. The seven strains not affiliated with the *Burkholderia* genus belonged to two *Bradyrhizobium* genospecies, *R. tropici* and a possibly new genus in the α-*Proteobacteria*. The *nodA* sequences of all the *Cyclopia* isolates corresponded to a large extent, indicating that different chromosomal genotypes harbour the same symbiotic genotype. All the isolates of the *Cyclopia* genus appear to be acid-tolerant, which is in agreement with the acidic nature of the soil from which the strains were isolated.

DIVERSITEIT VAN WORTELNODULERENDE BAKTERIEë GEASSOSIEER MET *CYCLOPIA* SPESIES

deur

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OPSOMMING

Die rhizobium-taksonomie het ingrypend oor die afgelope jare verander met die ontdekking van nuwe simbiotiese assosiasies tussen peulplante en nodulerende bakterieë. Hierdie ontdekkings is aangehelp deur die klemverskuiwing van die studie van slegs landbougewasse na inheemse peulplante. Hierdie verskuiwing was om nuwe entstofrasse te ontdek asook om die geheime van die rhizobium-peulplant simbiose te ontrafel. In die vorige diversiteit studies van Suid-Afrikaanse rhizobiums het dit duidelik geword dat ons land ryk is aan 'n verskeidenheid rhizobiums.

Cyclopia is 'n genus wat deel maak van die fynbos bioom van Suid-Afrika. Heuningbostee is 'n kruietee wat vervaardig word van die blare en stamme van sekere *Cyclopia* spp. Die kommersiële verbouing van hierdie potensiële landbougewas word ontwikkel om die natuurlike *Cyclopia* spp. populasies te beskerm teen uitwissing agv die oes van natuurlike populasies. Effektiewe entstofrasse is noodsaaklik vir hierdie kommersiële saailinge.

Die diversiteit van wortelnodulerende isolate afkomstig vanaf 14 *Cyclopia* spp. is ondersoek deur 16S-23S IGS-RFLP en gedeeltelike 16S rDNS basis volgorde-bepaling. Al die isolate,

CHAPTER 1

INTRODUCTION

Certain gram-negative bacteria, collectively called rhizobia, form a symbiotic association with legumes. It is a very special and specific association between the legume host and the rhizobium partner. This is also an agriculturally important association since the symbiotic bacteria fix nitrogen, which the plant can use, thus reducing the need for nitrogen fertiliser. Legumes enhance the productivity and sustainability of farming systems (Howieson *et al.*, 2000). The agricultural use of legumes can control cereal crop diseases and pests, which in turn reduces the reliance on pesticides and fungicides. This has economic and environmental significance for the sustainability of farming (Howieson *et al.*, 2000).

In South Africa Grobbelaar and co-workers, did a systematic survey on the nodulation status of the estimated 1350-1400 indigenous leguminous species grouped into 100 genera. Lists containing more than 1000 species of which 40 species were not nodulated were published. Most of the non-nodulating species belonged to the subfamily Caesalpinoideae. The bacteria were, unfortunately, not isolated from the nodules (Strijdom, 1998). Recent studies conducted on the rhizobia associated with indigenous South African legumes found a large diversity of root-nodulating bacteria (Dagutat, 1995; Kruger, 1998; Kock, 1999; Jaftha, 2002; Le Roux, 2003). The study of the indigenous bacterial diversity is important to discover new and better-adapted inoculant strains, as well as to improve our knowledge of South African root-nodulating bacteria (Dagutat, 1995).

Honeybush tea is a South African beverage with several health benefits and the potential to earn valuta on the foreign markets. In the early years of the manufacturing of this product, all the plant material was harvested from natural *Cyclopia* populations, mostly *C. intermedia* and *C. subternata* (De Nysschen *et al.*, 1996). In an effort to protect the natural populations, the commercial planting of several *Cyclopia* species is done in the Langkloof area near Port Elizabeth. This, which might save the natural populations from extinction is, however, still in the developmental phase and harvesting of the natural populations still occur.

The *Cyclopia* genus is part of the fynbos biome of South Africa. Most of the species of the genus have limited distribution ranges and special habitat preferences (Du Toit *et al.*, 1998). This genus is separate from the other genera in the tribe Podalyrieae, both phylogenetically and chemotaxonomically (De Nysschen *et al.*, 1996; Van der Bank, unpublished results). The tribe Podalyrieae also forms part of the genistoid alliance, which includes the tribes

Brongniartieae, Crotalarieae, Euchrestiae, Genistieae, Thermopsideae and part of the tribe Sophoreae (Van Wyk, 2003).

Recently, several new symbiotic associations have been discovered for members of this alliance. Sy *et al.* (2001) described *Methylobacterium nodulans* for methylotrophic root-nodulating bacteria associated with *Crotalaria* species. Jaftha *et al.* (2002) extended our knowledge of the methylotrophic root-nodulating bacteria with his report of such bacteria isolated from *Lotononis bainesii*. The root-nodulating *Burkholderia tuberum* was isolated from the South African legume, *Aspalathus carnosa* (Moulin *et al.*, 2001; Vandamme *et al.*, 2002). All the different legume genera involved in these novel associations belong to the tribe *Crotalarieae*. It could thus be assumed that still other interesting associations would be discovered in the genistoid alliance as more legumes are studied.

The main aim was to broaden our understanding and knowledge of the *Cyclopia*-rhizobia association, ultimately leading to the development of better inoculant strains. This study was conducted to determine the diversity of the root-nodulating bacteria associated with *Cyclopia* spp. with special reference to their identity and taxonomical positions within the rhizobia.

CHAPTER 2

NODULATION: PROCESS AND APPLICATION

1. Introduction

The use of nitrogenous fertilisers has increased after World War II to increase crop yield. Fertiliser use is higher in developed countries than in developing countries. The industrial process used to make fertilisers threatens the global ecology (Ishizuka, 1992). The Haber-Bosch process requires large energy inputs (in the form of fossil fuel) to drive the synthesis of ammonia from nitrogen and hydrogen gas under conditions of high temperature and pressure. It is estimated that 92.1096 x 10^6 kJ of energy / kg of fertiliser nitrogen is used to process, distribute and apply the fertiliser, or, 1.5 kg of fossil fuel is used for the production of 1 kg fertiliser (Bohlool *et al.*, 1992; Caetano-Anollés, 1997).

More than 20% of the applied fertiliser is lost due to nitrification in the soil, leaching of nitrates into groundwater or denitrification into volatiles (nitrous oxide) (Caetano-Anollés, 1997). In addition eutrophication of surface water is due to inorganic and organic nitrogen and the depletion of the ozone layer is caused by reactive gaseous oxides of nitrogen (Bohlool *et al.*, 1992). Human health is also endangered because of these toxic chemicals. Illnesses such as cancer and respiratory ailments can be attributed to excess levels of nitrogen containing compounds (Bohlool *et al.*, 1992).

The annual global cost of nitrogen fertiliser is US$20-60 billion. Biological nitrogen fixation (BNF) is by far a cheaper and more sustainable process. Inoculation of legume seed with root-nodule bacteria can result in a large benefit-cost ratio. The cost of the inoculant is about 1% of the total cost of input (Hardy, 1997).

Currently the demand for nitrogen is 23 x 10^6 T / year (Caetano-Anollés, 1997). The human population increases by 1.4% annually and is expected to reach 8.3 billion by 2025 (Graham and Vance, 2000). An increase in crop yields is important to feed the growing human population without harming the ecology and endangering human health. An environmentally friendly alternative is the use of BNF. Worldwide BNF is 17.2 x 10^7 T / year, three times the amount fixed industrially (Ishizuka, 1992). It is expected that BNF will not exceed the nitrogen requirements of the ecosystem, which limits possible nitrogen pollution (Kennedy and Tchan, 1992).

The ability of plants to photosynthesise resulted in nitrogen limitation. It was thus to the plant's advantage to be able to use soil nitrogen efficiently (Sprent, 1994). Plants need nitrogen at the highest amount of all nutrients. Plant yield as well as the quality of the product is affected by nitrogen availability in agriculture. Plants acquire nitrogen by the assimilation of nitrate and ammonium or through the symbiotic association with nitrogen-fixing bacteria (Stougaard, 2000).

In the case of symbiotic nitrogen fixation the plant supplies the carbon source for and protects the oxygen-sensitive nitrogenase enzyme of the bacterial symbiont. The symbiotic association between *Gunnera* sp. (Gunneraceae), *Azolla* sp. and cycads and the cyanobacteria *Nostoc* and *Anabaena* where the bacteria invade stem glands and form nitrogen-fixing heterocysts in the invaded cells, is an endosymbiotic association (Doyle, 1998; Gualtieri and Bisseling, 2000; Stougaard, 2000).

In the other symbiotic relationships a specialised organ, the nodule is formed. Woody plants from eight different families (Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae and Rosaceae) form actinorhizal nodules with the gram-positive genus *Frankia*. Legumes can form root nodules with gram-negative bacteria collectively called rhizobia. *Parasponia* (a non-legume) which belongs to the family Ulmaceae can also form nodules with rhizobia (Doyle, 1998; Gualtieri and Bisseling, 2000; Stougaard, 2000). There is an unconfirmed report of rhizobial nodules on the roots of *Roystonea regia*, a monocotyledonous tree (Basu *et al.*, 1997). The symbiotic relationships of *Frankia* spp. and rhizobia with plants is special, since most of the fixed nitrogen is transferred to the plant (Hirsch *et al.*, 2001).

2. Nodulation

Legumes can form root nodules with gram-negative bacteria collectively called rhizobia. In the nodule, the bacteria fix nitrogen, while the plant supplies the carbon source and protects the oxygen-sensitive nitrogenase enzyme (Doyle, 1998; Gualtieri and Bisseling, 2000; Stougaard, 2000).

2.1 The nodulation process

Legumes release many different compounds into the rhizosphere, such as carbohydrates, organic acids, vitamins, amino acids and phenolic derivatives. Rhizobia respond to the flavonoid compounds (2-phenyl-1, 4-benzopyrone derivatives) present in the seed and plant exudates which induce the expression of the genes for nodulation (*nod*, *nol* and *noe*). However, in some symbiotic relationships flavonoids inhibit induction. In these cases betaines, erythronic and tetronic acids may act as inducers (Perret *et al.*, 2000).

The rhizobia react by releasing Nod-factors (lipo-chitooligosaccharide signals) (Fig. 2.1) which consist of an oligosaccharide backbone of ß-1, 4-linked N-acetyl-D-glucosamine varying in length from three to five, even six sugar units with a structurally varied fatty acid group attached to the nitrogen group of the non-reducing amino sugar part. The presence of other substitutions is dependent on the species and strain. The Nod-factors elicit nodule formation in the host (Van Rhijn and Vanderleyden, 1995; Caetano-Anollés, 1997). Many rhizobia are capable of synthesising more than one type of Nod-factor molecule (Hirsch and LaRue, 1997).

Typically, the Nod-factors of the rhizobia cause the root hair to branch, deform and curl. The curled root hairs trap the bacterial cells in a pocket of the host cell wall. The bacteria enter the roots at the sites where the root hair cell walls are hydrolysed and penetrate through an invagination of the plasmamembrane. The plant host reacts by depositing new cell wall material around the lesion in the form of an inwardly growing tube. In the tube, the multiplying bacterial cells are surrounded by a matrix and the tube becomes an infection thread (Van Rhijn and Vanderleyden, 1995; Hirsch and LaRue, 1997; Gage and Margolin, 2000; Gualtieri and Bisseling, 2000).

The rhizobia penetrate the root tissue via infection threads (*Phaseolus* spp. and *Medicago* spp.) or through wounds in the root caused by lateral root emergence (*Sesbania rostrata*) or via middle lamellae [*Arachis hypogaea* and *Stylosanthes* spp.] (Van Rhijn and Vanderleyden, 1995; Caetano-Anollés, 1997; Gualtieri and Bisseling, 2000). At the same time, a nodule primordium is produced through cell division in the outer or inner root cortex. The position of the nodule primordium depends on the type of nodule that is formed by the plant. The infection thread grows toward the primordium. Within the growing nodule, the bacteria are

released from the infection threads into the host cytoplasm, but the bacteria remain within a host-derived membrane [peri-bacteroid membrane] (Van Rhijn and Vanderleyden, 1995; Caetano-Anollés, 1997). Not all bacteria are released from the infection thread (Hirsch and LaRue, 1997), they fix nitrogen within specialized fixation threads (Gualtieri and Bisseling, 2000; Hirsch *et al.*, 2001).

The spherical (determinate) or elongate (indeterminate) morphology of a nodule is determined by the plant host and not the by rhizobia (Van Rhijn and Vanderleyden, 1995; Caetano-Anollés, 1997). Since the host plant determines the nodule shape, it can be assumed that the host possesses the genetic information for symbiotic infection and nodulation. The role of the bacteria is to switch the genes of the host on (Van Rhijn and Vanderleyden, 1995). The legumes can sense the amount of external nitrogen and thus regulate the symbiotic process with the rhizobia (Caetano-Anollés, 1997). The number of nodules are limited by the plant host and this regulation might be integrated in the mechanisms which control lateral root development (Stougaard, 2000).

The bacteria differentiate into nitrogen-fixing bacteroids. In mature nodules nitrogen fixation and ammonia assimilation occur (Caetano-Anollés, 1997). Ineffective nodules are relatively small with pale or colourless interiors (Pueppke, 1996). The reaction in which biological nitrogen is formed by the conversion of dinitrogen to ammonia, catalysed by the enzyme nitrogenase can be summarised by the following reaction [Fd = ferredoxin] (Kennedy and Tchan, 1992):

$$N_2 + 8H^+ + 8Fd^- + 16MgATP^{2-} + 18H_2O \rightarrow 2NH_4^+ + 2OH^- + H_2 + 8Fd + 16MgADP^- + 16H_2PO_4^-$$

The energy requirement for nitrogen fixation is nearly the same, as the energy required for nitrate assimilation. The nitrogenase enzyme is very sensitive to oxygen, but several protective mechanisms exist which protect the enzyme against high oxygen levels (Kennedy and Tchan, 1992).

2.2 Genetics of nodulation

Most *Rhizobium* species carry the symbiotic genes on megaplasmids. The symbiotic plasmids carry the *nod* (nodulation), *fix* (nitrogen fixation) and *nif* (nitrogenase reductase) genes. In the case of *Mesorhizobium loti*, *Bradyrhizobium* and *Azorhizobium* the symbiotic genes are situated on the chromosome (Van Rhjin and Vanderleyden, 1995). The rhizobia can harbour from two to six plasmids, including the megaplasmid with the symbiotic genes (Pueppke, 1996).

2.2.1 The nodulation genes

The nodulation genes (*nod*, *noe*, *nol*) encode a set of proteins involved in the establishment of the symbiotic relationship with the legume host (Perret *et al.*, 2000; Loh and Stacey, 2003). The host-specific genes are linked to the common *nod* genes (*nodABC*) (Van Rhjin and Vanderleyden, 1995; Pueppke, 1996). The *nodD* gene is constitutively expressed and the product can detect minute amounts of flavonoids and then induce the expression of the *nod* operon (Pueppke, 1996).

2.2.2 Regulation of *nod* gene expression

The flavonoids of the plant host activate the transcription of the *nod* genes by changing the conformation of the NodD protein. The NodD protein belongs to the LysR-like transcriptional regulators which bind to conserved 47-bp DNA motifs (*nod* boxes) in the promoter regions upstream of the inducible *nod* operons which initiate the transcription of the *nod* genes. NodD proteins can bind to the *nod* boxes in the absence of an inducer, but the plant flavonoids are needed for expression of the *nod* genes (Perret *et al.*, 2000).

Some rhizobia, have only one *nodD* gene, while other have two to five copies of the *nodD* gene. The nodD proteins of different rhizobia vary in their response to different flavonoids. In the same strain the nodD products of different genes can differ in their response to different flavonoids (Perret *et al.*, 2000; Loh and Stacey, 2003).

NodD is central to *nod* gene expression, but additional regulators can help to achieve the correct output of the *nod* genes. In the case of *Bradyrhizobium japonicum* NodV is the

flavonoid sensor and NodW the regulator for the nodulation of *Macroptilum atropurpureum*, *Vigna radiata* and *V. unguiculata*. SyrM which is a NodD homologue can activate the *nod* genes in a flavonoid-independent manner (Perret *et al.*, 2000; Loh and Stacey, 2003) and can induce exopolysaccharide synthesis (Perret *et al.*, 2000).

The expression of the *nod* genes is also under negative control, since there is an optimal Nod-factor concentration for successful nodulation. NolR has been identified as a repressor in several rhizobia (Perret *et al.*, 2000; Loh and Stacey, 2003). NolA induces NodD2 expression under certain conditions, which then represses the expression of the nodulation genes. NolA is required by *B. japonicum* to nodulate restrictive soybean genotypes (Loh and Stacey, 2003). The *nod* genes are repressed before the rhizobia differentiate into bacteriods (Perret *et al.*, 2000; Loh and Stacey, 2003). The *nod* genes are regulated in a population-density manner, since maximum induction of the genes occur at low population densities (Loh and Stacey, 2003).

2.2.3 The common nodulation genes

The basic structure of the Nod-factor is determined by the products of the common genes (*nodABC*). NodC is an N-acetylglucosaminyltransferase, which is responsible for chain elongation of the oligosaccharide backbone at the nonreducing terminus. The product of *nodB* is a deacetylase, which removes the N-acetyl moiety from the nonreducing terminus of the N-acetylglucosamine oligosaccharide. NodA, an acyltransferase transfers an acyl chain to the acetyl-free C-2 carbon of the non-reducing terminus of the molecule (Perret *et al.*, 2000). However, it has been shown that NodA is host-specific, since it recognizes specific acyl chains to be transferred to the oligosaccharide backbone (Ritsema *et al.*, 1996). NodC determines the length of the chitin backbone, which also influences host-specificity (Schultze and Kondorosi, 1998).

2.2.4 Host-specific nodulation genes

Host-specific genes are responsible for substituents to the basic Nod-factor which enhance the basic structure for recognition by a specific legume hosts, but the substituents are not needed for all the plant responses (Gage and Margolin, 2000; Perret *et al.*, 2000). Special additions to the basic structure (N-acetylglucosamine oligosaccharide) can be glycosylation,

arabinosylation, fucosylation, sulfation, acetylation, methylation and carbamoylation (Perret *et al.*, 2000). NodA can transfer common saturated or monosaturated fatty acids or highly unsaturated fatty acids to the oligosaccharide backbone. In the case of the tribes Galegeae, Hedysareae, Trifolieae and Viciae of the 'galegoid group' the legume respond to highly unsaturated fatty acids and the *nodFE* genes are needed for this specific interaction (Yang *et al.*, 1999; Perret *et al.*, 2000; Geiger and López-Lara, 2002).

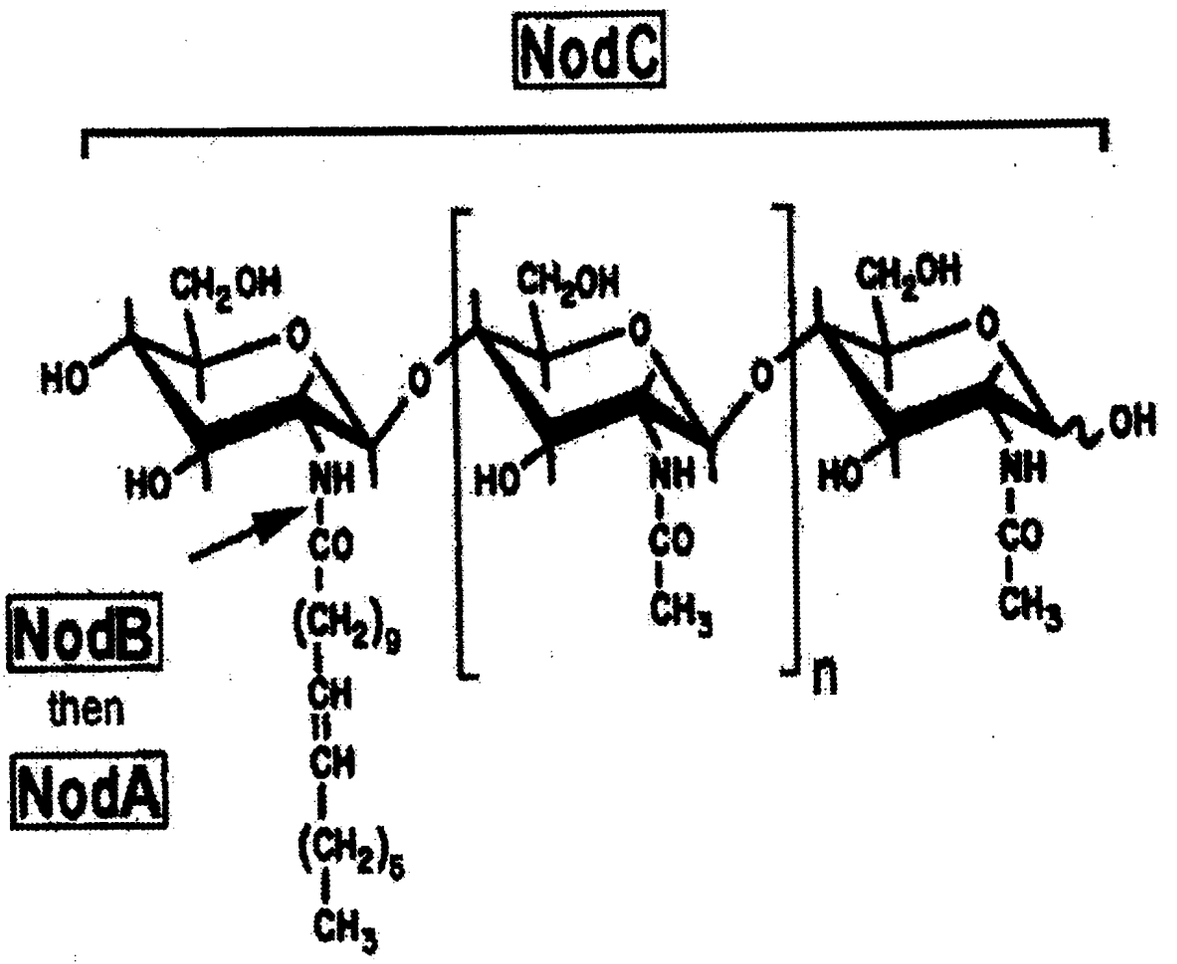


Figure 2.1: **General structure of a Nod-factor. NodC links the glucosamine monomers to form a chitin-like backbone. NodB removes an acetyl group from the non-reducing terminal residue of the oligosaccharide backbone. NodA transfers a fatty acyl chain to the acetyl-free C-2 carbon of the non-reducing terminus of the oligosaccharide molecule (Hirsch *et al.*, 2001)**

2.3 Factors unrelated to Nod-factor structure, which influence nodulation

The host range of rhizobia cannot be predicted by the Nod-factor structure alone, since rhizobia which nodulate the same legume host can produce different Nod-factors (Perret *et al.*, 2000). The concentration of the Nod-factors is important host-range determinants and the levels can be regulated by the plant host. Nod-factors are important keys for the nodulation of legume hosts, but other important host determinants are polysaccharides and other surface components of the rhizobia as well as secreted proteins, which are necessary for effective nodulation. The formation of functional bacteriods in plants require different components such as exopolysaccharides, lipopolysaccharides, capsular polysaccharides and cyclic β-glucans in addition to the correct Nod-factor. The polysaccharides may play a role in suppressing host plant defense and formation of the infection thread. Proteins, such as PlyA and PlyB, which are glycanases involved in polysaccharide processing, are symbiotically secreted. Other secreted proteins, such as a type II secretion system (TTSS) proteins play a role in lowering the host defense response (Perret *et al.*, 2000).

3. Evolution of the ability to nodulate in legumes

According to Soltis *et al.* (1995), it appears that there is a single origin for the predisposition to nodulate and fix nitrogen in plants. The symbiotic nitrogen-fixing clade (legumes and actinorhizal plants) is found in the "eurosid I" lineage (Doyle and Luckow, 2003). The ability to nodulate arose in the legumes after the earliest legume lineages diverged (Doyle *et al.*, 1997; Doyle and Luckow, 2003). Doyle *et al.* (1997) postulated based on a parsimonious interpretation of the distribution of nodulation that the ability to nodulate arose independently at least three times in the legume family. First in ancestors of the Papilionoideae, then in an ancestor of the lineage that includes the Mimosoideae and some Caesalpinoideae and finally in the genus *Chamaecrista* in the caesalpinoid line. A loss of the ability to nodulate might have occurred in the earlier diverging legumes (Doyle *et al.*, 1997).

However, in another study Doyle (1998) questioned the concept of several independent origins, since a loss of the ability to nodulate would be easier. In a phylogenetic study on the major nodule types found in the legume family the presence of the caesalpinoid (indeterminate and unbranched) nodule type was found in the Mimosoideae, Caesalpinoideae and the basal Papilionoideae. This distribution is rather in agreement with a single origin of

nodulation. The caesalpinoid nodule type would then be the ancestor of all the nodule types in the legumes (Doyle, 1998).

In the legume family, the Mimosoideae and Papilionoideae are nearly all nodulated; while in the Caesalpinoideae many non-nodulating genera occur. It can thus be assumed that symbiosis developed at a relatively late stage during legume evolution (Sprent, 1994). Van Rhjin and Vanderleyden (1995) argued that since legumes are so diverse in morphology and ecology, symbiosis is not an adaptation to a specialised ecological niche, but is more dependent on a genetic peculiarity of legumes.

Sprent (1994) proposed that the ability of plants to photosynthesise resulted in nitrogen limitation. It was thus to the plant's advantage to be able to use soil nitrogen efficiently. The early ancestors of the Fabaceae could have had nitrogen-fixing bacteria in their roots (Sprent, 1994). Early forms of the nodules are believed to be more parasitic than mutualistic (Sprent, 1994; Hirsch and LaRue, 1997).

3.1 Possible explanations for nodule development in legumes

It has been suggested that the processes needed for nodule development have been recruited from processes that are common to most higher plants. The necessary machinery for perception and recognition of Nod-factors has been shown to exist in non-legumes (Gualtieri and Bisseling, 2000). Genes, which might have been recruited from non-symbiotic pathways, are involved in the infection process, since some nodulin genes are also expressed in non-symbiotic plant tissues (Gualtieri and Bisseling, 2000; Mathesius *et al.*, 2000; Szczyglowski and Amyot, 2003).

Nodule formation could have initially evolved from the same developmental pathways activated during lateral root formation, since lateral roots and nodules share a number of similarities (Mathesius *et al.*, 2000). Mathesius *et al.* (2000) proposed that nodule formation from modified lateral roots might have been the initial strategy of the rhizobia. The ability to induce responses at the zone of emerging root hairs might have evolved later.

Studies conducted indicated that the legume-rhizobium symbiosis and the symbiotic interaction of vesicular arbuscular fungi and plants share signaling pathways, since some

legumes which are Nod^- are also Myc^-. It has been found that the early nodulin genes are expressed in both mycorrhizal and rhizobium symbiosis. (Hirsch and LaRue, 1997). Hirsch *et al.* (2001) suggested that nodulation by rhizobia has evolved from the older mycorrhizal symbiosis. However, many species of *Lupinus* are naturally Nod^+ and Myc^- which could indicate that the ability of legumes to be nodulated by either group might be associated with the legume's taxonomic position (Sprent, 2002).

It is estimated that the mycorrhizal association originated 400 million years ago. Estimations of the split between the slow-growing and fast-growing rhizobia suggested that it occurred 500 million years ago. Mono- and dicotyledonous plants diverged 156-171 million years ago and the brassicas and legumes separated 125-136 million years ago (Turner and Young, 2001). Rhizobia already diverged before the existence of legumes and it can thus be concluded that nodulation ability was acquired much later. Nodulation ability spread through horizontal transfer to different genera. The discovering of the origin of the three common *nod* genes (*nodABC*) might explain how symbiosis started. The G + C content of the nodulation genes differ from that of the chromosomal genes and the codon usage differ from that of most chromosomal genes. Homologues of NodB (a deacetylase) and NodC (a transferase) exist in databases. The origin of NodA is, however, unknown, since it has only been found in rhizobia. NodA has the unusual ability to transfer a fatty acyl chain to an already formed polysaccharide (Hirsch *et al.*, 2001).

3.2 Evolution of the bacterial symbiont

Perret *et al.* (2000) argued that rhizobia which have broad host ranges such as *Sinorhizobium* sp. NGR234 and *S. fredii* USDA257 are closer to the ancestral form of symbiosis and that narrow host range is an adaptation, which developed for certain legumes in restricted niches. The authors argued that the nodulation of the non-legume *Parasponia andersonii* by both *Sinorhizobium* sp. NGR234 and *S. fredii* USDA 257 supported the theory. *Sinorhizobium* sp. NGR234 was isolated from the same area where *Parasponia* spp. grew, which indicated that the symbiosis evolved in the same environment (Perret *et al.*, 2000).

The photosynthetic *Bradyrhizobium* isolates from *Aeschynomene* species is highly host-specific and form true stem-nodules (Boivin *et al.*, 1997b). The ability to nodulate the stems could have evolved together with the photosynthetic ability of the strains. These strains can

also grow on N_2 as the sole N source and photosynthesize heterotrophically. Unlike other rhizobia, these strains are epiphytic like *Azorhizobium* strains. *Azorhizobium* is phylogenetically closer related to *Xanthobacter*, than to other classic rhizobia, which suggest that *Azorhizobium* might have evolved from a diazotrophic to a symbiotic bacterium or *Azorhizobium* could be a more primitive form of rhizobia (Boivin *et al.*, 1997b). Based on the intermingled state of rhizobia with other non-symbiotic bacteria, lateral gene transfer of nodulation genes might have been a result of plant-bacteria co-evolution (Martínez-Romero and Caballero-Mellado, 1996; Boivin *et al.*, 1997b).

4. Applications of the rhizobium-legume symbiosis

4.1 Inoculation of legumes

It is necessary to apply commercial inoculants to nitrogen deficient fields especially when the indigenous rhizobial population is limited or has a poor nitrogen-fixing ability (Barran *et al.*, 1991). Three factors influence the outcome of inoculation: the bacteria, the host plant and the environment. The number of rhizobia added to the legume seed and the number of indigenous rhizobia capable of nodulating the host influences the inoculant success. The ability of an inoculum strain to adapt to the soil conditions and persist into the next growing season are important factors to consider when choosing an inoculum (Dowling and Broughton, 1986).

A major problem encountered is that inoculated rhizobia do not survive in the field. The inoculum strains are often displaced within a year or two by indigenous strains (Henzell, 1988). The soil populations of rhizobia are not stable, since genotypes change over time (Streeter, 1994). Genetic exchange takes place in the field mainly by conjugation (Dowling and Broughton, 1986). It can take as long as four years before an introduced strain becomes highly successful in nodule formation (Streeter, 1994). The characteristics of indigenous rhizobial populations that may affect the inoculation response are population density, effectiveness and competitive ability of strains. If there is even just a small population of indigenous rhizobia (10 rhizobia / g soil) and some of them are effective strains, the indigenous population can meet the nitrogen requirement of the host (Thies *et al.*, 1991).

A commercial inoculant should be more competitive than the indigenous rhizobia of the soil for nodulation (Gandee *et al.*, 1999). The quality of the commercial inoculant should be of a high standard, since the viability of the rhizobia in the inoculum carriers may change and rhizobia may lose their nitrogen-fixing effectiveness (Streeter, 1994). In South Africa, commercial inoculants should contain at least 5 x 10^8 rhizobial cells / g of peat (Strijdom, 1998). It was found that sterilisation by means of steam and gamma irradiation resulted in excellent inoculants (Strijdom, 1998). According to Strijdom (1998), a new patented carrier developed by Dagutat appears to be superior to peat carriers. The survival of the bacterial cells, adherence ability and suspension characteristics of the carrier are better.

The response of the legume host to inoculation is determined by several factors. The presence and quality of indigenous rhizobial populations, the availability of soil nitrogen, physical and chemical constraints of the soil (acidity, toxicity and low fertility) influence the nodulation process. Climatic conditions such as low rainfall, inadequate soil and air temperatures and insufficient solar radiation will affect nodulation efficiency (Thies *et al.*, 1991; Dowling and Broughton, 1986).

Extreme soil acidity has a negative effect on the host plants, the rhizobia and the symbiosis (Zahran, 1999). Low pH is linked with high aluminium and manganese levels and reduced calcium availability (Hungria and Vargas, 2000). The rhizobia are usually more pH sensitive than the host plant, but some strains are more acid-tolerant (Zahran, 1999; Hungria and Vargas, 2000). Acid-tolerance is dependent on the maintenance of a neutral intracellular pH (Hungria and Vargas, 2000). The use of strains and host plants adapted to grow in low pH soil is ways of improving nitrogen fixation (Graham and Vance, 2000; Hungria and Vargas, 2000). Acid soils can be improved by liming to achieve a pH where aluminium and manganese levels are no longer toxic, which will improve the nodulation and nitrogen fixation of the host plants (Graham and Vance, 2000; Hungria and Vargas, 2000).

Drought, salinity and possibly high soil temperatures limit the rhizobium-legume symbiosis (Zahran, 1999; Zahran, 2001). The rhizobial strains from arid regions are adapted to such adverse environmental conditions. These strains may be effective inoculant strains for crops growing in adverse conditions (Zahran, 2001) and they are genetic reservoirs for the improvement of other strains. The host plants and the nodulation process are more sensitive than the rhizobial strains (Zahran, 1999; Zahran, 2001). The best rhizobium-legume symbiosis

for arid conditions where drought and salinity prevail would be the ones that grow rapidly under ideal temperature and moisture conditions, with high tolerance levels to unfavourable conditions (Zahran, 2001). The increased breeding of legumes tolerant to drought and salinity is crucial (Graham and Vance, 2003).

Certain host plant genotypes exist that have preferences for specific rhizobial strains, while preventing infection by other rhizobia. The inoculum strain and the legume host must be compatible (Ishizuka, 1992; Thies *et al.*, 1991). The host plant can be improved by breeding to select for host plants, which successfully exploit the inoculant strains or the strains already present in the soil (Peoples and Craswell, 1992; Herridge and Rose, 2000). Enhanced nitrate tolerance and maximising the legume yield are other possibilities of plant breeding (Ishizuka, 1992; Peoples and Craswell, 1992; Herridge and Rose, 2000). Van Kessel and Hartley (2000) cautioned against the development of host plants with increased tolerance to nitrate, except if there is an improved use of the nitrogen through intercropping with non-legumes. Increasing the nitrogen demand of the plant through sound agricultural management practices would increase nitrogen fixation (Van Kessel and Hartley, 2000). Kiers *et al.* (2002) pointed out that crop plants, which possess the ability to enhance the reproduction and release of truly mutualistic rhizobia present in the nodules into the soil, would be more beneficial than inoculation of the plants with exceptional inoculants.

4.2 Co-inoculation

Legumes commonly interact with the fungi *Acaulospora*, *Gigaspora*, *Glomus* and *Sclerocytis* to produce vesicular arbuscular mycorrhizal (VAM) associations. VAM is an obligate symbiont dependent on the plant for fixed carbon, while it provides usable phosphates in turn for the plant. The association of the legume host, VAM and rhizobia are more efficient than the association of just two partners (Dowling and Broughton, 1986).

Badr El-Din and Moawad (1988) reported a significant increase in the plant dry weight, nitrogen and phosphorus content of lentil and faba bean as well as an increase in the seed yield of soybean after dual inoculation with rhizobia and VAM. Inoculation with mycorrhizae increased nodulation of the plants. The increased nodulation and nitrogen fixation of the legumes after co-inoculation is due to the ability of the mycorrhizae to improve the uptake of phosphorus, sulphur and minor elements such as cobalt, copper and zinc by

increasing the area of absorption of the roots in soil deficient in both nitrogen and phosphor (Badr El-Din and Moawad, 1988; Dela Cruz *et al.*, 1988). It is important to use effective VAM fungi for co-inoculation with the rhizobia inoculum since not all VAM fungi are equally efficient in improving plant nodulation status (Dela Cruz *et al.*, 1988).

Nodulation can be improved by co-inoculation with several rhizobacteria (Gupta *et al.*,1998). The use of plant growth promoting rhizobacteria (PGPR) (*Enterobacter* sp. and *Bacillus* sp.) together with a *Bradyrhizobium* inoculum increased the nodule occupancy of the inoculum. The PGPR increased the competitive ability of the *Bradyrhizobium* strains (Gupta *et al.*,1998).

Parmar and Dadarwal (1999) reported that rhizobacteria (*Pseudomonas* and *Bacillus*) might have a direct influence on the production of the root flavonoids. This might be one of the reasons for the improvement of chickpea nodulation by co-inoculated rhizobia. Co-inoculation improved nodule weight, root and shoot biomass and the total plant nitrogen when grown under laboratory conditions (Parmar and Dadarwal, 1999).

5. Other nitrogen fixation associations

5.1 *Frankia* and actinorhizal symbiosis

The actinorhizal trees (*Casaurina* species) are used to produce constructional timber, furniture, firewood and charcoal in temperate regions (Ishizuka, 1992). The actinorhizal trees can also be used to rehabilitate mine spoils and to stabilise recent flood deposits and landslide areas (Ishizuka, 1992; Peoples and Craswell, 1992). Approximately 220 plant species covering eight plant families and 25 genera in the tropics and sub-tropics fix nitrogen symbiotically with nitrogen-fixing actinomycetes (*Frankia*). The actinorhizal plants are pioneer plants, which can grow in poor and harsh conditions. If a compatible strain of *Frankia* is not present in the soil it is necessary to inoculate the plant to increase the establishment and growth of the plant (Sprent and Parsons, 2000).

5.2 *Gunnera* sp. - *Nostoc* symbiosis, *Azolla* sp. - *Anabaena* symbiosis and cycads (*Encephalartos* spp.)- *Nostoc* symbiosis

In rice paddies, BNF sustains the fertility of the fields. BNF contributes to rice production in paddy fields where the application of nitrogen fertiliser is not sufficient (Ishizuka, 1992). The use of *Azolla* and *Sesbania rostrata* as green manure improves rice production. The addition of green manure from *S. rostrata* is equivalent to the application of 60-120 kg nitrogen / ha / year as urea in a rice ecosytem (Danso *et al.*, 1992).

The water fern *Azolla* and the cyanobacterium *Anabaena azollae* can fix 2-4 kg nitrogen / ha / day in symbiosis. The symbiosis is of great value in rice production. However, it is not the only useful characteristic of *Azolla*. The water fern is a weed suppressor, potassium scavenger in floodwater, animal feed, fish feed, phosphorus scavenger in sewage-treatment plants and suppressor of ammonia volatilisation (Bohlool *et al.*, 1992).

In South Africa, the 31 species of cycads have cyanobacteria in their roots, which fix nitrogen. In the summer months, the nitrogenase activity and the respiration rate of the cycads are higher than in the other months. However, this could not be explained by the variation in the sugar concentration, the respiratory quotient or water and chlorophyll contents of the roots (Strijdom, 1998).

5.3 Associative nitrogen fixation

Some plants such as forage grasses, sugarcane, maize and rice associate with different nitrogen-fixing bacteria. In grasses BNF is most likely due to *Acetobacter diazotrophicus*, in sugar cane *Herbaspirillum* spp. and *Azoarcus* spp. in kallar grass. In rice and maize BNF are due to species of *Alcaligenes*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Rhizobium* (James, 2000). These associations do not form nodules and the improvement of growth is mostly attributed to the production of plant growth promoting substances (Hirsch *et al.*, 2001). The bacteria do transfer fixed nitrogen to the plants, but not in high amounts (Hirsch *et al.*, 2001), though some associations show high levels of nitrogen fixation (James, 2000). The associations might be quite specific since Berge *et al.* (1991) isolated similar strains of *Bacillus circulans* from maize roots at sites 500

km apart. The inoculation effects of associative symbiosis will thus vary with the host plant and the bacterial symbiont used (Ishizuka, 1992)

The interest of scientists in the associative biological nitrogen fixation process has led to the publishing of several articles describing new associations and new diazotrophic species. Eckert *et al.* (2001) reported a new *Azospirillum* species associated with a perennial grass, *Miscanthus*, where it was found that the plant obtained substantial nitrogen amounts from associative nitrogen fixation. Fuentes-Ramírez *et al.* (2001) described two new *Gluconacetobacter* species associated with coffee plants. In a previous study, Fuentes-Ramírez and colleagues found the presence of *Gluconacetobacter diazotrophicus*, a nitrogen contributor to sugar cane, associated with coffee plants as well. *Paenibacillus brasilensis*, a newly described nitrogen-fixing species isolated from the maize rhizosphere in Brazil was described by Von der Weid *et al.* (2002).

5.3.1 Plant growth-promoting rhizobia

It has been found that rhizobia can occur as endophytes in the roots of cereals such as rice, wheat and maize. Improved rice yields have been found with the plant growth promoting rhizobia (Chaintreuil *et al.*, 2000; Tan *et al.*, 2001a). The rhizobia can exhibit the following plant growth promoting characteristics: phytohormone production, siderophore production, increasing phosphorous availability and antagonistic activity against plant pathogens (Antoun *et al.*, 1998; Lodewyckx *et al.*, 2002). Antoun *et al.* (1998) showed the potential of *Bradyrhizobium* and *Rhizobium* as plant growth promoting rhizobia of non-legumes in a study on radishes. It is vital that the inoculated rhizobia establish a significant population for the interaction between rhizobia and cereal to be useful (Tan *et al.*, 2001a).

CHAPTER 3

THE PLANT PARTNER: THE GENUS *CYCLOPIA*

1. Introducing the legume family Fabaceae

The plant family Fabaceae consists of approximately 20 000 species in nearly 700 genera. It is the third largest flowering plant family, which displays variation both ecologically and morphologically (Doyle and Luckow, 2003). The Fabaceae belongs to the order Fabales based on molecular data. A small tropical family Surianaceae, the genus *Quillaja* and the family Polygalaceae are the closest neighbours of the family Fabaceae in the order Fabales (Doyle *et al.*, 1997; Doyle and Luckow, 2003). The family Fabaceae is divided into three subfamilies, the Mimosoideae, the Caesalpinoideae and the Papilionoideae (Fig. 3.1) [Allen and Allen, 1981; Doyle *et al.*, 1997; Doyle and Luckow, 2003]. Based on molecular data the Mimosoideae and Papilionoideae appear to be monophyletic (common ancestor) lineages, while the Caesalpinoideae appears to be paraphyletic (several unrelated ancestors) (Doyle *et al.*, 1997; Doyle and Luckow, 2003).

The Mimosoideae contains trees, scrubs, woody vines and a few perennial herbs. In the subfamily Caesalpinoideae, the plants are mainly trees and scrubs. The subfamily Papilionoideae contains a diverse collection of trees, scrubs and annual or perennial herbs. Many of the species in the subfamilies Mimosoideae and Caesalpinoideae are valuable for their timber, dye, tannins, resins, gums, insecticides, medicines and fibres. Some of the plants are exceptionally beautiful flowering trees, vines and scrubs. Members of the Papilionoideae, especially in the temperate zone are of economic importance. They include edible nutritional crops for human and animal consumption, for forage, fodder, ground cover, green manures, erosion control and major honey sources (Allen and Allen, 1981).

The Papilionoideae is the largest and most diverse subfamily including the major tribes Amorpheae, Bossiaeae, Crotalarieae, Dalbergieae, Desmodieae, Galegeae, Genisteae, Indigofereae, Loteae, Podalyrieae, Robinieae, Sophoreae, Swartzieae, Thephrosieae, Thermopsideae and Trifolieae (Polhill, 1994). The genistoid alliance as it is currently known comprise a part of the tribe Sophoreae, the whole of the tribes Brongniartieae, Crotalarieae, Euchrestiae, Genisteae, Podalyrieae and Thermopsideae (Van Wyk, 2003). All the tribes in the "genistoid alliance" in the Papilionoideae produce characteristic quinolizidine alkaloids (Polhill, 1994; Van Wyk and Schutte, 1995).

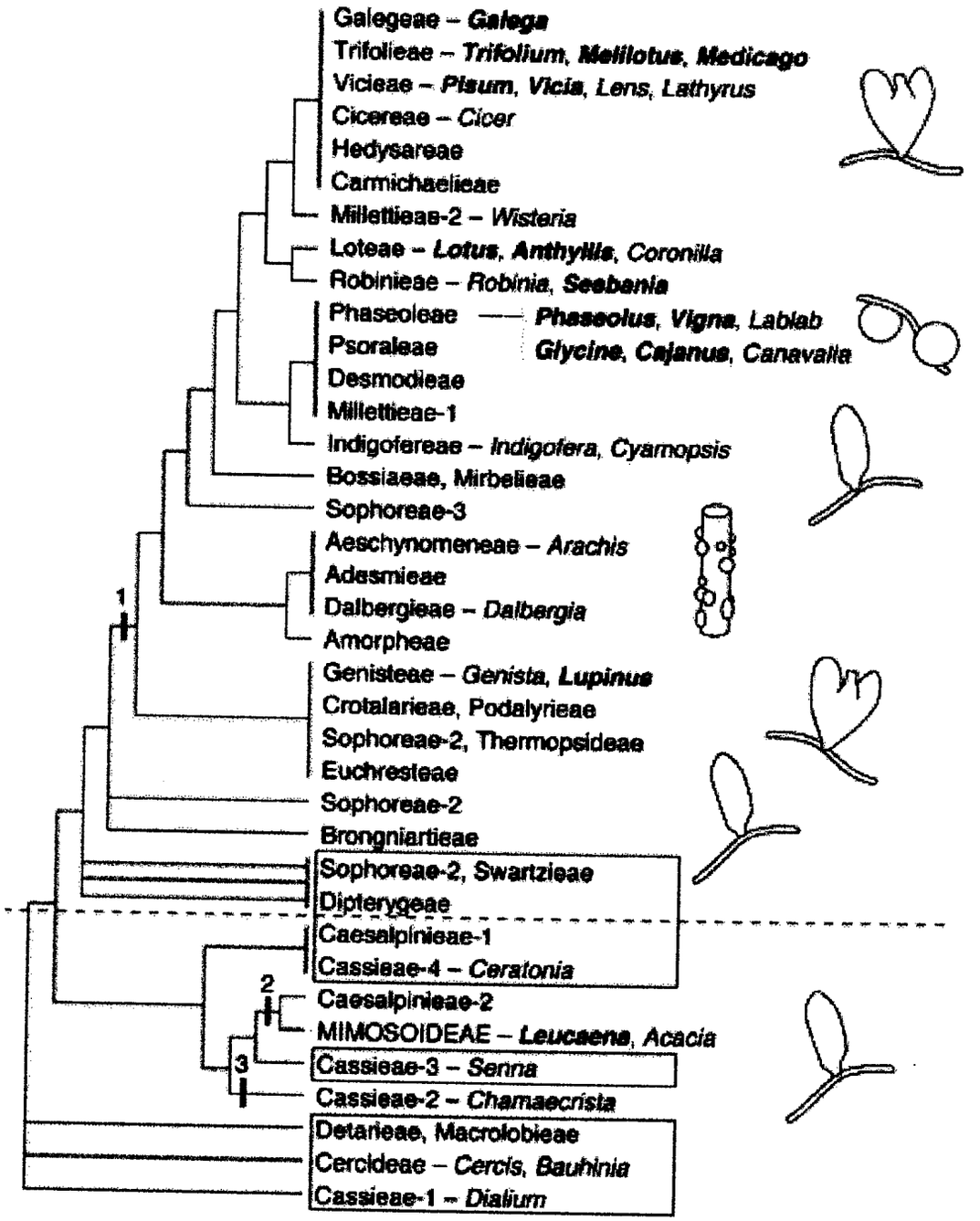


Figure 3.1: **Phylogeny of Fabaceae as obtained from chloroplast gene ribulose-1-5-bisphosphate carboxylase / oxygenase (*rbc*L) sequence data. All the tribes above the line belong to the Papilionoideae. Tribes in boxes do not nodulate. The numbers indicate possible origins of nodulation based on parsiminious analysis of the *rbc*L tree. The nodule sketches depict the distribution of nodule morphology in the family Fabaceae (Doyle, 1998).**

2. The tribe Podalyrieae

Schutte and Van Wyk (1998) amalgated the tribe Liparieae with Podalyrieae and described two subtribes Xiphothecinae and Podalyriinae based on morphological, cytological and chemical data. This change in the taxonomy of the tribes was clear from the phylogenetic results of Käss and Wink (1997). Schutte and Van Wyk (1998) included nine genera in the tribe Podalyrieae. The subtribe Xiphothecinae comprise the genera *Amphithalea*, *Coelidium* and *Xiphotheca*, while the subtribe Podalyriinae consist of *Calpurnia*, *Cyclopia*, *Liparia*, *Podalyria*, *Stirtonanthus* and *Virgilia* (Schutte and Van Wyk, 1998). All the genera are restricted and endemic to the winter rainfall Cape fynbos region of South Africa, except *Calpurnia* which is a widespread summer rainfall genus occurring from the southern Cape region in South Africa northwards along the highlands of Africa to India, (Schutte and Van Wyk, 1998).

In a comprehensive study by Van der Bank *et al.* (unpublished results), the phylogenetic relationships among the nine genera and 31 species of the Podalyrieae were determined based on sequence data of the nuclear internal transcribed spacers of the ribosomal genes (ITS1 and ITS2), as well as chemical and morphological data. The trees inferred from the ITS sequence data, as well as the combined data (ITS, chemical and morphological) identified three clades within the Podalyrieae. The first clade included all the species from subtribe Xiphothecinae. In the second clade all the species except the species of *Cyclopia* grouped. The *Cyclopia* species formed the third clade (Van der Bank *et al.*, unpublished results). This clearly indicates the uniqueness of the genus *Cyclopia*.

3. The genus *Cyclopia*

Cyclopia is a genus consisting of 19 accepted species (List of accepted names: http:/www.ildis.org/LegumeWeb/6.00/names/npall/npall_201.shtml) endemic to the fynbos region of South Africa. Van Wyk and Schutte (1995) reported 21 ± species in the genus *Cyclopia*. However, three species, namely *C. latifolia*, *C. subternata* and *C. tenuifolia* are included in the list of accepted names as synonyms of *C. buxifolia*, *C. falcata* and *C. maculata* respectively (http:/www.ildis.org/LegumeWeb/6.00/names/npall/npall_201.shtml), which supports the report of Van Wyk and Schutte (1995). The plants are woody fynbos shrubs, which are usually 1.5 m high, but can grow as high as 3 m. The leaf-to-stem ratio is low and

it is necessary to scarify the seeds to aid germination. The plants have hairless trifoliate leaves [each is divided into three leaflets] (Schutte and Van Wyk, 1998). All the plants have yellow fragrant (sweet honey scent) flowers (Van Wyk and Gericke, 2000).

3.1 Distribution of the genus *Cyclopia*

The plants grow in the coastal regions of the Western and Eastern Cape Provinces, from Darling to Port Elizabeth, bounded on the north by the Cederberg, Koue Bokkeveld, Klein Swartberg, Groot Swartberg and Kouga mountain ranges. Most of the species have limited distribution ranges and special habitat preferences (Du Toit *et al.*, 1998). Some species are restricted to the mountain peaks (*C. glabra*), others to the perennial streams (*C. maculata* and *C. longifolia*), marshy areas (*C. pubescens*), shalebands (*C. plicata*) and wet southern slopes (*C. bowieana*) (http://www.rooibos.ch/honeybush.html). *C. intermedia* is distributed over a wide geographical region (with reference to altitudinal distribution and mean annual rainfall) (Schutte *et al.*, 1995). *C. maculata* displays the most morphologically variation depending on locality. The plants on Paarlberg are resprouters, smaller plants than those from other localities and the leaves are broader. *C. maculata* plants from Riversdale and Jonkershoek are reseeders. The morphological variation of populations is restricted to *C. maculata* (De Villiers and Bosman, 1997).

3.2 Honeybush tea

A traditional herbal infusion, commonly referred to as honeybush tea is manufactured from the leaves, stems and flowers of mainly *C. intermedia* (Kouga bush tea) and *C. subternata* (synonym of *C. falcata*) (bush tea) [De Nysschen *et al.*, 1996]. These plants are now developed as commercial crop plants in the Langkloof area near Port Elizabeth. Originally, the tea was made from the leaves of *C. genistoides*, a small shrub known as coastal tea restricted to the Cape Peninsula of South Africa. However, the species is no longer used, since it is very scarce (Van Wyk and Gericke, 2000).

Most of the honeybush tea is harvested from natural populations, which places an enormous pressure on the environment, leading to the decline and extinction of *Cyclopia* populations (Du Toit *et al.*, 1998). The commercial cultivation of several species (*C. intermedia*, *C.*

subternata, *C. maculata*, *C. sessiliflora* and *C. genistoides*) is investigated to guard against the overexploitation of the natural populations (Du Toit and Joubert, 1998).

3.2.1 The presence and possible health benefits of phenolic compounds in *Cyclopia* species

De Nysschen *et al.* (1996) isolated a xanthone *C*-glycoside, mangiferin and two flavanones as *O*-glycosides of hesperitin and isosakuranetin as the major phenolic compounds of *Cyclopia* species leaves. *Cyclopia* is a chemically unique group in the tribe Podalyrieae, since the phenol compounds are restricted to the genus *Cyclopia*, although these compounds are distributed through the plant kingdom (De Nysschen *et al.*, 1996).

Ferreira *et al.* (1998) performed a study on the phenolic compounds of the fermented leaves and stems of *C. intermedia*. The fermented leaves and stems contained the inositol (+) pinitol, the flavone luteolin, the coumestans medicagol, flemichapparin and sophoracoumestan B, the isoflavones formononetin, afrormosin, calycosin, pseudobaptigen and fujikinetin, the flavanones naringenin, eriodictyol, hesperitin and hesperidin as well as 4-hydroxycinnamic acid (Ferreira *et al.*, 1998).

Pinitol is an expectorant (Beecher *et al.*, 1989) and displays anti-diabetic activity (Narayanan *et al.*, 1987). Luteolin displays antispasmodic activities (Ferreira *et al.*, 1998). Shimoi *et al.* (1996) indicated that luteolin has anti-oxidative activity in mice, which shows that the flavonoid can protect human cells against harmful free radicals, thus displaying antimutagenic abilitiy. Xanthones display several pharmacological properties; mangiferin has antiviral, antifungal, anti-inflammatory properties (Ferreira *et al.*, 1998). The isoflavones as well as the coumestans might have anticancer, estrogenic and antimicrobial activities in humans, while the flavanones may have antimicrobial, antiviral and anti-inflammatory activities (Ferreira *et al.*, 1998). Honeybush tea contains no caffeine and has a low tannin content (Du Toit *et al.*, 1998). As in the case of black tea and green tea further research on the bioavailability of the tea polyphenols in relation to their chemopreventive activity are warranted (Lambert and Yang, 2003).

CHAPTER 4

POLYPHASIC TAXONOMY

1. What is polyphasic taxonomy?

Colwell (1970) first used the term polyphasic taxonomy. Polyphasic taxonomy is a consensus type of taxonomy, which combines different data sets and information on microorganisms (Vandamme *et al.*, 1996). The information should be phenotypic and genomic (Rosselló-Mora and Amann, 2001). Taxonomy encompasses the classification, nomenclature and identification of microorganisms. Classification is the grouping of similar organisms into taxonomic units. In nomenclature the taxonomic units are named. The identification of an organism is the process of determining whether an organism belongs to a specific taxonomic unit (Vandamme *et al.*, 1996).

Different molecules of the cells can be used to determine the taxonomic position of isolates. The total chromosomal DNA or parts of it are used in such techniques as DNA base composition, DNA homology, DNA typing techniques and DNA sequencing. The chromosomal DNA can thus be used to resolve from intraspecies to genus rank and above depending on the technique used. The ribosomal RNA can resolve from species to genus rank and above with a technique such as sequencing (Priest and Austin, 1993, Vandamme *et al.*, 1996).

Proteins are valuable since a technique such as multilocus enzyme electrophoresis (MLEE) is extremely sensitive and can resolve intraspecies relationships. Other techniques such as electrophoretic patterns and serological comparisons can also resolve relationships within species (Priest and Austin, 1993; Vandamme *et al.*, 1996).

The use of different components of the cell using techniques to study the cell wall, membranes and metabolic products to obtain taxonomic information could resolve relationships between species and genera (Priest and Austin, 1993; Vandamme *et al.*, 1996). Some techniques are more appropriate to use in certain organisms than in other. The use of cell wall composition is more applicable for the classification of Gram-positive organisms, since more information can be found in the cell walls of these organisms (Vandamme *et al.*, 1996; Rosselló-Mora and Amann, 2001).

2. Genomic techniques

The determination of guanidine (G) + cytosine (C) content, DNA-DNA hybridisation, rRNA homology studies and DNA based typing target the genome, which content is unaffected by growth conditions and other external conditions (Vandamme *et al.*, 1996; Rosselló-Mora and Amann, 2001).

2.1 Determination of the DNA base ratio

The determination of the G + C content are regarded as part of the description of new species or genera. The G + C content within a well-defined species varies in the range of 3% (Vandamme *et al.*, 1996) to 5% (Rosselló-Mora and Amann, 2001). In the case of a well-defined genus the range is 10% (Vandamme *et al.*, 1996 and Rosselló-Mora and Amann, 2001).

2.2 DNA homology

A species is defined as a group of isolates which have 70% or more DNA-DNA homology and which ΔT_m values are 5 °C or less (Wayne *et al.*, 1987). DNA-DNA homology studies provide a consolidated measure to delineate bacterial species and the technique can be used to identify unknown isolates (Rosselló-Mora and Amann, 2001). Stackebrandt and Goebel (1994) noted that species that have 70% or more DNA-DNA homology usually have more than 97% 16S rDNA sequence similarity. However, there have been reports of organisms, which share 100% 16S rDNA sequence similarity, but have DNA-DNA homology values below 70%. The other side is also true, if the organisms share less than 97% 16S rDNA sequence similarity, the hybridization values will be less than 70% (Stackebrandt and Goebel, 1994). Ludwig and colleagues (1998) proposed that even though there is no official statement for genus delineation 95% sequence similarity might be regarded as the border for genus description.

2.3 DNA typing techniques

Rapid DNA typing methods such as the techniques which target the whole genome, gene clusters, individual genes and spacer regions enable the differentiation between strains of the

same species and between strains of different species (Stackebrandt *et al.*, 2002). The use of stringent PCR conditions rather than low stringency PCR increases the reproducibility of DNA typing with PCR. In the case of restriction fragment profiles, which are not highly complex, the profiles of different enzymes should be combined. The authors suggested the use of bootstrap to verify the statistical significance of the branching pattern (Stackebrandt *et al.*, 2002).

2.3.1 PCR DNA typing techniques

An identification technique should be reproducible and insensitive to previous manipulation of the strains (Santamaria *et al.*, 1999). DNA fingerprints can be generated by random primers as well as by using pairs of primers derived from repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences (De Bruijn, 1992). A primer, BOXA1 targeting the BOXA subunit of the BOX element a repetitive element first identified in *Streptococcus pneumoniae* can also be used to generate fingerprints (Rademaker *et al.*, 2000).

Random amplified polymorphic DNA (RAPD) is based on the use of short random primers (10-20 mer) which anneal to chromosomal DNA sequences at low annealing temperatures and then initiate amplification of regions of the genome (De La Puente-Redondo *et al.*, 2000). RAPD is a fast and easy method, but the reproducibility of the method is low.

PCR DNA fingerprinting has been widely used to study the relatedness and epidemiology of pathogens of animal, human and plant origin (Woods *et al.*, 1992; Liu *et al.* 1995; Alam *et al.*, 1999; Huang *et al.*, 1999; Van der Zee *et al.*, 1999; Al-Ghamdi *et al.*, 2000; De La Puente-Redondo *et al.*, 2000; Gunawardana *et al.*, 2000 and Scortichini *et al.*, 2000) and to type environmental isolates from soil and water (De Bruijn, 1992; Brim *et al.*, 1999; Santamaria *et al.*, 1999; Bastiaens *et al.*, 2000 and Heinaru *et al.*, 2000). De Bruijn (1992) used rep-PCR to type *Rhizobium meliloti* and other soil bacteria and found the method sensitive and extremely powerful. The different fingerprint techniques were found informative, easy, fast and reproducible if done under highly standardised conditions. Santamaria *et al.* (1999) used ERIC-PCR to identify *Bradyrhizobium* sp. strains of *Lotus* in a comparative study with lipopolysaccharide electrophoresis. Vinuesa *et al.* (1998), in their study of *Bradyrhizobium* isolates, first reported the use of the combined BOX-, ERIC and REP patterns to maximise

strain discrimination and to obtain phylogenetically more coherent groups. Rademaker *et al.* (2000) concluded that the combined analysis of BOX-, ERIC- and REP-PCR fingerprints of the genus *Xanthomonas* is in good correlation with DNA-DNA homology studies. Laguerre *et al.* (1996) found that DNA banding patterns of the same isolate could differ depending on the supplier of primers, batches of primers, different *Taq* enzymes and for random primers the type of thermal cycler used. However, Laguerre *et al.* (1996) concluded that these techniques could be used to rapidly type a large number of strains under well-standardised conditions to improve the reproducibility of results.

2.3.2 Use of the rRNA molecules

Ribosomal RNA molecules can be used as indicators of relatedness due to the following considerations:

* They are present in all living organisms (except viruses) [Woese, 1987].
* They have a conserved function (protein synthesis) and subsequently have changed little during evolution (Woese, 1987).
* They consist of variable regions, used for comparing or grouping of more closely related organisms, and more conserved regions, for comparing distantly related organisms (Woese, 1987).
* Phylogenetic lines of descent can be inferred from rRNA sequences. Changes in the nucleic acid sequences happen randomly, becoming fixed over time. The rate of change acts as a molecular chronometer and permits estimation of the elapsed time between evolutionary events. This provides an evolutionary clock (Woese, 1987).

The 5S rRNA molecule is too small to be of any use to measure evolutionary relatedness between organisms. The 23S rRNA molecule contains a higher information content than the 16S rRNA molecule and can be used for phylogenetic reconstruction and for the confirmation of phylogenetic trees based on the small subunit molecule (Priest and Austin, 1993). The 16S rRNA molecule has nine variable regions (Woese *et al.*, 1983; Neefs *et al.*, 1993) and can be used for phylogenetic studies. The molecule can be sequenced with the use of universal primers, which bind to conserved regions (Lane *et al.*, 1985; Weisburg *et al.*, 1991). The use of sequencing made the classification of unculturable organisms possible (Amann *et al.*, 1995).

The 16S-23S intergenic spacer (IGS) regions are variable [at least 4 times more variable than the 16S (Grundmann *et al.*, 2000)] and this can be used to identify, type and perform evolutionary studies on bacteria (Gürtler, 1999). The region can be divided into conserved and variable regions; the rate of evolution varies in these regions. The conserved regions might be involved in the formation of double-stranded processing stems, which are involved in the maturation of 16S and 23S rRNA. The tRNA genes: $tRNA^{ala}$, $tRNA^{ile}$ and $tRNA^{glu}$ might be present in the region (Gürtler, 1999). The presence or absence of these genes varies between species and within operons (Nagpal *et al.*, 1998; Gürtler and Stanisich, 1996; Gürtler, 1999). In the alpha-*Proteobacteria* the length of the IGS region varies from 800-1200 bp (Grundmann *et al.*, 2000). Length polymorphism of the 16S-23S IGS products might lead to the overestimation of distances between genotypes, because the differences does not always correspond to differences in restriction sites (Laguerre *et al.*, 1996). It is important to use IGS products of the same size or type in the case of multiple operons to differentiate between closely related strains (Nagpal *et al.*, 1998).

* **16S rDNA sequencing**

The evolutionary position of an isolate can be determined with 16S rDNA sequencing, since it contains both conserved and highly variable regions (Leblond-Bourget *et al.*, 1996; Vandamme *et al.*, 1996). It is used to determine inter- and intrageneric relationships. The method is unable to resolve the position of closely related strains of a species (Leblond-Bourget *et al.*, 1996; Vandamme *et al.*, 1996) and recently diverged species (Vandamme *et al.*, 1996). The use of full-length 16S rDNA sequences is vital to reconstruct phylogenetic trees. Partial sequences can be used to identify and assign organisms to phylogenetic groups, but not to draw phylogenetic conclusions (Ludwig *et al.*, 1998). The use of a single gene for the determination of taxonomic purposes assumes that the evolution of the whole genome proceeds at a constant rate and that no lateral transfer of genes occur (Broughton, 2003). Young (2001) therefore cautioned that the use of sequence data from a single gene could lead to the description of taxa, which are not stable, but only an interim solution.

Even though 16S rDNA sequencing can no longer be regarded as the ultimate method to study the phylogenetic relationships of organisms, the method still provides a framework for bacterial classification (Rosselló-Mora and Amann, 2001). Stackebrandt *et al.* (2002)

reported the use of other housekeeping (protein-coding) genes in conjunction with 16S sequencing and DNA-DNA hybridisation to delineate species.

* **16S rDNA restriction length polymorphism analysis**

In their study, Laguerre *et al.* (1994) found that 16S-RFLP data correspond well with the taxonomic data based on DNA-rDNA hybridisation and 16S rDNA sequence analysis. Rhizobial strains can be characterised and grouped by 16S-RFLP into species and above species level due to the conserved nature of the 16S rDNA gene. In another study, Laguerre *et al.* (1997) improved their results by performing mapped restriction site polymorphism analysis of the 16S rRNA genes of several rhizobial isolates. RFLP analysis of the 16S rDNA gene has been successfully used to differentiate between species of other bacterial genera (*Aeromonas*) (Figueras *et al.*, 2000) and genera and species of families (*Acetobacteraceae*) (Ruiz *et al.*, 2000). Odee *et al.* (2002) studied 41 rhizobial strains isolated from tree and herbaceous legumes grown in Kenyan soils. They found 12 distinct genotypes with 16S RFLP analysis and only 10 with partial (230bp) 16S sequence analysis. They were capable to differentiate to genus level with the use of 16S rDNA-RFLP. Mhamdi *et al.* (2002) used 16S RFLP to determine the identity of rhizobia nodulating *Phaseolus vulgaris* in Tunisian soils.

* **16S-23S IGS sequencing**

The evolutionary rate of the IGS is much higher than that of the 16S rDNA which make it possible to detect only recent evolutionary events by an IGS tree (Leblond-Bourget *et al.*, 1996). Leblond-Bourget and colleagues (1996) found the tree obtained from the IGS sequence data supported the tree reconstructed from 16S sequence data. This showed the usefulness of IGS sequence data for phylogenetic studies. The IGS tree also supported previous DNA-DNA hybridisation results (Leblond-Bourget *et al.*, 1996).

Van Berkum and Fuhrmann (2000) used IGS sequencing in conjunction with 16S sequencing to determine the phylogenetic relationships among soybean rhizobia. The 16S rRNA genes of the *Bradyrhizobium* strains were too conserved to resolve the relationship of closely related strains. The researchers achieved better resolution of the relationships with the IGS sequence data (Van Berkum and Fuhrmann, 2000).

Willems *et al.* (2001a) sequenced the 16S-23S IGS of *Bradyrhizobium* isolates to compare the grouping of the isolates with that obtained with DNA-DNA hybridisations and previous AFLP analysis. The researchers found that IGS sequencing is a useful tool to determine the taxonomic position of a *Bradyrhizobium* isolate, not replacing DNA-DNA hybridisation, but decreasing the number of hybridisations necessary to unequivocally determine the taxonomic position of an isolate (Willems *et al.*, 2001a).

Mehta and Rosato (2001) used IGS sequencing in conjunction with 16S sequencing to determine the phylogenetic position of the plant pathogen, *Xylella fastidiosa*. Interestingly the authors found that the IGS region of most of the isolates did not contain more substitutions than that found in the 16S rRNA gene. In their study, Aakra *et al.* (1999) found that the IGS sequence data improved and corroborated their understanding of the phylogenetic position of the ammonia-oxidising isolates.

* **16S-23S IGS-RFLP**

RFLP analysis of the 16S-23S rDNA gene can be used to differentiate between strains at the intraspecies level (Laguerre *et al.*, 1996; LeBlond-Bourget *et al.*, 1996). Grundmann *et al.* (2000) reported that the results obtained from the RFLP of the IGS region were in agreement with 16S sequencing as well as DNA-DNA hybridisation results, confirming that the technique is discriminative and suitable for routine use in monitoring natural populations and diversity studies. Guo *et al.* (1999) characterised *Astragalus sinicus* rhizobia with 16S-23S IGS-RFLP and found that a specific IGS type did not necessarily correspond to a specific 16S genotype. The researchers concluded as a cautionary measure that IGS-RFLP might not be the best method to characterise a large heterogeneous population (Guo *et al.*, 1999). Doignon-Bourcier *et al.* (2000) used 16S-23S IGS-RFLP to genotypically characterise *Bradyrhizobium* strains isolated from the nodules of small Senegalese legumes. Guasp *et al.* (2000) used 16S-23S IGS-RFLP to differentiate and identify the genomovars based on DNA-DNA hybridisation of *Pseudomonas stutzeri*. Guasp *et al.* (2000) concluded that IGS-RFLP is the most inexpensive and quick way to identify different isolates. In their study on rhizobia which nodulate *Acacia* spp. in Morocco, Khbaya *et al.* (1998), could differentiate the strains into groups based on the IGS region lengths. No finer resolution of the strains was achieved with IGS-RFLP (Khbaya *et al.*, 1998).

2.3.3 Other genes useful for phylogenetic deductions

Hunter-Cevera (1998) reported that phylogenetic trees based on functional genes, such as *recA*, are congruent with trees based on 16S rDNA data. Phylogenetic trees based on molecules such as 23S rRNA, ATPase subunits, elongation factors and RNA polymerases have been tested and found to give similar results to that obtained with 16S rRNA (Rosselló-Mora and Amann, 2001). Gaunt *et al.* (2001) found that the phylogenetic trees reconstructed from *atpD* (β-subunit of the membrane ATP synthase) and *recA* (part of the DNA recombination and repair system) supported the 16S rDNA-based classification of rhizobia. In another study using *glnI* (glutamine synthetase found in all prokaryotes) and *glnII* (glutamine synthetase found in all eukaryotes and some prokaryotes) to reconstruct phylogenetic relationships of rhizobia, Turner and Young (2000) found broad agreement between the *glnI* and 16S rDNA phylogenies.

3. Phenotypic techniques

Classical phenotypic traits, substrate utilisation tests, cellular fatty acids and whole-cell protein analysis are some of the phenotypic techniques employed as part of a polyphasic approach. Morphological, physiological and biochemical features are the classical phenotypic features, which can be used to supply descriptive information of a species (Vandamme *et al.*, 1996).

Numerical taxonomy of phenotypic features has been used to compare the phenotypic characteristics of a large number of bacterial strains. Automated systems such as, Biolog (Biolog Inc., Hayward, California, USA) and API (Analytab Products, Plainview, New York, USA) have replaced classical phenotypic studies, since several features can rapidly be tested and compared against data in a database (Vandamme *et al.*, 1996). McInroy *et al.* (1999) successfully used Biolog and partial 16S rRNA sequencing to characterise rhizobia from African acacias and other tropical woody legumes. The two techniques were in good agreement in the grouping of the isolates up to genus level (McInroy *et al.*, 1999). Schneider *et al.* (1998) used Biolog as a phenotypic fingerprinting technique to monitor upsets in wastewater treatment systems. They concluded that Biolog could be further developed as a management tool.

Whole-cell protein analysis is a standardised and reliable method to group large numbers of closely related isolates. The method can differentiate at and below species level (Vandamme *et al.*, 1996). Chen and colleagues (1991) used SDS-PAGE in conjunction with G + C content and DNA homology studies to study root-nodulating isolates from *Astragalus sinicus*. Yao *et al.* (2002) used SDS-PAGE of whole-cell proteins to characterise rhizobial isolates from *Lespedeza*. The researchers found that the clusters obtained from the protein analysis, DNA-DNA hybridisation data and the numerical taxonomic analysis all agreed, which supported their description of a new *Bradyrhizobium* species, *B. yuanmingense* (Yao *et al.*, 2002).

Multilocus enzyme electrophoresis (MLEE) is a high-resolution phenotypic method which can determine the amount of genetic recombination between members of a population (Schloter *et al.*, 2000). Wang and colleagues (1999) used MLEE as one of the techniques to characterise the rhizobia associated with *Amorpha fruticosa*, which led to the description of *Mesorhizobium amorphae*. MLEE showed variation within a genus (Wang *et al.*, 1999).

The characterisation of the cellular fatty acids of bacteria is a phenotypic method, which can differentiate between strains of species (Vandamme *et al.*, 1996). Now, with the automation (Sherlock microbial identification system) of the procedure it is possible to analyse a large number of strains (Tighe *et al.*, 2000). So *et al.* (1994) found it a good indicator of the phylogenetic relationships of bacteria as well as able to resolve the taxonomic position of bacteria of the family *Rhizobiaceae*.

CHAPTER 5

TAXONOMY OF THE ROOT-NODULATING BACTERIA

1. The polyphasic taxonomy of root-nodule bacteria

Previous classification of rhizobia were based on plant infection tests or the ability to nodulate, but after it became clear that in most, but not all, instances the genes for nodulation, nitrogen fixation and host specificity are located on transmissible plasmids, this method became absolute as the only tool for taxonomic purposes. Graham *et al.* (1991) proposed as minimal criteria for the description of new rhizobia species and genera the use of both phylogenetic and phenotypic (symbiotic, cultural, morphological and physiological) traits. The description should be based on a large number of strains, chosen from different geographical origins focusing on the original habitat of the host legume. Any new species should be supported by phenotypic differences, enabling the non-taxonomist to identify the new species (Graham *et al.*, 1991).

In recent publications, molecular symbiotic data (nodulation and nitrogen fixation genes) were more frequently used in conjunction with other genomic and phenotypic methods to further our knowledge of the rhizobial diversity. Graham *et al.* (1991) suggested that methods, such as the RFLP analysis of *nod* and *nif* genes would support nodulation differences. Trees reconstructed from nodulation genes differ from 16S rDNA phylogenetic trees. The nodulation trees have been reported to be more related to the host plant taxonomy (Guo *et al.*, 1999). Laguerre *et al.* (2001) proposed that the characterization and phylogenetic classification of symbiotic genes should be included in the minimal standards to describe a new species, enabling the definition of the broad host range of isolates.

Several authors have used a polyphasic approach to study the diversity of rhizobia associated with particular legumes and to describe new genera or species (De Lajudie *et al.*, 1994; Van Berkum *et al.*, 1998; Wang *et al.*, 1998; Nick *et al.*, 1999; Tan *et al.*, 1999; Wang *et al.*, 1999b; Velázquez *et al.*, 2001; Sylla *et al.*, 2002; Wang *et al.*, 2002; Wei *et al.*, 2002; Yao *et al.*, 2002 and several other authors). Nick et al. (1999) used a polyphasic approach to distinguish between the two species, *Sinorhizobium arboris* and *Sinorhizobium kostiense* both isolated from nodules of *Acacia senegal* and *Prosopis chilensis*. Sy and colleagues (2001) conducted a polyphasic study on methylotrophic root-nodulating bacteria from *Crotalaria* spp. culminating in the proposal of *Methylobacterium nodulans*. *M. nodulans* was the first described species of root-nodulating isolates which did not belong to the genera: *Allo-*, *Azo-*, *Brady-*, *Mesorhizobium*, *Rhizobium* or *Sinorhizobium*. This broadened our understanding of

the bacteria capable of forming root-nodules on legumes. The species status of *Sinorhizobium xinjiangense* was unequivocally proven by Peng and colleagues (2002) in a polyphasic approach where *S. xinjiangense* was clearly differentiated from the other Chinese soybean-nodulating species *S. fredii*. The new species *Devosia neptuniae* was only described after extensive research showed that this organism is truly a new species and capable of root-nodulating *Neptunia natans* (Rivas *et al.*, 2002; Rivas *et al.*, 2003).

2. Horizontal gene transfer and the implications for rhizobial taxonomy

The transfer of genes between lineages, referred to as horizontal gene transfer, complicates phylogenetic reconstruction, because some species are chimeric, with different phylogenetic histories for different parts of the genome (Eisen, 2000). Genes can be transferred by conjugation, transformation or transduction (Schloter *et al.*, 2000; Dutta and Pan, 2002). Horizontal gene transfer changes the microbial genome. Novel metabolic capabilities are conferred to the genome through horizontal gene transfer and this enable the recipient organism to explore new ecological niches (Dutta and Pan, 2002). Horizontal gene transfer could initiate diversification of bacterial lineages (Lawrence, 1999; Schloter *et al.*, 2000, Dutta and Pan, 2002).

It is now clear that the genes for pathogenic or symbiotic interactions with eukaryotes are acquired through horizontal gene transfer (Finan, 2002). The genes can be located on plasmids or on the chromosome as genomic islands. In most cases the genomic islands loose their transmissibility (Finan, 2002), but in the case of *M. loti* strain R7A, it was shown that this strain still had the necessary machinery to transfer the symbiosis island (Sullivan *et al.*, 1995; Sullivan and Ronson, 1998). Rosselló-Mora and Amann (2001) argued that if it is known that characters, especially extrachromosomally coded characters, which affect phenotype have been acquired through horizontal gene transfer, these characters should be excluded from taxonomic studies.

Functional or essential genes like the ribosomal genes are not usual candidates for horizontal gene transfer, since the recipient genome already has functional copies (Lawrence, 1999). The native genes have co-evolved with the rest of the cellular machinery and it is thus unlikely to be displaced, which makes it difficult for the novel genes to persist in the recipient genome. However, horizontal gene transfer of essential genes does occur. *Thermomonospora*

has two functional copies of rRNA genes; one of the copies was obtained by horizontal gene transfer (Lawrence, 1999). *Bacillus megaterium* harbours a functional rRNA operon on a small plasmid, which might be transferable to other bacteria (Broughton, 2003).

In the family, *Rhizobiaceae* the horizontal transfer of rRNA genes and recombination among species of the same genus did occur (Eardly *et al.*, 1996 and Parker, 2001). Gaunt *et al.* (2001) reconstructed phylogenetic trees from *atpD* (β-subunit of the membrane ARP synthase) and *recA* (encodes part of the DNA recombination and repair system) sequence data. The researchers found that the trees supported the 16S rDNA sequence based classification of rhizobia, but possible recombination events within the genera were found (Gaunt *et al.*, 2001). Turner and Young (2000) found that *glnI* (glutamine synthetase found in all prokaryotes) phylogeny and 16S rDNA phylogeny were in broad agreement. However, incongruence between the 16S rDNA phylogeny and *glnII* (glutamine synthetase found in eukaryotes and some prokaryotes) phylogeny were found. The incongruence might be explained by horizontal gene transfer to *Bradyrhizobium*, *Rhizobium galegae* and *Mesorhizobium huakuii*. The researchers also found evidence of horizontal gene transfer within the genus *Mesorhizobium* (Turner and Young, 2000).

A new report by Van Berkum *et al.* (2003) showed incongruence between phylogenetic trees reconstructed from 16S rDNA and those reconstructed from IGS and 23S rDNA sequence data. Analysis of the sequence data identified possible recombination events between short segments of the 16S rRNA genes of *B. elkanii* with *Mesorhizobium* species and between species of *Sinorhizobium* and *Mesorhizobium* species (Van Berkum *et al.*, 2003). It is clear that phylogenetic trees based on 16S rDNA sequence data should be used with caution, but the trees can still give a basic idea of the taxonomic position of an isolate.

Broughton (2003) suggested that authors should wait for the sequence data of other conserved genes to become available before new changes are made to the taxonomic groupings of rhizobia. This opinion was also shared by Van Berkum *et al.* (2003). Rhizobial taxonomists should thus strive for a taxonomy that is stable: a name should not be a source of error, a name should be maintained for as long as possible, a name should be accepted by every rhizobial scientist and finally the name should be unambiguous (Broughton, 2003).

3. The current taxonomy of the root-nodulating bacteria

Jordan (1984) included all rhizobia in the family *Rhizobiaceae* in the α-2-subgroup of the *Proteobacteria*. The rhizobial taxonomy changed significantly since 1984 with the development of new techniques and the study of more diverse legumes. On the website (http://www.cme.msu.edu/bergeys/outline.prn.pdf) of Bergey's manual rhizobia are included in several different families. *Rhizobium*, *Allorhizobium* and *Sinorhizobium* are placed in the family *Rhizobiaceae*. *Mesorhizobium* is grouped in the family "Phyllobacteriaceae", however Gaunt *et al.* (2001) found little support for this in their study on the *atpD*, *recA* and 16S rDNA sequence data of the α-*Proteobacteria*. *Bradyrhizobium* are placed in the family "Bradyrhizobiaceae", while *Azorhizobium* and the genus *Devosia* in which a newly nodulating species have been described belong to the family *Hyphomicrobiaceae*. The other nodulating species of the α-*Proteobacteria* belong to the genus *Methylobacterium* in the family "Methylobacteriaceae" (Bergey's manual taxonomic list: http://www.cme.msu.edu/bergeys/outline/prn.pdf).

Recently, it became clear that the ability to nodulate and fix nitrogen is not restricted to the α-*Proteobacteria*, but that several species in the β-*Proteobacteria* acquired the ability as well. The two genera involved *Burkholderia* and *Ralstonia* belong to the families "Burkholderiaceae" and "Ralstoniaceae" respectively in the order "Burkholderiales" (Bergey's manual taxonomic list: http://www.cme.msu.edu/bergeys/outline/prn.pdf).

In 2001, Young *et al.* proposed the emended description of *Rhizobium* (Fig. 5.1) to include the previous genera *Allorhizobium*, *Agrobacterium* (genus of plant pathogens) and *Rhizobium*. This proposal was based on high 16S rDNA sequence similarity values and no clear phenotypic differences between the three genera (Young *et al.*, 2001). With specific reference to the inclusion of *Agrobacterium* in the genus *Rhizobium*, Broughton (2003) stated that it is important for a bacterial name to reflect the reality of its symbiotic or pathogenic nature. Even if the true nature (symbiotic or pathogenic) of the organism is not reflected in its 16S rDNA sequence (Broughton, 2003). The International Committee on Systematics of Prokaryotes (2002) stated that the latest proposal of a new name does not mean that it has preference over the older name. The committee suggested that individual experts decide which name they want to use.

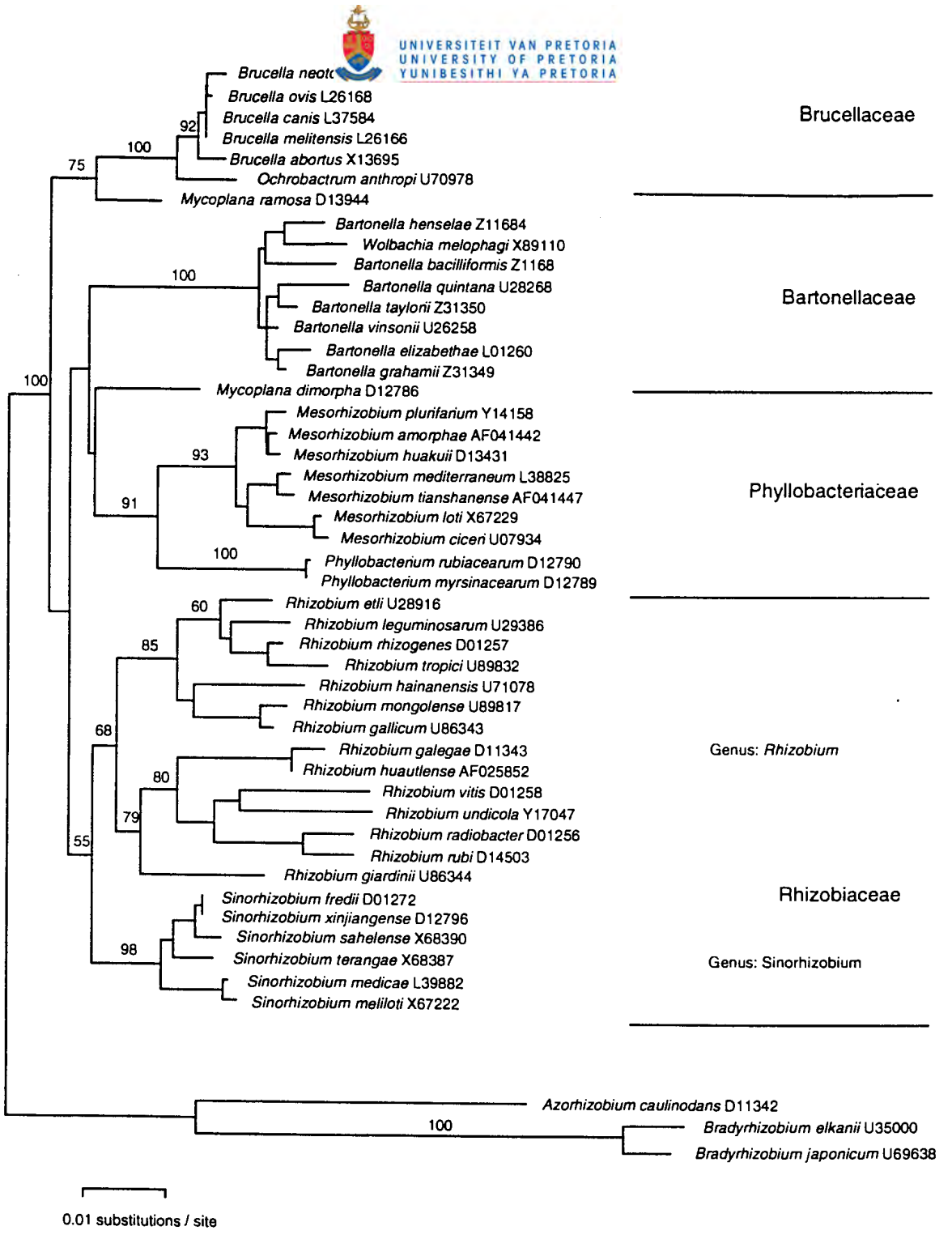


Figure 5.1: **Neighbour-joining tree showing the relationships of the *Rhizobiaceae* and closely-related families in the order Rhizobiales reconstructed from 16S rDNA sequence data. The branch lengths are proportional to the estimated number of nucleotide substitutions and bootstrap probabilities (percentages) were determined from 1000 resamplings (Young *et al.*, 2001).**

3.1. The genus *Allorhizobium*

De Lajudie *et al.* (1998a) described the new genus *Allorhizobium* for rhizobia isolated from nodules from the aquatic legume, *Neptunia natans*. Phylogenetically, the closest relatives of the genus are *Agrobacterium vitis* and *Rhizobium galegae*, but the 16S similarity values are relatively low and the bootstrap support for the group is not significant, which led the authors to conclude that the description of a new genus is warranted.

The genus contains one species, *Allorhizobium undicola*. The G + C content of the DNA is 60.1 mol%. Strains form effective nodules on their original host and induce nodules on *Acacia senegal*, *A. seyal*, *A. tortilis* subsp. *raddiana*, *Faidherbia albida*, *Lotus arabicus*, *Medicago sativa*, which are not always effective (De Lajudie *et al.*, 1998a).

3.2 The genus *Azorhizobium*

The genus was described by Dreyfus *et al.* (1988) for isolates that nodulate the stem and root of *Sesbania rostrata*. The strains are capable of fixing atmospheric nitrogen under microaerobic conditions and grow well with vitamins present in a nitrogen-free medium. Dreyfus *et al.* (1988) described only one species, *Azorhizobium caulinodans* based on identical protein electrophoregrams, high levels of DNA-DNA binding and phenotypic similarities. The G + C content of the strains is 66.5 mol%.

Adebayo *et al.* (1989) indicated that *Azorhizobium* strains are present as epiphytic bacteria on their host plants (10^5-10^7 bacteria per gram dry weight of leaves and flowers). The epiphytic survival of the *Azorhizobium* strains and their relative insensitivity to root inoculation is an advantage for the organisms. It may explain their greater competitiveness for stem nodulation (Adebayo *et al.*, 1989; Boivin *et al.*, 1997a).

3.3 The genus *Bradyrhizobium*

Jordan (1984) described *Bradyrhizobium* as slow-growing bacteria that nodulate some tropical- and temperate-zone legumes. The G + C content of the DNA is 61-65 mol%. Only one species, *B. japonicum*, was included in the genus described by Jordan (1984). Jordan (1984) concluded that the genus *Bradyrhizobium* represents an extremely heterogeneous

collection of strains. Two other species were later described namely, *B. elkanii* (Kuykendall *et al.*, 1992) and *B. liaoningense* (Xu *et al.*, 1995). In 2002, Yao *et al.* described *B. yuanmingense* for slow-growing rhizobia that nodulate *Lespedeza cuneata*. However, the taxonomy of the genus *Bradyrhizobium* will change significantly as more unknown *Bradyrhizobium* isolates from new legume sources are characterised. The increased availability of new techniques and knowledge will aid the unraveling of the taxonomic difficulties of the genus *Bradyrhizobium*.

Willems *et al.* (2001b) conducted a DNA-DNA hybridisation study of *Bradyrhizobium* isolates from *Faidherbia* (*Acacia*) *albida* and *Aeschynomene* species, as well as the *Bradyrhizobium* reference strains (excluding *B. yuanmingense*). The researchers found that the genus consisted of at least 11 genospecies. The genospecies formed four subgeneric groups that were more related to each other than to other genospecies (Willems *et al.*, 2001b).

One of the first phototrophic symbiotic strains (BTAil) isolated from the stem nodules of *Aeschynomene indica* belonged to *Bradyrhizobium* based on 16S rRNA and fatty acid methyl ester analyses (FAME) (So *et al.*, 1994). In the extensive study of Willems et al. (2001b), it was found that the photosynthetic isolates form *Aeschynomene* spp. belonged to at least two distinct genospecies in one subgeneric group.

3.3.1 *Bradyrhizobium japonicum*

The species was first described as *Rhizobacterium japonicum* by Kirchner in 1896 and then changed to *Rhizobium japonicum* by Buchanan in 1926. In 1982 Jordan proposed the genus *Bradyrhizobium* and thus the species name was changed to *Bradyrhizobium japonicum* as quoted by Jordan (1984). Jordan (1984) described the species as capable of nodule formation on species of *Glycine* (soybean) and *Macroptilium atropurpureum*.

3.3.2 *Bradyrhizobium elkanii*

Two distinct groups in the soybean nodulating *Bradyrhizobium* group were described by Hollis *et al.* (1981) based on DNA homology analysis. Kuykendall *et al.* (1992) described *B. elkanii* for the group II strains isolated from soybeans. The two groups differed in phenotype (fatty acid profiles, antibiotic resistance profiles, EPS and cytochrome composition) and

genotype (DNA homology, RFLP analysis and 16S rDNA sequence analysis) (Kuykendall *et al.*, 1992).

3.3.3 *Bradyrhizobium liaoningense*

The species, *B. liaoningense* was described for the extra slow growing rhizobia isolated from soybean in Liaoning, a province in the People's Republic of China (Xu *et al.*, 1995). Strains of this species nodulate *Glycine max* and *G. soja* effectively. The G + C content of the strains varies between 60-64 mol% (Xu *et al.*, 1995).

3.3.4 *Bradyrhizobium yuanmingense*

Yao *et al.* (2002) described the species for slow-growing rhizobia that nodulate *Lespedeza cuneata*. The genus belongs to the cowpea cross-inoculation group. The researchers followed a polyphasic approach including both phenotypic and genomic techniques to show that the species is a separate lineage within the *Bradyrhizobium* genus. The strains nodulate their original host, as well as *Vigna unguiculata* and *Glycyrrhiza uralensis*. G + C content ranges from 61.8-64.1 mol% Yao *et al.* (2002).

3.4. The genus *Rhizobium*

Jordan (1984) described the genus as fast-growing bacteria able to form nodules on the roots of temperate-zone and some tropical-zone leguminous plants. The G + C content of the DNA of this genus is 59-64 mol%. The type species of the genus is *R. leguminosarum*. Jordan (1984) included three species in the genus, *R. leguminosarum*, *R loti* and *R. meliloti*. The last two species have been assigned, respectively, to *Mesorhizobium* (Jarvis *et al.*, 1997) and *Sinorhizobium* (De Lajudie *et al.*, 1994).

Several new species are included in the genus: *R. galegae* (Lindström, 1989), *R. tropici* (Martínez-Romero *et al.*, 1991), *R. etli* (Segovia *et al.*, 1993), *R. hainanense* (Chen *et al.*, 1997), *R. gallicum* (Amarger *et al.*, 1997), *R. giardinii* (Amarger *et al.*, 1997), *R. mongolense* (Van Berkum *et al.*, 1998), *R. huautlense* (Wang *et al.*, 1998), *R. yanglingense* (Tan *et al.*, 2001b), *R. indigoferae* (Wei *et al.*, 2002), *R. sullae* (Squartini *et al.*, 2002) and *R. loessense* (Wei *et al.*, 2003).

3.4.1 *Rhizobium leguminosarum*

According to Jordan (1984), *R. leguminosarum* was first described by Frank in 1879 as *Schinzia leguminosarum*. Jordan (1984) included three biovars: trifolii, phaseoli and viceae in the species. The biovars differ according to the host plants that they nodulate (Jordan, 1984). The different genes coding for nodulation are plasmid-borne (Martínez-Romero *et al.*, 1991). Jordan (1984) described the species as strains capable of nodule formation on some species of *Lathyrus*, *Lens*, *Phaseolus*, *Pisum*, *Trifolium* and *Vicia*.

3.4.2 *Rhizobium galegae*

Lindström (1989) described *R. galegae* for fast-growing root nodule bacteria isolated from *Galega orientalis* and *G. officinalis*. Strains form effective nodules only on their original host plant, while other rhizobia might occasionally form ineffective nodules on *Galega* spp. The G + C content of *R. galegae* is 63 mol% (Lindström, 1989). Radeva *et al.* (2001) proposed two biovars for the strains of *R. galegae*, *R. galegae* bv. officinalis and *R. galegae* bv. orientalis corresponding to the host plant which they effectively nodulated.

3.4.3 *Rhizobium etli and Rhizobium tropici*

Rhizobium etli was described by Segovia *et al.* (1993) for the former *R. leguminosarum* bv. phaseoli Type I strains. The researchers included one biovar in the species, namely *R. etli* bv. phaseoli, which nodulated and fix nitrogen only with *Phaseolus vulgaris*. Non-symbiotic strains were also included in the species (Segovia *et al.*, 1993). Wang *et al.* (1999a) proposed *R. etli* bv. mimosae to include *R. etli* strains obtained from *Mimosa affinis*. These strains differed from *P. vulgaris R. etli* strains in their plasmid-borne symbiotic traits. The strains nodulated *Leucaena leucocephala*, *M. affinis* and *P. vulgaris* effectively, while *R. etli* bv. *phaseoli* strains cannot form nodules on *L. leucocephala*. In a study on indigenous South African legumes, Dagutat (1995) isolated putative *R. etli* strains from nodules of *Desmodium*, *Melolobium*, *Indigofera*, *Acacia melanoxylon* and *Chamaecrista stricta*.

Martínez-Romero *et al.* (1991) described *Rhizobium tropici* for the strains previously known as *R. leguminosarum* bv. phaseoli Type II. Two distinct subgroups, Type IIA and Type IIB were reported. The subgroups differ in phenotypic and genotypic characteristics (Martínez-

Romero *et al.*, 1991). Geniaux *et al.* (1995) reported the presence of a specific megaplasmid in each subgroup. Strains from *R. tropici* form effective nodules on *Leucaena leucocephala*, *L. esculenta* and *Phaseolus vulgaris* (Martínez-Romero *et al.*, 1991). Van Berkum *et al.* (1998) isolated strains belonging to *R. tropici* from *Medicago ruthenica*, which it nodulated effectively. Dagutat (1995) isolated strains corresponding to *R. tropici* from nodules of *Bolusanthus* and *Spartium*.

3.4.4 *Rhizobium gallicum*

Amarger *et al.* (1997) described *Rhizobium gallicum* for isolates from the nodules of *Phaseolus vulgaris* grown in France. Two biovars, *R. gallicum* bv. gallicum and *R. gallicum* bv. phaseoli were included in the species. *R. gallicum* bv. gallicum strains nodulate the following legumes: *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Phaseolus* spp. and *Onobrychis viciifolia* and fix nitrogen with *Phaseolus vulgaris*. The strains of the above-mentioned biovar have a single copy of the *nifH* gene (Amarger *et al.*, 1997). The strains of *R. gallicum* bv. phaseoli nodulate *Phaseolus* spp. and nodulate *Macroptilium atropurpureum* after one month. *Phaseolus vulgaris* is effectively nodulated by the biovar. The strains of *R. gallicum* bv. phaseoli contain three copies of the *nifH* gene (Amarger *et al.*, 1997).

Sessitsch *et al.* (1997) included Austrian isolates from *Phaseolus vulgaris* as well as the Mexican isolate FL27 in the species. The isolates were confirmed as *R. gallicum* strains based on phenotypic and phylogenetic analyses, belonging to the biovar, *R. gallicum* bv. gallicum. The isolates nodulated not only bean plants, but also cowpea plants, *Gliricidia* and *Leucaena*. The Austrian isolates and the Mexican isolate differ in their geographic origin, but are similar in their nodulation host range, which showed that the isolates contain a similar or related symbiotic plasmid (Sessitsch *et al.*, 1997). Laguerre *et al.* (1997) reported the isolation of *R. gallicum* strains in Canada from *Onobrychis viciifolia* and *Oxytropis riparia*. Mhamdi *et al.* (2002) reported the isolation of *R. gallicum* bv. gallicum from beans in Tunisia. Silva *et al.* (2003) reported the isolation of *R. gallicum* bv. gallicum isolates from *P. vulgaris* and *P. coccineus* in Mexico. These results indicate that several hosts and geographic distributions exist for *R. gallicum*.

3.4.5 *Rhizobium giardinii*

The species *Rhizobium giardinii* was described by Amarger *et al.* (1997) for *Phaseolus vulgaris*-nodulating bacteria in France. The closest neighbour of the species is *R. galegae* based on phenotypic and genotypic results. The strains were divided into two biovars namely, *R. giardinii* bv. giardinii and *R. giardinii* bv. phaseoli.

R. giardinii bv. giardinii nodulates *Leucaena leucocephala*, *Macroptilium atropurpureum* and *Phaseolus* spp. The strains are not able to form nitrogen-fixing nodules on *Phaseolus vulgaris*. The researchers found no hybridisation of the strains with the *nifH* probe used even under conditions of low stringency (Amarger *et al.*, 1997). Mhamdi *et al.* (2002) reported the presence of *R. giardinii* bv. giardinii in Tunisian soil which was the first report of the biovar outside of French soil.

The strains of *R. giardinii* bv. phaseoli nodulate *Phaseolus* spp. and form nodules on *Macroptilium atropurpureum* after a month or more. The nitrogen fixation level of the strains with *Phaseolus vulgaris* is low. Three copies of the *nifH* gene were detected in these strains (Amarger *et al.*, 1997).

3.4.6 *Rhizobium hainanense*

Chen *et al.* (1997) described *R. hainanense* for rhizobia isolated from trees, herbs and vines in the tropical Hainan province in the People's Republic of China. In their study, Chen *et al.* (1997) isolated rhizobial strains from 12 legume species classified into nine different genera. These strains were isolated from nodules of *Acacia sinuata*, *Centrosema pubescens*, *Desmodium triquctrum*, *D. gyroides*, *D. heterophyllum*, *Macroptilium lathyroides*, *Stylosanthes guyanansis*, *Tephrosia candida* and *Zornia diphylla*. The type strain of the species can only nodulate its original host and not the hosts of the other strains, which showed that the legume hosts do not fall within a single cross-inoculation group. The G + C content of the DNA is 59-63 mol% (Chen *et al.*, 1997).

3.4.7 *Rhizobium mongolense*

R. mongolense was described by Van Berkum and colleagues (1998) for a new genotype of rhizobia isolated from nodules of *Medicago ruthenica*. *M. ruthenica* is a potential new forage crop, which is tolerant to stress since it is adapted to grow in dry conditions with cold winters. According to Van Berkum *et al.* (1998), *R. mongolense* strains nodulate *M. ruthenica* and *Phaseolus vulgaris* effectively, while ineffective nodules are formed on *M. sativa*.

3.4.8 *Rhizobium huautlense*

Rhizobial strains isolated from *Sesbania herbacea* growing in Sierra de Huautla, Mexico was described as the new species, *Rhizobium huautlense* (Wang *et al.*, 1998). *R. galegae* is closely related to *R. huautlense* based on 16S rRNA sequencing analysis. The G + C content of the DNA is 57-58.9 mol%. *R. huautlense* strains form effective nodules on *Leucaena leucocephala* and *S. herbacea* (Wang *et al.*, 1998).

3.4.9 *Rhizobium yanglingense*

A new *Rhizobium* species, *R. yanglingense*, was described for strains isolated from the wild legumes *Amphicarpaea trisperma*, *Coronilla varia* and *Gueldenstaedtia multiflora*, which grow in arid and semi-arid regions in north-western China (Tan *et al.*, 2001b). Phylogenetically, the species is most related to *R. gallicum* and *R. mongolense*. Isolates from different host plants differ in their nodulation genes. The G + C content of the DNA of this species is 59-63 mol% (Tan *et al.*, 2001b).

3.4.10 *Rhizobium indigoferae*

Wei *et al.* (2002) described *R. indigoferae* for rhizobia isolated from wild *Indigofera* spp. growing in the Loess Plateau in north-western China. Based on 16S rDNA sequence data the closest neighbours of *R. indigoferae* were *R. gallicum* and *R mongolense*. DNA G + C content of the species is 59-62 mol%. The isolates can nodulate *Kummerowia stipulacea* (from the same geographic region) under laboratory conditions, as well as the *Indigofera* hosts (Wei *et al.*, 2002).

3.4.11 *Rhizobium sullae*

Rhizobium sullae (formerly referred to as *'R. hedysari'*) include the rhizobia associated with *Hedysarum coronarium* (sulla) [Squartini *et al.*, 2002]. This plant is an important agronomic crop in Spain and Italy and forms a specific symbiotic relationship with *R. sullae*. The G + C content of the strains is 61.6 mol%. According to the phylogenetic tree reconstructed from 16S sequence data *R. gallicum* was the closest neighbour of *R. sullae* (Squartini *et al.*, 2002).

3.4.12 *Rhizobium loessense*

Wei *et al.* (2003) described *Rhizobium loessense* for strains isolated from *Astragalus scobwerrimus*, *A. complanatus* and *A. chrysopterus*, which can nodulate *A. adsurgens* under laboratory conditions. These strains have been isolated from plants growing in the Loess Plateau of China. After a polyphasic approach, which included both phenotypic and genotypic analyses, it was clear that the strains belong to a novel *Rhizobium* species. This new species is phylogenetically closely related to *R. galegae* and *R. huautlense*. The G + C content of the DNA is 59.1-60.3 mol% (Wei *et al.*, 2003).

3.5 The genus *Mesorhizobium*

Jarvis *et al.* (1997) proposed the new genus *Mesorhizobium* to include strains that are intermediate in both growth rate and in their phylogenetic position. The growth rate of the strains of this genus ranges from moderate- to slow-growing (produce colonies 2-4 mm in diameter after incubation on yeast mannitol agar for 3-7 days at 28 °C). These strains form nitrogen-fixing nodules on the roots of a restricted range of leguminous plants. There is no known cross-inoculation between the strains of one species on the plant hosts associated with another species of the genus (Jarvis *et al.*, 1997).

The analysis of the 16S rDNA sequence data of the *Rhizobium loti*-group (including *R. huakuii*, *R. ciceri* and *R. mediterraneum*) and *R. tianshanense* showed that the group is monophyletic and more related to each other than any other *Rhizobium* species (Jarvis *et al.*, 1997). Jarvis *et al.* (1997) thus included the species *R. loti* (Jarvis *et al.*, 1982), *R. huakuii* (Chen *et al.*, 1991), *R. ciceri* (Nour *et al.*, 1994b), *R. mediterraneum* (Nour *et al.*, 1995), *R. tianshanense* (Chen *et al.*, 1995) in the genus *Mesorhizobium* based on phylogenetic and

phenetic data. *M. plurifarium* (De Lajudie *et al.*, 1998b), *M. amorphae* (Wang *et al.*, 1999b) and *M. chacoense* (Velázquez *et al.*, 2001) are the most recent additions to the genus. The G + C content of the DNA is 59-64 mol%. The type species is *Mesorhizobium loti* (Jarvis *et al.*, 1982).

Kishinevsky *et al.* (2003) conducted a phenotypic and genetic diversity study of rhizobia isolated from *Hedysarum spinosissimum*, *H. coronarium* and *H. flexuosum*. The researchers found that the isolates from *H. spinosissimum* might constitute a new species in the *Mesorhizobium* genus, but DNA-DNA hybridisation and G + C content analyses have to be done to determine the position unequivocally (Kishinevsky *et al.*, 2003).

3.5.1 *Mesorhizobium loti*

Jarvis *et al.* (1982) described *Rhizobium loti* for the fast-growing *Lotus* rhizobia and related strains. The strains of the rhizobial species form nitrogen-fixing nodules on the roots of *Anthyllis vulneria*, *Lotus corniculatus*, *Lotus filicalius*, *Lotus japonicus*, *Lotus krylovii*, *Lotus schoelleri*, *Lotus tenuis* and *Lupinus densiflorus*. The G + C content of the DNA ranges from 59-64 mol% (Jarvis *et al.*, 1982).

3.5.2 *Mesorhizobium huakuii*

Rhizobium huakuii was described for rhizobia that nodulate the roots of *Astragalus sinicus* (Chen *et al.*, 1991). *Astragalus sinicus* is an important winter-growing green manure in the southern part of the People's Republic of China. The nodulation of this legume has only been found in China and Japan. Strains of this rhizobial species form effective nodules on *Phaseolus vulgaris*, *Sesbania* sp. and *Vicia villosa*. The G + C content of the DNA is in the 59-64 mol% range (Chen *et al.*, 1991).

3.5.3 *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*

Nour *et al.* (1994b) described the species *Rhizobium ciceri*. The strains of the species nodulate the legume *Cicer arietinum* (chickpea). *C. arietinum* is the third most widely grown grain legume in the world (Nour *et al.*, 1994a). In their study Nour *et al.* (1994a) used

MLEE, RFLP of the 16S-23S IGS and more than 150 phenotypic characteristics to study the chickpea rhizobia.

The study of Nour and colleagues (1994a) showed two distinct phylogenetically distant groups, group A and group B. Group A contained the slow-growing rhizobia. The group A isolates were thought to be *Bradyrhizobium* strains due to their slow growth rate. However, in the polyphasic study done by Nour *et al.* (1994b) the authors showed that the genomic diverse group A strains were *Rhizobium* isolates. Nour *et al.* (1994b) proposed the new species, *R. ciceri* for the isolates of group B. The G + C content of the DNA is 63-64 mol%.

Nour *et al.* (1995) described *Rhizobium mediterraneum*. Four genomic species could be distinguished in the group A strains. One of the genomic species differed significantly from the previously described *R. ciceri* and the name *R. mediterraneum* was subsequently proposed for the genomic species (Nour *et al.*, 1995).

3.5.4 *Mesorhizobium tianshanense*

Rhizobial isolates from *Caragana polourensis*, *Glycine max*, *Glycyrrhiza pallidiflora*, *G. uralensis*, *Halimodendron holodendron*, *Sophora alopecuroides* and *Swainsonia salsula* were included in the species, *Rhizobium tianshanense* (Chen *et al.*, 1995). These strains were isolated from saline alkaline desert soil from a region with low rainfall and a wide variation in temperature. *R. tianshanense* strains only nodulate their original hosts thus forming a single cross-inoculation group. The G + C content of the DNA is 59-63 mol% (Chen *et al.*, 1995).

3.5.5 *Mesorhizobium plurifarium*

De Lajudie *et al.* (1998b) described the species *Mesorhizobium plurifarium* for tropical rhizobia previously referred to as cluster U (De Lajudie *et al.*, 1994). Cluster U representatives grouped in the *Mesorhizobium* genus with its closest neighbour on the rRNA tree being *M. huakuii* (De Lajudie *et al.*, 1994). These isolates nodulate *Acacia nilotica*, *A. senegal*, *A. tortilis* subsp. *raddiana*, *A. seyal*, *Leucaena leucocephala* and *Neptunia oleracea* effectively. The G + C content of the strains is 62.6-64.4 mol% (De Lajudie *et al.*, 1998).

3.5.6 *Mesorhizobium amorphae*

The species *Mesorhizobium amorphae* was described by Wang *et al.* (1999b). The rhizobial strains were isolated from Chinese soils associated with the legume, *Amorpha fruticosa*. This is a leguminous scrub native to South-Eastern and Mid-Western United States. The polyphasic approach used by Wang and colleagues (1999b) showed three groups within the *A. fruticosa* group, but only the most distinct group (group 1) was proposed as a new species. The researchers reported that the symbiotic genes reside on a plasmid and all the strains displayed the same *nifH* gene hybridisation pattern (Wang *et al.*, 1999).

3.5.7 *Mesorhizobium chacoense*

Velázquez *et al.* (2001) described the species *Mesorhizobium chacoense*. This species was proposed for rhizobia associated with *Prosopis alba* from the Chaco Arido region in Argentina. The rhizobia form effective nodules on *P. alba*, *P. chilensis* and *P. flexuosa*. The G + C content of the DNA is 62 mol% for the *M. chacoense* strains (Velázquez *et al.*, 2001).

3.6 The genus *Sinorhizobium*

Chen *et al.* (1988) described the genus *Sinorhizobium* for fast-growing soybean bacteria from China. The results of numerical taxonomy, DNA-DNA hybridisation, serological analysis data, G + C content, soluble protein patterns, bacteriophage typing and the composition of extracellular gum showed that the new genus differed adequately from *Bradyrhizobium*, *Rhizobium* and *Azorhizobium* to warrant the description of the new genus. Chen and colleagues (1988) included two species in the genus: *S. fredii*, previously *R. fredii* (Scholla and Elkan, 1984) and a new species, *S. xinjiangense*. The type species of the genus is *S. fredii* (Chen *et al.*, 1988; De Lajudie *et al.*, 1994).

The genus was emended by De Lajudie *et al.* (1994) to include *S. meliloti*, previously *R. meliloti* (Jordan, 1984) and two new species, *S. saheli* and *S. terangae* from *Sesbania* and *Acacia* spp. The emendation was based on a polyphasic study including: SDS-PAGE of cellular proteins, auxanographic tests, host specificity tests, DNA-DNA hybridisation, DNA-rRNA hybridisation and 16S rRNA gene sequencing (De Lajudie *et al.*, 1994). Rome *et al.* (1996b) described *S. medicae* isolated from annual *Medicago* spp. Nick *et al.* (1999)

described two new species, *S. arboris* and *S. kostiense* for isolates from *Acacia senegal* and *Prosopis chilensis*. *S. morelense* was described by Wang *et al.* (2002) for *Leucaena leucocephala*-associated isolates. Wei *et al.* (2002) described *S. kummerowiae* for isolates from *Kummerowia stipulacea*. Rhizobial isolates from indigenous Mexican *Acacia* spp. were described as a new species, *S. americanum* (Toledo *et al.*, 2003). Willems *et al.* (2003) reported the isolation of new *Ensifer adhaerens* strains from nodules. The isolates belonged to the *Sinorhizobium* genus based on 16S rDNA and *recA* gene sequence data. Based on an extensive study of the new *Ensifer* isolates an opinion was requested for the transferal of *Ensifer adhaerens* to the genus *Sinorhizobium* as *Sinorhizobium adhaerens* (Willems *et al.*, 2003).

Chen *et al.* (1988) described the G + C content of the genus as 59.9-63.8 mol%. The G + C content of the emended genus is 57-66 mol% (De Lajudie *et al.*, 1994). The G + C content still falls in the range of a well-defined genus, since the G + C values do not differ by more than 10% (Vandamme *et al.*, 1996).

3.6.1 *Sinorhizobium fredii*

Rhizobium fredii was described by Scholla and Elkan (1984) for the fast-growing rhizobia isolated from China that nodulate soybeans. The researchers proposed two chemovars for the two different subgroups differentiated. The chemovar formation was based on DNA-DNA hybridisation, acid production on yeast extract mannitol (YEM) broth, kanamycin resistance and serology. According to Scholla and Elkan (1984), the chemovars were *R. fredii* chemovar fredii and *R. fredii* chemovar siensis.

Strains of *Sinorhizobium fredii* nodulate *Cajanus cajan*, *Glycine max*, *Glycine soja* and *Vigna* effectively (Chen *et al.*, 1988). The strains form nodules on wildtype soybeans, while forming ineffective nodules on commercial soybean cultures (Jordan, 1984).

3.6.2 *Sinorhizobium meliloti*

Rhizobium meliloti was described by Dangeard in 1926 as quoted by Jordan (1984). The strains of the species form nodules on species of *Medicago* (alfalfa), *Melilotus* (sweet clover) and *Trigonella* (fenugreek) (Jordan, 1984). In a study conducted by Eardly *et al.* (1990) on

isolates from various geographical regions and species of *Medicago*, the researchers showed the presence of two phylogenetically different evolutionary lineages within *Sinorhizobium* (*Rhizobium*) *meliloti*. One of the lineages was adapted to the annual medic species of the Mediterranean basin, now known as *S. medicae*.

Rome *et al.* (1996b) revised the species, *S. meliloti*. Results obtained in a previous study by Rome *et al.* (1996a) confirmed the findings of Eardly *et al.* (1990). The strains which corresponded to *S. meliloti* were capable of nodule-formation on the more promiscuous annual *Medicago* species (Rome *et al.*, 1996b). In a study on the symbiotic and taxonomic diversity of rhizobia isolated from *Acacia tortilis* subsp. *raddiana* in Africa, Ba *et al.* (2002) proposed a new biovar namely, *S. meliloti* bv. acaciae based on the host range and Nod-factor structure of the isolates.

3.6.3 *Sinorhizobium xinjiangense*

Chen *et al.* (1988) found two distinct taxonomic groups in the 33 strains of fast-growing soybean rhizobia isolated from soil and soybean nodules collected in China. The strains were compared by numerical taxonomy of 240 different characters (Chen *et al.*, 1988). The type strains of each chemovar of *S. fredii* (Scholla and Elkan, 1984) clustered in the same group. The second group of fast-growing soybean strains differed from *S. fredii* and other known species and the strains of the group were assigned to a new species, *S. xinjiangense* (Chen *et al.*, 1988).

Strains of this species nodulate *Cajanus cajan*, *Glycine max*, *Glycine soja* and *Vigna unguiculata* effectively (Chen *et al.*, 1988). In the emended description of the genus *Sinorhizobium*, De Lajudie *et al.* (1994) questioned the taxonomic position and validity of the species. Peng *et al.* (2002) identified *S. xinjiangense* strains isolated from soybeans in the *Xinjiang* region (China) and differentiated *S. xinjiangense* from *S. fredii* using phylogenetic, genotypic and phenotypic data. The G + C content of the DNA is 60.1-60.9 mol% (Peng *et al.*, 2002).

3.6.4 *Sinorhizobium saheli*

The rhizobial strains were isolated from *Sesbania* species in the Sahel region in Africa. Strains from the species can form nodules on different *Sesbania* species, *Acacia seyal*, *Leucaena leucocephala* and *Neptunia oleracea* (De Lajudie *et al.*, 1994). The strains of the species are also capable of stem nodulation of *Sesbania rostrata*. However, when the roots of the plant are already nodulated, ineffective stem nodules are formed (Boivin *et al.*, 1997a). Haukka *et al.* (1998) proposed two biovars, *S. saheli* bv. acaciae and *S. saheli* bv. sesbaniae based on phylogenetic studies of the *nodA* gene. G + C DNA content is 65-66 mol% (De Lajudie *et al.*, 1994).

3.6.5 *Sinorhizobium terangae*

The species name, *teranga*, refers to the fact that the strains of the species were isolated from different host plants. The strains of the species can nodulate *Acacia*, *Leucaena leucocephala*, *Neptunia oleracea* and *Sesbania* spp. DNA G + C content of the strains is 60.8-61.6 mol% (De Lajudie *et al.*, 1994).

Lortet *et al.* (1996) divided the species into two biovars, *S. teranga* bv. sesbaniae (*Sesbania*-nodulating strains) and *S. teranga* bv. acaciae (*Acacia*-nodulating strains). The strains of *S. teranga* bv. sesbaniae is capable of nodulating the stems of *Sesbania rostrata* (Lortet *et al.*, 1996). The formation of the stem nodules is inhibited if root nodules already exist, unlike the strains of *Azorhizobium*, which are not negatively affected by the existence of root nodules (Boivin *et al.*, 1997a).

3.6.6 *Sinorhizobium medicae*

S. medicae was described by Rome *et al.* (1996b) for the strains previously included in the second genomic species of *S. meliloti*. DNA-DNA hybridisation results of Rome *et al.* (1996a) showed that the DNA homology between the genomic species II and *S. meliloti* strains was 42 to 60%.

The strains of *S. medicae* have different host ranges from that of *S. meliloti* (Rome *et al.*, 1996b). The strains are adapted to form symbiosis with certain *Medicago* species, especially

the annual medics of the Mediterranean basin. *S. medicae* strains nodulate their host plant with more specificity than *S. meliloti* strains. The strains effectively nodulate *M. polymorpha* (selective nodulating plant species) and the more promiscuous species *M. minima*, *M. orbicularis*, *M. rigidula*, *M. sativa* and *M. truncatula*, (Rome *et al.*, 1996b).

3.6.7 *Sinorhizobium arboris* and *Sinorhizobium kostiense*

Nick *et al.* (1999) described *Sinorhizobium arboris* for isolates from *Acacia senegal* and *Prosopis chilensis*. Phylogenetically, based on 16S rDNA sequence data *S. medicae* (99.4% similarity) and *S. meliloti* (99.4% similarity) were the neighbours of *S. arboris*. DNA G + C content is 60.6-61.8 mol% (Nick *et al.*, 1999).

The species *Sinorhizobium kostiense* was also described for isolates from *Acacia senegal* and *Prosopis chilensis* (Nick *et al.*, 1999). The two species *S. arboris* and *S. kostiense* were differentiated with a polyphasic approach. The different techniques employed corroborated each other and showed the need to describe new species. The G + C content of *S. kostiense* strains is 57.9-61.6 mol%. In the phylogenetic tree, *S. kostiense* formed a branch on its own (Nick *et al.*, 1999).

3.6.8 *Sinorhizobium morelense*

Sinorhizobium morelense was described by Wang *et al.* (2002) for *Leucaena leucocephala*-associated isolates, which lack symbiotic plasmids and are thus unable to nodulate. Phylogenetically, the closest relative of *S. morelense* is *Ensifer adhaerens* (*S. adhaerens*) based on 16S rDNA sequence data. The two species form the most divergent branch in the *Sinorhizobium* genus. DNA G + C content is 61.7 mol% (Wang *et al.*, 2002).

3.6.9 *Sinorhizobium kummerowiae*

In their study, Wei *et al.* (2002) isolated rhizobia from *Indigofera* spp. and *Kummerowia stipulacea* growing wild in north-western China. The isolates from *K. stipulacea* was described as the new species *S. kummerowiae*. This species can nodulate *Indigofera* spp.,

K. stipulacea and *Medicago sativa* under laboratory conditions. Based on 16S rDNA sequence data the closest phylogenetic relatives of *S. kummerowiae* are *S. fredii* and *S. xinjiangense*. The G + C content of the DNA is 59.1-61.8 mol% (Wei *et al.*, 2002).

3.6.10 *Sinorhizobium americanum*

Toledo and colleagues (2003) described the species *S. americanum* for isolates from Mexican *Acacia* spp. The closest relative of the species *S. americanum* is *S. fredii*, a species originally described for soybean isolates from China. Strains from the species can nodulate *Acacia* spp., *Leucaena leucocephala* and *Phaseolus vulgaris*. This species is however not included in the list of bacterial names with standing in nomenclature (http://www.bacterio.cict.fr).

3.7 Root-nodulating species from other genera

Methylobacterium nodulans isolated from *Crotalaria* spp. was the first report of species not known to nodulate in the α-*Proteobacteria* (Sy *et al.*, 2001). *Devosia neptuniae* is another novel species which nodulate legumes and fix nitrogen (Rivas *et al.*, 2003). Recently, Van Berkum and Eardly (2002) reported the effective nodulation of *Aeschynomene indica* by the budding bacterium *Blastobacter denitrificans*.

The first report on β-*Proteobacteria* species capable of legume nodulation was from Chen and colleagues (2001) who described the species *Ralstonia taiwanensis*. Moulin *et al.* (2001) first reported the existence of *Burkholderia* spp. which nodulate legumes effectively. Vandamme *et al.* (2002) proposed two new *Burkholderia* species capable of effectively nodulating legumes (Fig. 4). The researchers also reported the isolation of *B. caribiensis* strains from nodules of *Mimosa diplotricha* and *M. pudica* and *B. cepacia* genomovar VI strains from *Alysicarpus glumaceus* nodules (Vandamme *et al.*, 2002).

3.7.1 *Methylobacterium nodulans*

Methylobacterium nodulans was described for facultative methylotrophic unpigmented isolates from *Crotalaria* spp. (Sy *et al.*, 2001). The symbiosis between the bacteria and the legume species is very specific. Jaftha *et al.* (2002) reported pigmented methylotrophic bacteria isolated from *Lotononis bainesii* closely related to *Methylobacterium nodulans* (98%

16S rDNA sequence similarity). The DNA-DNA homology of the isolates still has to be performed to determine the species status of the *Lotononis bainesii* isolates (Jaftha *et al.*, 2002).

3.7.2 *Devosia neptuniae*

The new species, *Devosia neptuniae* was first reported by Rivas *et al.* (2002) for root-nodulating isolates from *Neptunia natans*. Phylogenetic analysis with 16S rDNA showed that these isolates grouped closest to the genus *Devosia* in the family *Hyphomicrobiaceae*. The *nodD* and *nifH* genes of the isolates are similar to that of *R. tropici*, which suggest that the symbiotic genes were transferred from *R. tropici* to *Devosia neptuniae* (Rivas *et al.*, 2002). Rivas *et al.* (2003) conducted a polyphasic approach to characterise the new species and to formally describe the new species, *Devosia neptuniae*. The G + C content of the species *Devosia neptuniae* is 62.0-62.4 mol% (Rivas *et al.*, 2003).

3.7.3 *Ralstonia taiwanensis*

The first β-*Proteobacteria* to be described as capable of forming nodules was *Ralstonia taiwanensis*. After a polyphasic analysis, Chen *et al.* (2001) described the novel *Ralstonia* species for bacteria isolated from *Mimosa* spp., as well as one isolate from the sputum of a cystic fibrosis patient. All isolates effectively nodulate *Mimosa diplotricha* and *Mimosa pudica*. DNA G + C content is 66.7-67.7 mol% (Chen *et al.*, 2001).

3.7.4 *Burkholderia phymatum* and *Burkholderia tuberum*

Burkholderia phymatum was proposed by Vandamme *et al.* (2002) for the *Machaerium lunatum* isolate (STM815) which Moulin *et al.* (2001) reported as capable of forming nodules. DNA G + C content is 62.1 mol%. Based on 16S rDNA sequence data *B. caribiensis* (98.6% sequence similarity) is the closest neighbour of *B. phymatum* (Vandamme *et al.*, 2002).

Vandamme *et al.* (2002) described *Burkholderia tuberum* for the *Aspalathus carnosa* isolate (STM678) first reported by Moulin *et al.* (2001) as root-nodulating. Phylogenetically, *B.*

kururiensis is closest (97.0% 16S rDNA sequence similarity) to *B. tuberum*. The G + C content of the DNA is 62.8 mol% (Vandamme *et al.*, 2002).

CHAPTER 6

DETERMINATION OF THE DIVERSITY OF ROOT-NODULATING BACTERIA ASSOCIATED WITH *CYCLOPIA* SPP.

ABSTRACT

The diversity of root-nodulating isolates associated with 14 different *Cyclopia* spp. isolated from different localities in the geographic distribution of the *Cyclopia* genus were determined using 16S-23S IGS-RFLP analysis. With the exception of seven isolates, all the isolates grouped distantly from the α-*Proteobacteria* rhizobial reference strains. Partial 16S rDNA sequencing was performed to identify and classify the isolates. The sequencing data confirmed and corroborated the RFLP analysis. All the isolates except the seven α-*Proteobacteria* isolates belonged to the genus *Burkholderia*. A large number of the isolates belonged to the recently described root-nodulating species, *B. tuberum*. Several new *Burkholderia* genotypes were detected. The α-*Proteobacteria* isolates belonged to the genus *Bradyrhizobium*, *Rhizobium tropici* and one isolate displayed a novel genotype.

Keywords: honeybush tea, 16S-23S IGS-RFLP, partial 16S rDNA sequencing, *Burkholderia*

INTRODUCTION

The symbiotic association between legumes and the gram-negative bacteria collectively called rhizobia is an agricultural important association. The bacteria form nodules on the roots of the legumes and as a special adaptation for waterlogged regions on the stems of the legumes. In mature nodules nitrogen fixation and ammonia assimilation occur (Caetano-Anollés, 1997).

The taxonomy of the root-nodulating bacteria changed rapidly the last years as new techniques are employed and more legumes studied. Jordan (1984) included all rhizobia in the family *Rhizobiaceae* in the α-2-subgroup of the *Proteobacteria*. On the website (http://www.cme.msu.edu/bergeys/outline.prn.pdf) of Bergey's Manual rhizobia are included in several different families. *Rhizobium*, *Allorhizobium* and *Sinorhizobium* are placed in the family *Rhizobiaceae*, while *Mesorhizobium* is grouped in the family "Phyllobacteriaceae". *Bradyrhizobium* is placed in the family "Bradyrhizobiaceae", while *Azorhizobium* and the genus *Devosia* in which a newly nodulating species [*Devosia neptuniae* (Rivas *et al.*, 2003)] have been described, belong to the family *Hyphomicrobiaceae*. The other nodulating species of the α-*Proteobacteria* belong to the genus *Methylobacterium* in the family "Methylobacteriaceae" (http://www.cme.msu.edu/bergeys/outline.prn.pdf).

Recently, it became clear that the ability to nodulate and fix nitrogen is not restricted to the α-*Proteobacteria*, but that several species in the β-*Proteobacteria* acquired the ability as well (Chen *et al.*, 2001; Moulin *et al.*, 2001; Vandamme *et al.*, 2002). The two genera involved *Burkholderia* and *Ralstonia* belong to the families "Burkholderiaceae" and "Ralstoniaceae" respectively in the order "Burkholderiales" (Bergey's manual taxonomic list: http://www.cme.msu.edu/bergeys/outline/prn.pdf).

A traditional South African herbal infusion, commonly referred to as honeybush tea is manufactured from the leaves, stems and flowers of mainly *Cyclopia intermedia* (Kouga bush tea) and *C. subternata* (synonym of *C. falcata*) (bush tea) (De Nysschen *et al.*, 1996). The commercial cultivation of several species (*C. intermedia*, *C. subternata*, *C. maculata*, *C.sessiliflora* and *C. genistoides*) is investigated to guard against the overexploitation of the natural populations (Du Toit and Joubert, 1998).

Cyclopia is a genus consisting of 19 accepted species (List of accepted names: http:/www.ildis.org/LegumeWeb/6.00/names/npall/npall_201.shtml) endemic to the fynbos region of South Africa. The genus *Cyclopia* (tribe Podalyrieae, subtribe Podalyriinae) belong to the "genistoid alliance" in the Papilionoideae (Schutte and Van Wyk, 1998), of which the members produce characteristic quinolizidine alkaloids (Polhill 1994; Van Wyk and Schutte, 1995, Van Wyk, 2003). The plants grow in the coastal regions of the Western and Eastern Cape Provinces, from Darling to Port Elizabeth, bounded on the north by the Cederberg, Koue Bokkeveld, Klein Swartberg, Groot Swartberg and Kouga mountain ranges. Most of the species have limited distribution ranges and special habitat preferences (Du Toit *et al.*, 1998).

There is a need for good inoculant strains for the *Cyclopia* commercial plantings. The study of the root-nodulating bacteria associated with different natural populations of *Cyclopia* species covering the geographical distribution of the genus provides a collection of possible inoculant strains and knowledge of the diversity of these symbionts. The strains might also be used for other crop plants grown in similar environmental conditions, since the strains are adapted to such conditions. Some of the strains have been isolated from soil with a pH as low as 3.1 (J. Bloem, personal communication).

The aim of this study was to identify and determine the diversity of the *Cyclopia* nodule isolates. In this study, 16S-23S IGS-RFLP was used to identify, type and differentiate between closely related strains. This method has been used by several researchers (Laguerre *et al.*, 1996; LeBlond-Bourget *et al.*, 1996; Khbaya *et al.*, 1998; Guo *et al.*, 1999; Doignon-Bourcier *et al.*, 2000; Grundmann *et al.*, 2000; Bala *et al.*, 2002). Partial 16S rDNA sequences was used to identify and determine the taxonomic position of the isolates. This method has been used by several researchers to determine and confirm the identity of new isolates (Lafay and Burdon, 1998; Vinuesa *et al.*, 1998; McInroy *et al.*, 1999; Van Berkum and Fuhrmann, 2000; Mehta and Rosato, 2001; Odee *et al.*, 2002; Qian *et al.*, 2003).

MATERIALS AND METHODS

Bacterial strains used

The strains used in this study were received from the Agricultural Research Council-Plant Protection Research Institute (Private Bag X134, Pretoria, 0001, South Africa) [Table 6.1] and the Botany Department (University of Cape Town, Rondebosch, 7701, Cape Town, South Africa) [Table 6.2]. All the strains received from the University of Cape Town were authenticated nodulating strains. A selection of the strains received from the ARC was used in plant nodulation studies and confirmed as root-nodulating. Reference strains of the different rhizobial genera used in this study were obtained from the culture collections of the Laboratorium voor Microbiologie (LMG), University of Gent, Gent, Belgium, the United States Department of Agriculture (USDA), *Rhizobium* Culture Collection, Maryland, USA and the Laboratoire des Symbioses Tropicales et Méditerranéennes (STM), Montpellier, France.

Maintenance of bacterial cultures

The isolates were maintained on yeast mannitol agar (YMA) [1% (m/v) mannitol (UniVar), 0.5% (m/v) K_2HPO_4 (Merck), 0.02% (m/v) $MgSO_4.7H_2O$ (Merck), 0.01% (m/v) NaCl (NT Chemicals), 0.04% (m/v) yeast extract (Biolab) and 1.5% (m/v) bacteriological agar (Biolab)] slants and the long-term storage of the isolates was done in glycerol. The isolates were grown in yeast mannitol broth (YMB) for 5-7 d at 25-28°C with vigorous shaking. The broth cultures were mixed 1:1 with sterile 50% (v/v) glycerol (Merck) in sterile cryotubes and stored in duplicate at -20°C and -70°C.

Extraction of genomic DNA

A modified method for proteinase-K (Roche Molecular Biochemicals) treated cells as described by Laguerre *et al.* (1997) was used. A fresh culture of each strain, which had been checked for purity, was streaked on a tryptone yeast (TY) agar slant [0.5% (m/v) tryptone (Difco), 0.3% (m/v) yeast extract (Biolab), 0.13% (m/v) $CaCl_2.6H_2O$ (UniLab), 1.5% (m/v) bacteriological agar] in a screw-cap tube. TY reduces slime formation by the rhizobia. The

strains were incubated at 28 °C and checked for sufficient growth. Sterile 4.5 ml dH_2O was added to the slant growth to harvest the cells. An inoculation loop was used to aid the release of cells clinging to the agar. The volume of the water added was adjusted according to the amount of growth. Less water was used if the growth was poor and *vice versa*. The cell-suspension was collected in a clear plastic tube and vortexed to ensure a uniform suspension. The absorbancy of the suspension was measured with dH_2O as the spectophotometric blank at 620 nm. A formula was used to determine the volume of the cellsuspension to be treated further. The volume to be used in ml is equal to 0.2 divided by the absorbancy at 620 nm. Two tubes of the same strain were filled with the appropriate volume of cells and centrifuged at 13 000 *g* for 5 minutes at 4 °C. The supernatant was discarded and the excess media blotted dry. One of the tubes was stored at -20 °C for future use. In the second tube, 100 µl ddH_2O, 100 µl Tris-HCl (10 mM, pH 8.2) and 10 µl proteinase-K (15 mg/ml) (Roche Molecular Biochemicals) were added to the cell pellet. The mixture was incubated at 55 °C overnight. In order to inactivate the proteinase-K the mixture was boiled for 10 minutes. The cell lysates were stored at -20 °C until needed.

Table 6.1: Authenticated root-nodulating isolates of indigenous *Cyclopia* species included in this study received from the ARC-PPRI.

Isolate	Host species	Locality	Isolate	Host species	Locality
CS 1	*C. subternata*	Dennehoek, Joubertina	**Cses 1**	*C. sessiliflora*	Plattekloof, Heidelberg
CS 2	*C. subternata*	Dennehoek, Joubertina	**Cses 2**	*C. sessiliflora*	Plattekloof, Heidelberg
CS 3	*C. subternata*	Dennehoek, Joubertina	**Cses 3**	*C. sessiliflora*	Plattekloof, Heidelberg
CS 5	*C. subternata*	Dennehoek, Joubertina	**Cses 4**	*C. sessiliflora*	Plattekloof, Heidelberg
CS 6	*C. subternata*	Dennehoek, Joubertina	**Cses 5**	*C. sessiliflora*	Plattekloof, Heidelberg
CS 7	*C. subternata*	Dennehoek, Joubertina	**Cses 6**	*C. sessiliflora*	Plattekloof, Heidelberg
CI 1	*C. intermedia*	Dennehoek, Joubertina	**Cses 7**	*C. sessiliflora*	Plattekloof, Heidelberg
CI 2	*C. intermedia*	Dennehoek, Joubertina	**CF 1**	*C. falcata*	Large Winterhoek mountain Porterville
CI 2b	*C. intermedia*	Dennehoek, Joubertina	**CG 1**	*C. genistoides*	Silwerstroomstrand, Darlin
CI 3	*C. intermedia*	Dennehoek, Joubertina	**CG 4**	*C. genistoides*	Rondeberg, Darling
CI 4b	*C. intermedia*	Dennehoek, Joubertina	**Clong 1**	*C. longifolia*	Thornhill, Humansdorp
CI 6	*C. intermedia*	Onverwacht, Garcia Pass	**Clong 2**	*C. longifolia*	Thornhill, Humansdorp
CI 9	*C. intermedia*	Onverwacht, Garcia Pass	**Clong 3**	*C. longifolia*	Thornhill, Humansdorp
Cint S2*	*C. subternata* #	Dennehoek, Joubertina	**Clong 4**	*C. longifolia*	Thornhill, Humansdorp
Cint I1*	*C. intermedia* #	Dennehoek, Joubertina	**Clong 5**	*C. longifolia*	Thornhill, Humansdorp
Cint I2*	*C. intermedia* #	Dennehoek, Joubertina	**CM 1**	*C. maculata*	Paarlberg, Paarl
Cint I4*	*C. intermedia* #	Dennehoek, Joubertina	**CM 2**	*C. maculata*	Garcia Pass, Riversdal
Csub I1**	*C. intermedia* #	Dennehoek, Joubertina	**CM 3**	*C. maculata*	Garcia Pass, Riversdal
Csub I5**	*C. intermedia* #	unknown	**CB 2**	*C. buxifolia*	Helderberg, Somerset-Wes
Csub S1**	*C. subternata* #	Dennehoek, Joubertina	**CD 1**	*C. dregeana*	Du Toitskloof, Paarl
Csub S3**	*C. subternata* #	Dennehoek, Joubertina	**CD 4**	*C. dregeana*	Du Toitskloof, Paarl
Cses I1***	*C. intermedia* #	Dennehoek, Joubertina	**CD 9**	*C. dregeana*	Du Toitskloof, Paarl
Cses I2***	*C. intermedia* #	Dennehoek, Joubertina	**CD 10**	*C. dregeana*	Du Toitskloof, Paarl

Table 6.1: continued

Isolate	Host species	Locality	Isolate	Host species	Locality
Cses S1gr.***	*C. subternata* #	Dennehoek, Joubertina	**CD 11**	*C. dregeana*	Du Toitskloof, Paarl
Cses S1kl.***	*C. subternata* #	Dennehoek, Joubertina	**CD 12a**	*C. dregeana*	Du Toitskloof, Paarl
Cses S2gr.***	*C. subternata* #	Dennehoek, Joubertina	**CD 13**	*C. dregeana*	Du Toitskloof, Paarl
Cses S2kl.***	*C. subternata* #	Dennehoek, Joubertina	**Cpub 4**	*C. pubescens*	Next to N1, Port Elizabeth
Cses S3***	*C. subternata* #	Dennehoek, Joubertina	**Cpub 5**	*C. pubescens*	Next to N1, Port Elizabeth
Cses S7***	*C. subternata* #	Dennehoek, Joubertina	**Cpub 6**	*C. pubescens*	Next to N1, Port Elizabeth
Cmey 1	*C. meyeriana*	Kunje, Citrusdal	**Cplic 1**	*C. plicata*	Mannetjiesberg, Uniondale

* Re-isolated from plant inoculation test performed on *C. intermedia*

** Re-isolated from plant inoculation test performed on *C. subternata*

*** Re-isolated from plant inoculation test performed on *C. sessiliflora*

Original host plant of strain

Table 6.2: Authenticated root-nodulating strains isolated from indigenous *Cyclopia* species received from the Botany Department (UCT) and included in this study.

Isolate	Host species	Locality	Isolate	Host species	Locality
UCT 2	*C. genistoides*	Rein's Farms	**UCT 35**	*C. glabra*	Matroosberg
UCT 3	*C. genistoides*	Rein's Farms	**UCT 36**	*C. galioides*	Cape Point
UCT 4	*C. genistoides*	Rein's Farms	**UCT 37**	*C. galioides*	Cape Point
UCT 5	*C. genistoides*	Pearly Beach	**UCT 38**	*C. galioides*	Cape Point
UCT 6	*C. genistoides*	Pearly Beach	**UCT 39**	*C. galioides*	Cape Point
UCT 7	*C. genistoides*	Pearly Beach	**UCT 40**	*C. galioides*	Cape Point
UCT 8	*C. genistoides*	Betty's Bay	**UCT 41**	*C. plicata*	Kougaberg
UCT 9	*C. genistoides*	Betty's Bay	**UCT 42**	*C. plicata*	Kougaberg
UCT 10	*C. genistoides*	Rondeberg	**UCT 43**	*C. meyeriana*	Hottentots Holland mountains
UCT 11	*C. genistoides*	Rondeberg	**UCT 44**	*C. meyeriana*	Hottentots Holland mountains
UCT 13	*C. genistoides*	Rondeberg	**UCT 45**	*C. meyeriana*	Bains Kloof
UCT 14	*C. genistoides*	Rondeberg	**UCT 46**	*C. meyeriana*	Hottentots Holland mountains
UCT 15	*C. genistoides*	Constantiaberg	**UCT 47**	*C. glabra*	unknown
UCT 16	*C. genistoides*	Constantiaberg	**UCT 48**	*C. maculata*	Jonkershoek
UCT 17	*C. genistoides*	Constantiaberg	**UCT 49**	*C. genistoides*	Constantiaberg
UCT 18	*C. genistoides*	Constantiaberg	**UCT 50**	*C. sessiliflora*	Callie's farm, Heidelberg
UCT 19	*C. genistoides*	Constantiaberg	**UCT 52**	*C. plicata*	unknown
UCT 20	*C. genistoides*	Paardeberg	**UCT 53**	*C. plicata*	unknown
UCT 21	*C. genistoides*	Paardeberg	**UCT 55**	*C. plicata*	unknown
UCT 22	*C. maculata*	Jonkershoek	**UCT 56**	*C. meyeriana*	Hottentots Holland mountains
UCT 24	*C. maculata*	Jonkershoek	**UCT 57**	*C. subternata*	Port Alfred Pass
UCT 25	*C. intermedia*	Swartberg Pass	**UCT 58a**	*C. subternata*	Port Alfred Pass
UCT 26	*C. intermedia*	Swartberg Pass	**UCT 60**	*C. meyeriana*	Bains Kloof
UCT 27bii	*C. subternata*	Waboomskraal farm (wild tea)	**UCT 61**	*C. subternata*	Garcia Pass, Riversdal
UCT 28	*C. subternata*	Waboomskraal farm (wild tea)	**UCT 62**	*C. genistoides*	Pearly Beach

Table 6.2: continued

Isolate	Host species	Locality	Isolate	Host species	Locality
UCT 29	*C. sessiliflora*	Callie's farm, Heidelberg	**UCT 63**	*C. genistoides*	Betty's Bay
UCT 30	*C. sessiliflora*	Callie's farm, Heidelberg	**UCT 67**	*C. glabra*	unknown
UCT 31	*C. sessiliflora*	Grootvadersbosch	**UCT 69**	*C. glabra*	unknown
UCT 32	*C. buxifolia*	McGregor	**UCT 70**	*C. maculata*	Jonkershoek
UCT 33	*C. buxifolia*	McGregor	**UCT 71**	*C. glabra*	unknown
UCT 34	*C. glabra*	Matroosberg	**UCT 73**	*C. genistoides*	Betty's Bay

Table 6.3: Reference strains included in the IGS RFLP study

Reference strain	Strain number	Reference strain	Strain number
A. caulinodans	LMG 6465^T	***M. huakuii***	USDA 4779^T
A. caulinodans	USDA 4892^T	***M. huakuii***	LMG 14107^T
B. elkanii	USDA 76^T	***R. etli***	LMG 17827^T
B. japonicum	LMG 6138^T	***R. galegae***	LMG 6214^T
B. japonicum	USDA 6^T	***R. tropici***	LMG 9503^T
***Bradyrhizobium* sp.**	LMG 8319	***S. medicae***	LMG 18864^T
Burkholderia phymatum	STM 815^T	***S. meliloti***	LMG 6133^T
Burkholderia tuberum	STM 678^T	***S. saheli***	USDA 4893^T
M. ciceri	LMG 14989^T		

^{T} Type strain

Amplification of the 16S-23S IGS region and 16S rDNA gene

The 16S-23S IGS regions of the different strains including the reference strains (Table 6.3) were amplified with the primers FGPS1490 and FGPS132 (Table 6.4) as described by Laguerre *et al.* (1996). In a 50 µl PCR reaction mix the following were added: 5 µl of the cell lysate, 50 pmol of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Supertherm Taq DNA polymerase (Southern Cross Biotechnology). The amplification reaction was done in a Perkin Elmer GeneAmp PCR System 2400 thermocycler with the following profile: an initial 3 minutes of denaturation at 95 °C, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 1 minute followed by a final extension step at 72 °C for 5 minutes. Aliquots (5 µl) of the PCR reactions were examined to determine the size, purity and concentration of the products with horizontal agarose electrophoresis (Sambrook *et al.*, 1989) using 0.9% (m/v) agarose gels (Promega) in a 1 x TAE buffer (40 mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA pH 8.5) stained with ethidium bromide (10 mg/ml). IGS PCR products were visualised by UV fluorescence (results

not shown). The standard marker 1 Kb PLUS DNA Ladder (GibcoBRL®) was included on each gel.

Amplification of the 16S rDNA gene of selected strains (Table 5) were performed with the primers fD1 and rP2 (Table 6.4) as described by Weisburg *et al.* (1991). The linker sequences of the primers were not included in the primer synthesis, since cloning of the products was not anticipated. These shorter primers were thus designated fD1SHRT and rP2SHRT. The PCR mixture of each strain contained: 5 µl of the cell lysate, 50 pmol of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Gold Taq DNA polymerase (Southern Cross Biotechnology) in a 50 µl reaction volume. The PCR reactions were done on a Perkin Elmer GeneAmp PCR System 2400 thermocycler using the same thermal profile as used in the amplification of the IGS region. The concentration, purity and size of the products were evaluated by running an aliquot (5 µl) of each reaction on 0.9% (m/v) horizontal agarose gels (Promega) (results not shown). The standard marker, molecular marker VI (Roche Molecular Biochemicals), was included on each gel.

Table 6.4: Primers used in the amplification and/or sequencing of the different genes analysed in this study.

Primer name*	Primer sequence (5'-3')	Target gene	Reference
FGPS1490	5'-TGCGGCTGGATCACCTCCTT-3'	IGS	Laguerre *et al.*, 1996
FGPS132	5'-CCGGGTTTCCCCATTCGG-3'	IGS	Laguerre *et al.*, 1996
fD1SHRT	5'-AGAGTTTGATCCTGGCTCAG-3'	16S rDNA	Weisburg *et al.*, 1991
rP2SHRT	5'-ACGGCTACCTTGTTACGACTT-3'	16S rDNA	Weisburg *et al.*, 1991
16SRNAII-S	5'-GTGTAGCGGTGAAATGCGTAG-3'	16S rDNA	Kuhnert *et al.*, 1996

* All the primers were synthesised by Roche Molecular Biochemicals, Mannheim, Germany

The 16S PCR product of each strain was purified, since any traces of unincorporated dNTPs, primers, etc. can negatively influence the 16S sequencing reaction. The products were purified using a Qiagen QIAquick PCR Purification kit (Southern Cross Biotechnology). Purification reactions were done as prescribed by the manufacturer. The concentration and purity of each purification reaction was verified visually. An aliquot (1 µl) of each purified 16S PCR product was run on 0.9% (m/v) horizontal agarose gels (Promega) (results not shown). On each gel, a standard marker, molecular marker VI (Roche Molecular Biochemicals) was included.

16S-23S IGS-RFLP

The IGS PCR products without prior purification were restricted with four tetrameric restriction enzymes. The enzymes *Alu*I, *Cfo*I, *Hae*III and *Msp*I (Roche Molecular Biochemicals) were each used to digest an aliquot of the products. In each 10 µl reaction volume the following was added: 5 µl of the PCR product, 5 U of the enzyme as well as the optimal restriction buffer for each enzyme as prescribed by the manufacturer. The reactions were incubated overnight at 37 °C. The restricted products were analysed on a 3.5% (m/v) horizontal agarose gel in a Hybaid Maxi Gel System for 180 minutes at 80V. Molecular weight marker VIII (Roche Molecular Biochemicals) was loaded as a standard in specified lanes on each gel. All the profiles were analysed visually, grouping isolates with similar bands in the same enzyme profile type. Additionally the gel-files were analysed with GelcomparII (Applied Maths, Kortrijk, Belgium) using the molecular weight marker VIII as the standard lane. The Dice coefficient (Nei and Lei, 1979) was used to calculate a distance matrix for each enzyme and the unweighted pair group method with arithmetic mean (UPGMA) was used to construct a dendrogram. The data of all four enzymes were combined with GelcomparII (Applied Maths, Kortrijk, Belgium) as described by the manufacturer and presented as a UPGMA constructed dendrogram.

16S rDNA sequencing

The partial sequence of each purified amplified 16S rDNA product of the chosen isolates were determined with the internal forward primer 16SRNAII-S (Kuhnert *et al.*, 1996) and the reverse primer rP2SHRT (Weisburg *et al.*, 1991) using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (AmpliTaq® DNA Polymerase, FS) (PE Applied Biosystems). Each 5 µl sequencing reaction contained the following: 2 µl of the ready reaction mix supplied with the kit which contains the dye terminators, dNTP's, AmpliTaq® DNA polymerase, $MgCl_2$ and Tris-HCl buffer pH 9.0; 12.5 pmol primer and approximately 100 ng template DNA. The sequencing reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler with the following thermal profile: an initial denaturation at 96 °C for 5 seconds followed by 25 cycles of denaturation (96 °C for 10 seconds), annealing (50 °C for 5 seconds) and extension (60 °C for 4 minutes). The products were precipitated using the protocol as suggested by the manufacturer. The reaction tubes

were placed on ice, while 4 µl sterile ddH_2O and 16 µl ice cold absolute ethanol were added to the sequencing reaction mix. The tubes were vortexed briefly and placed in the dark for 30 minutes to aid the precipitation of the sequencing products. This was followed by centrifugation at maximum speed for 30 minutes. The supernatant was discarded and any excess moisture was removed. Washing of the pellet was done with 50 µl ice cold 70% (v/v) ethanol. The tubes were centrifuged for 5 minutes at maximum speed. After discarding the supernatant and removing any excess moisture the pellets was vacuum dried for 15 minutes. The tubes were then stored at -20 °C until used. For analysis, the purified products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer (Perkin Elmer Applied Biosystems). The loading buffer was prepared by combining deionised formamide and 25 mM EDTA (pH 8.0) containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran. The resuspended products were denatured for 2 min at 90°C and loaded onto the ABI Prism model 377 DNA sequencer gel.

Phylogenetic analysis of the 16S rDNA sequences

The sequencing gels were analysed and sequences edited with the ABI Prism Sequencing Analysis 3.1 and the ABI Prism Sequencing Navigator 1.0.1 computer programmes (Perkin Elmer Applied Biosystems). Both strands were sequenced with the primers used and the strands could be aligned to correct ambiguous positions. The ClustalX programme (Thompson *et al.*, 1997) was used to analyse the edited sequences as well as the reference sequences obtained from GenBank (Table 6.5). A distance matrix was constructed by pair-wise alignment of the sequences. The neighbour-joining method (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the distance matrix. Branch lengths were proportional to the estimated divergence along each branch. Confidence levels of the phylogenies were estimated with the bootstrap method (Felsenstein, 1985). The phylogenetic tree was visualised with NJplot (Perrière and Gouy, 1996).

Table 6.5: Reference sequences obtained from Genbank[1] included in the partial 16S rDNA sequence analysis.

Reference strain	Strain number	Host plant or relevant characteristics	Genbank[1] Accession number
Agrobacterium rhizogenes	LMG 152	NS	X67224
Allorhizobium undicola	LMG 11875^T	*Neptunia natans*	Y17047
Azorhizobium caulinodans	LMG 6465^T	*Sesbania rostrata*	X67221
Bradyrhizobium elkanii	USDA 76^T	*Glycine max*	U35000
Bradyrhizobium japonicum	LMG 6138^T	*Glycine max*	X66024
Bradyrhizobium liaoningense	LMG 18230^T	*Glycine max*	AJ250813
Bradyrhizobium yuanmingense	CCBAU 10071^T	*Lespedeza cuneata*	AF193818
Burkholderia ambifaria	MVPC 1/4	*B. cepacia* complex	AY028444
Burkholderia andropogonis	ATCC 23061^T	Sorghum (*Sorghum bicolor)*	X67037
Burkholderia anthina	R-4183^T	Rhizosphere soil, *B. cepacia* complex	AJ420880
Burkholderia brasilensis	M130	Plant-associated N_2-fixer	AJ238360
Burkholderia caledonica	LMG 19076^T	Rhizosphere soil	AF215704
Burkholderia caribiensis	LMG 18531^T	Vertisol microaggregates	Y17009
Burkholderia caryophylli	ATCC 25418^T	Carnation (*Dianthus caryophyllus)*	AB021423
Burkholderia cepacia	LMG 12615	Cystic fibrosis sputum, plant associated genomovar III	AF265235
Burkholderia fungorum	LMG 16225^T	*Phanerochaete chrysosporium*	AF215705
Burkholderia gladioli	LMG 11626	Fermented coconut	U96934
Burkholderia glathei	LMG 14190^T	Fossil lateritic soil	U96935
Burkholderia glumae	LMG 2196^T	Rice (*Oryza sativa)*	U96931
Burkholderia graminis	AUS 35	Rhizosphere	U96941
Burkholderia kururiensis	LMG 19447^T	Trichloroethylene degrader	AB024310
Burkholderia mallei	NCTC 10260	NS	AF110187
Burkholderia multivorans	LMG 13010^T	*B. cepacia* complex	Y18703
Burkholderia phenazinium	LMG 2247^T	Soil enriched with threonine	U96936
Burkholderia phymatum	STM 815^T	*Machaerium lunatum*	AJ302312
Burkholderia plantarii	LMG 9035^T	*Oryza sativa* pathogen	U96933
Burkholderia pseudomallei	V686	Soil	AF093052
Burkholderia pyrrocinia	LMG 14191^T	NS	U96930
Burkholderia sacchari	LMG 19450^T	Soil from sugarcane plantation	AF263278
Burkholderia sordicola	SNU 020123	Associated with white rot fungus *Phanerochaete sordicola*	AF512827
Burkholderia stabilis	LMG 14294^T	Formerly *B. cepacia* complex IV	AF148554
Burkholderia thailandensis	E264^T	*Pseudomallei* group	U91838
Burkholderia tropicalis	Ppe8^T	Plant-associated N_2-fixer	AJ420332
Burkholderia tuberum	STM 678^T	*Aspalathus carnosa*	AJ302311
Burkholderia ubonensis	GTC-P3-415	NS	AB030584
Burkholderia vietnamensis	LMG 10929^T	N_2-fixer from rice rhizophere	AF097534
Devosia neptuniae	J1^T	*Neptunia natans*	AF469072
Ensifer adhaerens	LMG 20582	NS	AY040360
Kaistia adipata	Chj 404^T	Rhizobiaceae group	AY039817
Mesorhizobium amorphae	ACCC 19665	*Amorpha fruticosa*	AF041442
Mesorhizobium chacoense	PR-5^T	*Prosopis alba*	AJ278249
Mesorhizobium ciceri	UPM-Ca7^T	*Cicer arietinum*	U07934
Mesorhizobium huakuii	IAM 14158^T	*Astragalus sinicus*	D12797
Mesorhizobium loti	LMG 6125^T	*Lotus corniculatus*	X67229
Mesorhizobium mediterraneum	UPM-Ca36^T	*Cicer arietinum*	L38825
Mesorhizobium plurifarium	LMG 11892^T	*Acacia senegal*	Y14158
Mesorhizobium tianshanense	A-1BS^T	*Glycyrrhiza pallidiflora*	Y71079
Methylobacterium nodulans	ORS 2060^T	*Crotalaria podocarpa*	AF220763

Table 6.5: continued

Reference strain	Strain number	Host plant or relevant characteristics	Genbank[1] Accession number
Pandoraea norimbergensis	NS	Alkaliphilic sulphur oxidiser	Y09879
Ralstonia picketti	MSP 3	Rhizosphere, soil	AB004790
Ralstonia solanacearum	ATCC 11696	*Lycopersicon lycopersicum*	X67036
Ralstonia taiwanensis	LMG 19424^T	*Mimosa pudica*	AF300324
Rhizobium etli	CFN 42^T	*Phaseolus vulgaris*	U28916
Rhizobium galegae	USDA 3394	*Galega officinalis*	AF025853
Rhizobium gallicum	R602sp^T	*Phaseolus vulgaris*	U86343
Rhizobium giardinii	H152^T	*Phaseolus vulgaris*	U86344
Rhizobium hainanensis	I66^T	*Desmodium sinuatum*	U71078
Rhizobium huautlense	USDA 4900^T	*Sesbania herbacae*	AF025852
Rhizobium indigoferae	CCBAU 71042^T	*Indigofera amblyantha*	AY034027
Rhizobium leguminosarum	LMG 8820	*Phaseolus vulgaris*	X67227
Rhizobium mongolense	USDA 1844^T	*Medicago ruthenica*	U89817
Rhizobium sullae	IS123^T	*Hedysarum coronarium*	Y10170
Rhizobium tropici	CIAT 899^T	*Phaseolus vulgaris*	U89832
Rhizobium yanglingense	CCBAU 71462	*Coronilla varia*	AF195031
Sinorhizobium arboris	HAMBI 1552^T	*Prosopis chilensis*	Z78204
Sinorhizobium fredii	LMG 6217^T	*Glycine max*	X67231
Sinorhizobium kostiense	HAMBI 1489^T	*Acacia senegal*	Z78203
Sinorhizobium kummerowiae	CCBAU 71714^T	*Kummerowia stipulacea*	AY034028
Sinorhizobium medicae	A321^T	*Medicago truncatula*	L39882
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*	X67222
Sinorhizobium morelense	Lc04^T	*Leucaena leucocephala*	AY024335
Sinorhizobium saheli	LMG 7837^T	*Sesbania pachycarpa*	X68390
Sinorhizobium terangae	LMG 6463	*Sesbania rostrata*	X68387
Sinorhizobium xinjiangensis	IAM 14142	*Glycine max*	D12796

[1] Genbank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi.nlm.nih.gov/Genbank/]

T Type strain

ACCC Agricultural Center of Culture Collection, Chinese Academy of Agriculture, Beijing, China

ATCC American Type Culture Collection, Rockville, Maryland, USA

CCBAU Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China

CFN Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico

CIAT Rhizobium Collection, Centro International de Agricultura Tropical, Cali, Colombia

DSM Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany

HAMBI Culture Collection of the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland

IAM Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan

IFO Institute for Fermentation, Osaka, Japan

LMG BCCM™/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium

NCIMB National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, UK

NCPPB National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Hertfordshire, UK

NCTC	National Collection of Type Cultures, Central Public Health Laboratory, London, UK
ORS	ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal
STM	Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France
UPM	Universidad Politécnica Madrid, Spain
USDA	United States Department of Agriculture, *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, MD, USA

RESULTS

IGS PCR

The IGS products of the isolates were successfully amplified with the primers FGPS1490 and FGPS132 as used by Laguerre *et al.* (1997) [results not shown]. Amplification products of the *Cyclopia* isolates varied in the range of approximately 700 bp to 1250 bp. The isolates in cluster 2A and 2B (see Fig. 6.1) had amplification products of approximately 730-780 bp. The amplification products of the isolates of cluster 2C are larger and range from 820 to 860 bp. All the IGS products of the isolates in cluster 4A and 4B have bands in the range of 710-780 bp. For the isolates in cluster 4C two additional less prominent bands of approximately 700 bp and 600 bp were amplified in addition to the approximately 800 bp prominent band. However, isolate UCT 30 which groups into cluster 4C has only the approximately 800 bp band. Isolates of cluster 4D have a band in the range of 800 bp. The IGS products of the isolates: UCT 42 (~ 970 bp), UCT 50 (~ 1250 bp), UCT 53 (~ 900 bp), UCT 55 (~ 970 bp), Cmey 1 (~950 bp), Cplic 1(~ 1250bp) and Cpub 4 (~ 950 bp) which group into cluster 1 were significantly larger in comparison with the *Cyclopia* isolates of cluster 2 and cluster 4.

The reference strains included in this study gave different size products. The root-nodulating *Burkholderia* species, *B. tuberum* STM 678^T and *B. phymatum* STM 815^T gave IGS products of approximately 750 bp. The IGS products of the *Azorhizobium caulinodans*, *Bradyrhizobium* and the *Mesorhizobium huakuii* reference strains were in the range of approximately 900 bp. In the *M. ciceri* strain a larger product than that of *M. huakuii* in the magnitude of 1000 bp was amplified. The *Rhizobium* and *Sinorhizobium* species strains included in the study gave larger products than the other rhizobial strains. *R. etli*, *R. galegae*, *S. medicae*, *S. meliloti* and *S. saheli* strains gave an IGS product in the range of approximately

1250 bp. The IGS product of *R. tropici* LMG 9503^T was approximately 100 bp smaller than that of the other *Rhizobium-Sinorhizobium* strains.

IGS-RFLP

The enzymes *Alu*I, *Cfo*I, *Hae*III and *Msp*I generated 17, 20, 9 and 22 different restriction profiles respectively, excluding the profiles generated for the rhizobial reference strains. The discrimination level of *Hae*III was the lowest. Four clusters could be distinguished on the dendrogram constructed from the combined profiles of the four restriction enzymes (Fig. 6.1). All the root-nodulating α-*Proteobacteria* reference strains included in this study grouped in cluster 1, while cluster 2 contained most of the *Cyclopia* isolates and *Burkholderia tuberum* STM 678^T. *Burkholderia phymatum* STM 815^T was the only isolate of cluster 3. No reference strains clustered in cluster 4. Cluster 1, which contained the different rhizobial genera, is the most heterogeneous collection of strains. Cluster 2 and cluster 4 are relatively homogeneous clusters, sharing similar restriction profiles for specific enzymes.

Table 6.6: Restriction enzyme (*Alu*I, *Cfo*I, *Hae*III and *Msp*I) profiles of the amplified intergenic spacer regions of the *Cyclopia* isolates and the reference strains included in the IGS RFLP study.

Isolate number	Host plant / species designation	*Alu*I	*Cfo*I	*Hae*III	*Msp*I
CS 1	*C. subternata*	b	g	NS	k
CS 2	*C. subternata*	b	g	NS	k
CS 3	*C. subternata*	f	j	a	h
CS 5	*C. subternata*	b	g	NS	k
CS 6	*C. subternata*	b	h	NS	NS
CS 7	*C. subternata*	b	g	NS	k
CI 1	*C. intermedia*	c	a	NS	NS
CI 2	*C. intermedia*	f	j	a	h
CI 2b	*C. intermedia*	b	g	NS	k
CI 3	*C. intermedia*	f	i	a	a
CI 4b	*C. intermedia*	e	k	b	l
CI 6	*C. intermedia*	c	a	NS	NS
CI 9	*C. intermedia*	f	i	a	a
Cint S2	*C. subternata*	b	g	NS	k
Cint I1	*C. intermedia*	f	i	a	a
Cint I2	*C. intermedia*	f	j	a	a
Cint I4	*C. intermedia*	e	k	b	l
Csub I1	*C. intermedia*	f	i	a	a
Csub S1	*C. subternata*	b	g	NS	k
Csub S3	*C. subternata*	b	g	NS	k
Cses 1	*C. sessiliflora*	f	i	a	a
Cses 2	*C. sessiliflora*	b	b	NS	j

Table 6.6: continued

Isolate number	Host plant / species designation	*Alu*I	*Cfo*I	*Hae*III	*Msp*I
Cses 3	*C. sessiliflora*	f	i	a	a
Cses 4	*C. sessiliflora*	b	b	NS	j
Cses 5	*C. sessiliflora*	b	b	NS	j
Cses 6	*C. sessiliflora*	b	b	NS	j
Cses 7	*C. sessiliflora*	b	b	NS	j
Cses I1	*C. intermedia*	f	i	a	a
Cses I2	*C. intermedia*	f	j	a	a
Cses S1gr.	*C. subternata*	f	j	a	a
Cses S1kl.	*C. subternata*	b	g	NS	k
Cses S2gr.	*C. subternata*	b	g	NS	k
Cses S2kl.	*C. subternata*	b	g	NS	k
Cses S3	*C. subternata*	c	a	NS	NS
Cses S7	*C. subternata*	b	k	b	l
CF 1	*C. falcata*	b	g	NS	k
CG 1	*C. genistoides*	f	j	a	m
CG 4	*C. genistoides*	f	e	a	m
Clong 1	*C. longifolia*	c	a	NS	NS
Clong 2	*C. longifolia*	c	a	NS	NS
Clong 3	*C. longifolia*	f	j	a	a
Clong 4	*C. longifolia*	c	a	NS	NS
Clong 5	*C. longifolia*	c	a	NS	NS
CM 1	*C. maculata*	a	a	NS	NS
CM 2	*C. maculata*	c	a	NS	NS
CM 3	*C. maculata*	c	a	NS	NS
CB 2	*C. buxifolia*	q	a	NS	NS
CD 1	*C. dregeana*	e	k	d	l
CD 4	*C. dregeana*	e	k	d	l
CD 9	*C. dregeana*	b	s	NS	NS
CD 10	*C. dregeana*	e	l	c	n
CD 11	*C. dregeana*	e	l	a	n
CD 12a	*C. dregeana*	e	l	a	n
CD 13	*C. dregeana*	c	a	NS	b
Cpub 4	*C. pubescens*	i	m	h	r
Cpub 5	*C. pubescens*	f	n	a	m
Cpub 6	*C. pubescens*	f	i	a	m
Cmey 1	*C. meyeriana*	n	c	g	q
Cplic 1	*C. plicata*	j	o	f	s
Csub I5	*C. intermedia*	f	j	a	a
UCT 2	*C. genistoides*	f	e	a	m
UCT 3	*C. genistoides*	f	j	a	m
UCT 4	*C. genistoides*	f	j	a	d
UCT 5	*C. genistoides*	f	e	a	e
UCT 6	*C. genistoides*	f	j	a	e'
UCT 7	*C. genistoides*	f	j	a	e
UCT 8	*C. genistoides*	f	e	a	c
UCT 9	*C. genistoides*	f	j	a	c
UCT 10	*C. genistoides*	f	j	a	c
UCT 11	*C. genistoides*	f	a	a	m
UCT 13	*C. genistoides*	f	a	a	m
UCT 14	*C. genistoides*	f	a	a	m
UCT 15	*C. genistoides*	f	j	a	h
UCT 16	*C. genistoides*	f	j	a	h
UCT 17	*C. genistoides*	f	j	a	h
UCT 18	*C. genistoides*	f	j	a	h

Table 6.6: continued

Isolate number	Host plant / species designation	*Alu*I	*Cfo*I	*Hae*III	*Msp*I
UCT 19	*C. genistoides*	f	j	a	d
UCT 20	*C. genistoides*	d	a	NS	NS
UCT 21	*C. genistoides*	d	a	NS	NS
UCT 22	*C. maculata*	p	a	NS	NS
UCT 24	*C. maculata*	f	j	a	i
UCT 25	*C. intermedia*	f	i	a	a
UCT 26	*C. intermedia*	e	l	c	n
UCT 27bii	*C. subternata*	f	i	a	a
UCT 28	*C. subternata*	g	t	a	o
UCT 29	*C. sessiliflora*	b	k	a	p
UCT 30	*C. sessiliflora*	k	p	NS	t
UCT 31	*C. sessiliflora*	f	j	a	h
UCT 32	*C. buxifolia*	f	i	a	a
UCT 33	*C. buxifolia*	f	i	a	a
UCT 34	*C. glabra*	a	a	NS	b
UCT 35	*C. glabra*	a	q	NS	NS
UCT 36	*C. galioides*	f	e	a	d
UCT 37	*C. galioides*	f	j	a	c
UCT 38	*C. galioides*	f	j	a	e
UCT 39	*C. galioides*	f	j	a	e
UCT 40	*C. galioides*	f	j	a	e′
UCT 41	*C. plicata*	b	l	NS	NS
UCT 42	*C. plicata*	m	r	g	f
UCT 43	*C. meyeriana*	a	a	NS	b
UCT 44	*C. meyeriana*	a	a	NS	b
UCT 45	*C. meyeriana*	a	a	NS	b
UCT 46	*C. meyeriana*	a	a	NS	b
UCT 47	*C. glabra*	b	a	NS	b
UCT 48	*C. maculata*	a	a	NS	NS
UCT 49	*C. genistoides*	f	j	a	h
UCT 50	*C.sessiliflora*	h	d	e	g
UCT 52	*C. plicata*	g	l	a	v
UCT 53	*C. plicata*	l	m	g	u
UCT 55	*C. plicata*	m	r	g	r
UCT 56	*C. meyeriana*	a	a	NS	b
UCT 57	*C. subternata*	c	a	NS	b
UCT 58a	*C. subternata*	c	a	NS	b
UCT 60	*C. meyeriana*	a	a	NS	b
UCT 61	*C. subternata*	p	a	NS	NS
UCT 62	*C. genistoides*	f	e	a	e
UCT 63	*C. genistoides*	f	e	a	h
UCT 67	*C. glabra*	a	a	NS	b
UCT 69	*C. glabra*	a	a	NS	b
UCT 70	*C. maculata*	f	e	a	i
UCT 71	*C. glabra*	b	a	NS	b
UCT 73	*C. genistoides*	f	j	a	e
LMG 6465^T	*Azorhizobium caulinodans*	A	U	J	D
USDA 4892^T	*Azorhizobium caulinodans*	A	U	J	D
USDA 76^T	*Bradyrhizobium elkanii*	I	L	R	A
LMG 6138^T	*Bradyrhizobium japonicum*	K	C	g	O
USDA 6^T	*Bradyrhizobium japonicum*	K	S	g	F
LMG 8319	*Bradyrhizobium* sp.	J	M	g	N
STM 815^T	*Burkholderia phymatum*	o	f	i	b
STM 678^T	*Burkholderia tuberum*	f	e	a	d
LMG 14989^T	*Mesorhizobium ciceri*	V	Q	C	X

Table 6.6: continued

Isolate number	Host plant / species designation	*Alu*I	*Cfo*I	*Hae*III	*Msp*I
USDA 4779^T	*Mesorhizobium huakuii*	P	T	A	H
LMG 14107^T	*Mesorhizobium huakuii*	P	H	A	T
LMG 17827^T	*Rhizobium etli*	R	J	E	U
LMG 6214^T	*Rhizobium galegae*	Q	I	F	S
LMG 9503^T	*Rhizobium tropici*	T	O	D	V
LMG 18864^T	*Sinorhizobium medicae*	D	X	M	J
LMG 6133^T	*Sinorhizobium meliloti*	F	Z	O	L
USDA 4893^T	*Sinorhizobium saheli*	C	W	L	E

NS no site for the restriction enzyme used, product remained uncut

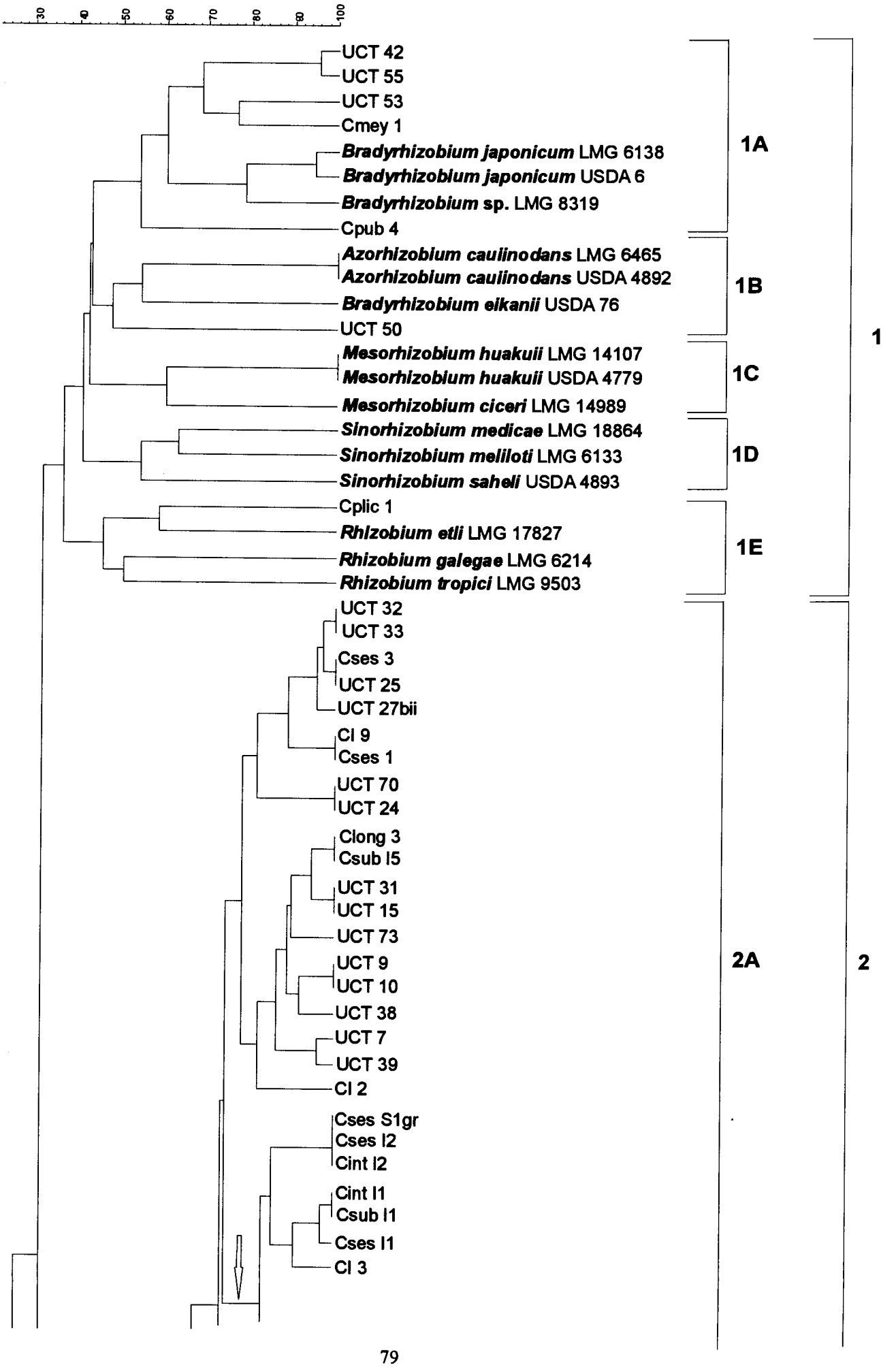
Cluster 1

Cluster 1 was divided into five sub-clusters to aid the discussion of the results. In this cluster all the rhizobial reference strains of the α-*Proteobacteria* grouped. The cluster contained seven *Cyclopia* isolates, which grouped in cluster 1A, 1B and 1E. In cluster 1C, the *Mesorhizobium* reference strains clustered, while the *Sinorhizobium* strains clustered in 1D.

Cluster 1A

The isolates UCT 42, UCT 53, UCT 55, Cmey1 and Cpub 4 grouped in cluster 1A together with the *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. reference strains. The isolates were isolated from three different *Cyclopia* species: *C. plicata* (UCT 42, UCT 53 and UCT 55), *C. meyeriana* (Cmey 1) and *C. pubescens* (Cpub 4). UCT 42 and UCT 55 have the same restriction profiles for the enzymes *Alu*I, *Cfo*I, *Hae*III and *Msp*I and thus most likely have similar genotypes. The restriction profiles of UCT 53 show that the *Hae*III profile of the isolate is similar to that of UCT 42 and UCT 55. However, the resolution power of *Hae*III and *Msp*I has been found to be lower than that of the other two enzymes. Inspection of the profiles showed that Cmey 1 shares some similar bands with UCT 53, but not similar profiles. Isolate Cpub 4 clustered with a low similarity value (~55%) in cluster 1A. The isolate has different enzyme profiles for all four enzymes, which differ from that of the other isolates in cluster 1A.

Figure 6.1 (next page): **UPGMA dendrogram constructed from the combined restriction profiles of the amplified 16S-23S IGS PCR products generated with the enzymes *Alu*I, *Cfo*I, *Hae*III and *Msp*I. The x-axis shows the correlation values between the isolates and displays similarity values for convenience.**


30
40
50
60
70
80
90
100
UCT 42
UCT 55
UCT 53
Cmey 1
Bradyrhizobium japonicum LMG 6138
Bradyrhizobium japonicum USDA 6
Bradyrhizobium sp. LMG 8319
Cpub 4
Azorhizobium caulinodans LMG 6465
Azorhizobium caulinodans USDA 4892
Bradyrhizobium elkanii USDA 76
UCT 50
Mesorhizobium huakuii LMG 14107
Mesorhizobium huakuii USDA 4779
Mesorhizobium ciceri LMG 14989
Sinorhizobium medicae LMG 18864
Sinorhizobium meliloti LMG 6133
Sinorhizobium saheli USDA 4893
Cplic 1
Rhizobium etli LMG 17827
Rhizobium galegae LMG 6214
Rhizobium tropici LMG 9503
UCT 32
UCT 33
Cses 3
UCT 25
UCT 27bii
CI 9
Cses 1
UCT 70
UCT 24
Clong 3
Csub I5
UCT 31
UCT 15
UCT 73
UCT 9
UCT 10
UCT 38
UCT 7
UCT 39
CI 2
Cses S1gr
Cses I2
Cint I2
Cint I1
Csub I1
Cses I1
CI 3
1A
1B
1C
1D
1E
1
2A
2

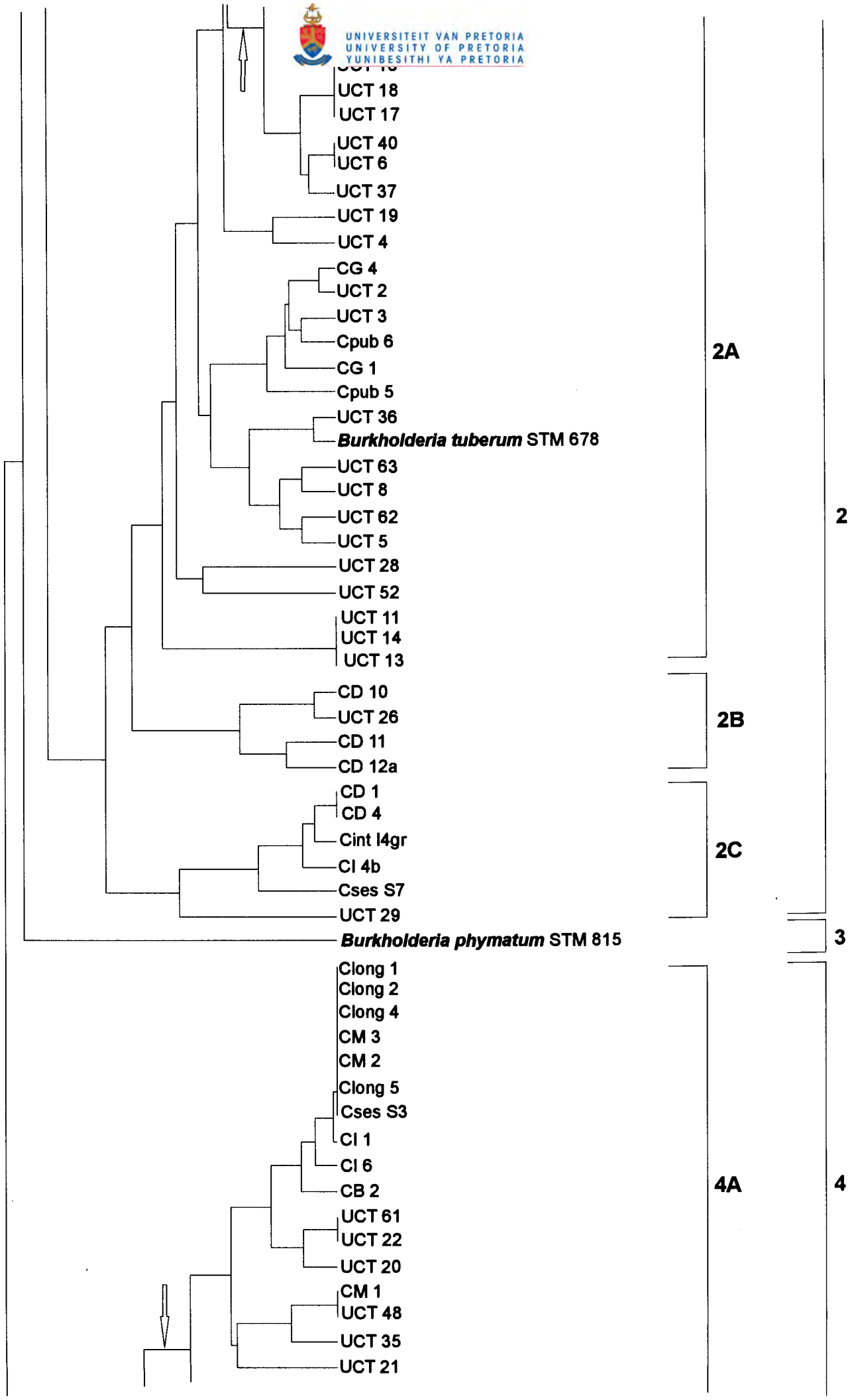


Figure 6.1: continued

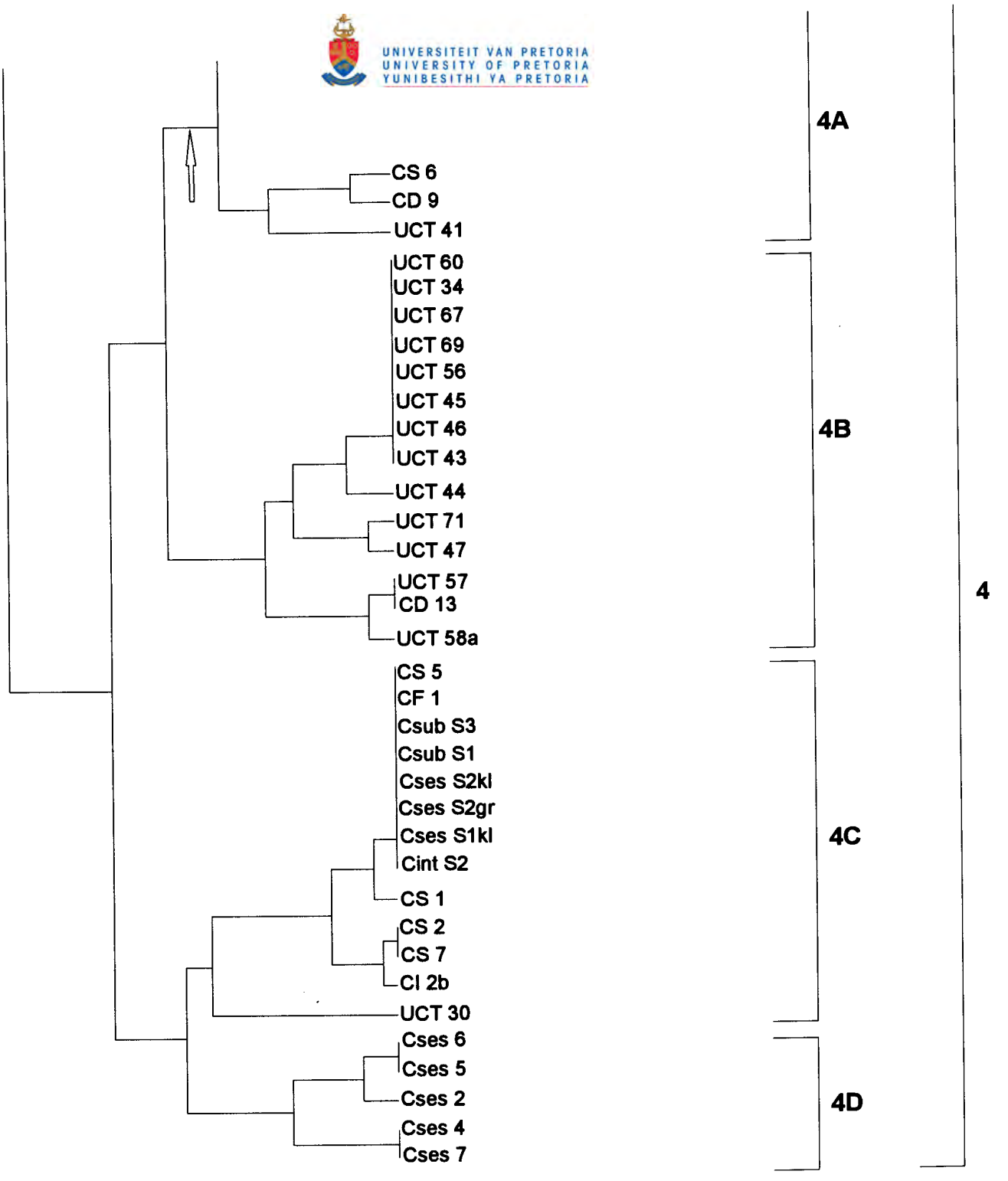


Figure 6.1: continued

Cluster 1B

In cluster 1B the *Azorhizobium caulinodans* and *Bradyrhizobium elkanii* reference strains clustered, though the association between the *A. caulinodans* strains and the *B. elkanii* strain is low (~55%). As an outgroup the *C. sessiliflora* isolate UCT 50 grouped distantly (~ 45%) in cluster 1B.

Cluster 1E

The reference strains of *R. etli*, *R. galegae* and *R. tropici* grouped in cluster 1E forming a *Rhizobium* group. Isolate Cplic 1 from *C. plicata* grouped in the *Rhizobium*-group having a different IGS profile type, but sharing some bands with the other isolates. The closest relative of Cplic 1 in the cluster was *R. etli* LMG 17827^T sharing 60% similarity.

Cluster 2

Cluster 2 consisted of the largest collection of *Cyclopia* strains and the sub-clusters 2A, 2B and 2C can be distinguished based on the IGS-RFLP type. The reference strain *Burkholderia tuberum* STM 678^T grouped in cluster 2. Root-nodulating isolates from ten different *Cyclopia* species grouped in this cluster. Most of the isolates have identical IGS *Alu* I and *Hae*III restriction profiles. However, the relationship of the isolates was resolved with the restriction enzymes *Cfo*I and *Msp*I and 20 different IGS-RFLP types can be distinguished.

Cluster 2A

Cluster 2A represents the largest collection of homogeneous *Cyclopia* isolates from nine different *Cyclopia* species (*C. buxifolia*, *C. galioides*, *C. genistoides*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. plicata*, *C. pubescens*, *C. sessiliflora* and *C. subternata*). All the isolates of cluster 2A displayed the same restriction profile for *Hae*III. With the exception of UCT 28 (*C. subternata*) and UCT 52 (*C. plicata*) all the isolates had the same *Alu*I restriction enzyme profile. These isolates had unique profiles for *Cfo*I and *Msp*I. The isolates UCT 11, UCT 13 and UCT 14, all from *C. genistoides* shared a *Cfo*I profile with isolates of cluster 4.

Cluster 2B

The isolates of this cluster were isolated from *C. dregeana* and *C. intermedia*. Only UCT 26 was isolated from *C. intermedia*. Cluster 2B is a highly related cluster having a similarity value of 80%. The profiles of most the isolates in this cluster differ from those of the other isolates in cluster 2. However, all the isolates of cluster 2B, except isolates CD 10 and UCT 26 have the common *Hae*III restriction profile of cluster 2.

Cluster 2C

Four *Cyclopia* species (*C. dregeana*, *C. intermedia*, *C. sessiliflora* and *C. subternata*) are represented in this cluster. Except for isolates Cint I4gr, CI 4b and Cses S7, which have the common *Hae*III restriction profile, the other isolates of this cluster have different IGS-RFLP restriction patterns. However, some of the bands are shared with the profiles of the other isolates of cluster 2.

Cluster 3

The reference strain *Burkholderia phymatum* STM 815^T is the only isolate in this cluster. The strain has the common *Alu*I restriction profile of cluster 2 and displayed the same *Msp*I restriction profile as the isolates in cluster 4C, but differed from all the isolates analysed in the restriction profiles obtained with *Cfo*I and *Hae*III.

Cluster 4

No reference strain grouped in cluster 4. Four sub-groups can be distinguished in cluster 4. The isolates of cluster 4 had been isolated from twelve different *Cyclopia* species. All the isolates in cluster 4 lacked an enzyme site for *Hae*III.

Cluster 4A

Cluster 4A represents isolates from nine different *Cyclopia* spp. (*C. buxifolia*, *C. dregeana*, *C. genistoides*, *C. glabra*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. plicata* and *C. subternata*). All the isolates of cluster 4A lacked an enzyme site for *Msp*I in addition to

having no *Hae*III restriction site. The isolates formed a relatively homogeneous group where the genotypic differences of the isolates could be resolved with the aid of *Alu*I and/or *Cfo*I in some instances.

Cluster 4B

All the isolates in cluster 4B have the same *Msp*I and *Cfo*I profiles in addition to the common characteristic of cluster 4, the absence of any *Hae*III restriction sites. Three different genotypes could be distinguished in this cluster based on the profiles created with *Alu*I. The isolates were isolated from four different *Cyclopia* species (*C. dregeana*, *C. glabra*, *C. meyeriana* and *C. subternata*).

Cluster 4C

Most of the isolates in cluster 4C displayed the same *Alu*I restriction profile and all shared the characteristic lack of a cleavage site for *Hae*III. Two genotypes could be resolved in this group based on the profiles created with the enzymes *Cfo*I and *Msp*I. Isolate UCT 30 (*C. sessiliflora*) which clustered on a separate branch in the cluster displayed different restriction profiles for *Alu*I, *Cfo*I and *Msp*I from that of the other isolates in cluster 4C, though some bands are shared with the other profiles. The isolates in this cluster were isolated from four *Cyclopia* species (*C. falcata*, *C. intermedia*, *C. sessiliflora* and *C. subternata*).

Cluster 4D

Cluster 4D is a homogeneous collection of isolates from *Cyclopia sessiliflora*. The isolates displayed the same profiles for all four enzymes and the differences shown on the dendrogram could be attributed to different size IGS amplification products. The isolates had the same *Alu*I profile as the isolates in cluster 4C, but differed in the profiles generated with *Cfo*I and *Msp*I.

16S PCR

The primers fD1 and rP2 (Weisburg *et al.*, 1991) were able to amplify the 16S rDNA gene of the selected isolates (Table 6.7). The size of the amplification products of the 16S rDNA gene were in the range of approximately 1500 bp (results not shown).

Table 6.7 ***Cyclopia*** **isolates included in the determination of the partial 16S sequence data.**

Isolate	GenBank Accesion number	IGS cluster	Isolate	GenBank Accesion number	IGS cluster
CB 2	AY178059	4A	Cses 5	AY178106	4D
CD 1	AY178094	2C	UCT 11	AY178107	2A
CD 10	AY178083	2B	UCT 15	AY178068	2A
CD 12a	AY178096	2B	UCT 2	AY178073	2A
CD 13	AY178095	4B	UCT 21	AY178057	4A
CD 9	AY178076	4A	UCT 27bii	AY178084	2A
CG 4	AY178097	2A	UCT 28	AY178085	2A
CI 1	AY178060	4A	UCT 29	AY178062	2C
CI 2	AY178069	2A	UCT 30	AY178067	4C
CI 3	AY178072	2A	UCT 31	AY178074	2A
CI 4b	AY178098	2C	UCT 34	AY178056	4B
CI 6	AY178099	4A	UCT 42	AY178077	1A
CI 9	AY178100	2A	UCT 43	AY178055	4B
Clong 1	AY178061	4A	UCT 50	AY178082	1B
Clong 3	AY178070	2A	UCT 52	AY178086	2A
CM 1	AY178058	4A	UCT 53	AY178078	1A
Cmey 1	AY178079	1A	UCT 56	AY178054	4B
Cplic 1	AY178081	1E	UCT 57	AY178087	4B
Cpub 4	AY178080	1A	UCT 58a	AY178088	4B
Cpub 5	AY178101	2A	UCT 61	AY178089	4A
Cpub 6	AY178071	2A	UCT 62	AY178090	2A
CS 2	AY178065	4C	UCT 63	AY178092	2A
CS 3	AY178102	2A	UCT 67	AY178091	4B
CS 6	AY178066	4A	UCT 70	AY178075	2A
Cses 1	AY178103	2A	UCT 71	AY178064	4B
Cses 2	AY178104	4D	UCT 73	AY178093	2A
Cses 3	AY178105	2A	UCT 8	AY178108	2A
Cses 4	AY178063	4D			

16S rDNA sequence analysis

The sequencing reactions conducted with the internal forward primer 16SRNAII-S (Kuhnert *et al.*, 1996) and the reverse primer rP2SHRT (Weisburg *et al.*, 1991) were able to give an unambiguous DNA sequence for each isolate of approximately 700 bp. The last part of each strand had ambiguous positions, since the sequencer had problems to distinguish the correct signal. However, the ambiguous positions could be resolved using the other strand. The

sequences were deposited in the GenBank database. The relevant accession numbers and IGS cluster type can be seen in Table 6.7 for the *Cyclopia* isolates included in this study.

Phylogenetic relationship of the *Cyclopia* isolates within the α- and β-*Proteobacteria*

In order to simplify the dendrogram only the relevant rhizobial reference strains and several *Burkholderia* species were included in the analysis to reveal the possible affinities of the *Cyclopia* isolates. All the sequences of the reference strains used were edited to include the same part of the 16S rDNA gene in the sequence analysis.

The tree reconstructed with the partial 16S rDNA sequence data revealed two prominent lineages corresponding to the α- and β- subclass of the *Proteobacteria*. The isolates Cplic 1 (*C. plicata*), UCT 50 (*C. sessiliflora*), UCT 53 (*C. plicata*), Cmey 1 (*C. meyeriana*), UCT 42 (*C. galioides*) and Cpub 4 (*C. pubescens*) grouped in the α-*Proteobacteria* cluster. All the other *Cyclopia* isolates belonged to the β-*Proteobacteria*.

Isolates Cplic 1 and UCT 50 clustered in the *R. tropici-Agrobacterium rhizogenes* branch within the *Rhizobium* lineage. Within the *Bradyrhizobium* lineage the three isolates UCT 53 (*C. plicata*), Cmey1 (*C. meyeriana*) and UCT 42 (*C. galioides*) grouped. The closest neighbour of isolate UCT 53 is *B. japonicum* LMG 6138^T, while Cmey 1 formed a separate branch and isolate UCT 42 is closer related to *B. liaoningense*. Comparison of the sequence data of Cpub 4 (*C. pubescens*) with that of the data available in GenBank using the BLAST algorithm (Altschul *et al.*, 1990) revealed that the sequence is most related to "*Kaistia adipata*", which led to the inclusion of this isolate in the sequence analysis. The phylogenetic tree revealed that *Kaistia adipata* is the closest neighbour of isolate Cpub 4 sharing 96.9% sequence similarity.

The *Cyclopia* isolates belonged to six different clusters in the β-*Proteobacteria* (see Fig. 6.2). All the isolates in cluster I corresponded to IGS RFLP cluster 2. Isolates of cluster II belonged to IGS cluster 2C and cluster 4D. The isolates from the different IGS clusters grouped on two separate branches in cluster II. In cluster III, all the isolates displayed the genotype of IGS cluster 4. Isolate CS 2 (*C. subternata*) belonged to cluster IV. Two isolates

Figure 6.2 (next page): **Unrooted neighbour-joining tree reconstructed from partial 16S rDNA sequence data to show the phylogenetic relationships between the *Cyclopia* isolates and some reference strains of the α- and β-*Proteobacteria*. Horizontal branch lengths are proportional to the phylogenetic distances, while the vertical branches are non-informative. The scale bar indicates 2% nucleotide difference and bootstrap values higher than 600 are indicated. Abbreviations: *B.* = *Burkholderia*, *M.* = *Mesorhizobium*, *R.* = *Rhizobium* and *S.* = *Sinorhizobium***

UCT 71 (*C. glabra*) and CS 6 (*C. subternata*) both from IGS cluster 4, formed cluster V, a separate cluster lacking any reference isolates. Another isolate from IGS cluster 4 CD 9 (*C. dregeana*) grouped in cluster VI. The tree was simplified by excluding the sequences of all the α-*Proteobacteria* in a separate analysis and thus enabling a better understanding of the phylogenetic position of the *Cyclopia* isolates within the β-*Proteobacteria*, specifically the genus *Burkholderia*.

Phylogenetic relationship of the *Cyclopia* isolates within the β-*Proteobacteria*

The *Cyclopia* isolates belonged to the same six different clusters (see Fig. 6.3) in the simplified tree as seen in the more complex tree (see Fig. 6.2). Clusters I and II shared a common ancestor and more distantly, a common ancestor was shared by clusters I, II and III. Most of the *Cyclopia* isolates belonged to these clusters. In clusters IV, V and VI only four *Cyclopia* isolates grouped.

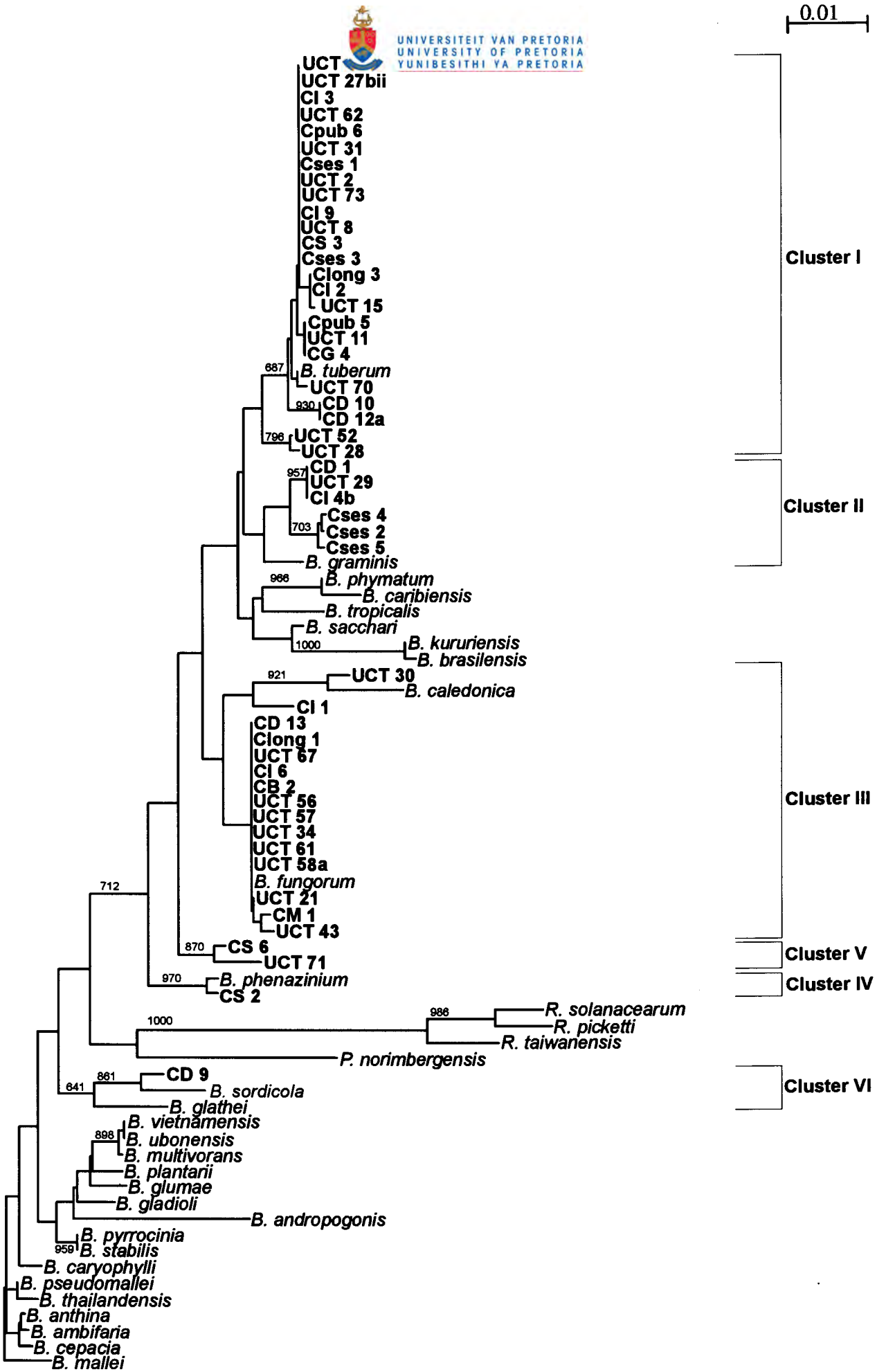
Cluster I

Isolates from nine *Cyclopia* species (*C. dregeana*, *C. genistoides*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. plicata*, *C. pubescens*, *C. sessiliflora* and *C. subternata*) belonged to this cluster. The cluster is a highly related group of isolates sharing high 16S rDNA sequence similarity values. A single reference strain, *Burkholderia tuberum* STM 678^T was included in this cluster. Isolates CD 10 and CD 11a (both from *C. dregeana*) formed a separate branch in cluster I sharing 99.5% sequence similarity with *B. tuberum*. Another separate branch was formed by the isolates UCT 52 (*C. plicata*) and UCT 28 (*C. subternata*) sharing 99.2% and 99.1% sequence similarity respectively with *B. tuberum*.

Cluster II

Isolates from two different IGS clusters belonged to cluster II. The isolates from IGS cluster 2C and 4D grouped on separate branches in cluster II. Sequence similarities between the isolates of the two branches ranged from 99.3%-99.4%. *Burkholderia graminis* AUS 35, a rhizosphere organism was the reference strain, which belonged to this cluster. However, *B. graminis* grouped as an outgroup of the cluster. *B. graminis* was closer related to the isolates

Figure 6.3 (next page): **Unrooted neighbour-joining tree reconstructed from the partial 16S rDNA sequence data of the *Cyclopia* isolates and some reference strains of the genera *Burkholderia*, *Pandoraea* and *Ralstonia* to show the phylogenetic relationship of the *Cyclopia* isolates within the β-*Proteobacteria*. Branch lengths reflect phylogenetic distances between the isolates, while the vertical branches are non-informative. The scale bar shows 1% sequence difference. Bootstrap probabilities higher than 600 are indicated at the respective nodes. Numbering of clusters as found in Fig. 6.2 were retained in this tree.**



CD1 (*C. dregeana*), UCT 29 (*C. sessiliflora*) and CI 4b (*C. intermedia*) than to the other isolates sharing 99% sequence similarity with the previously mentioned isolates.

Cluster III

Three lineages corresponding to two *Burkholderia* species, *B. fungorum* LMG 16225^T and *B. caledonica* LMG 19076^T can be seen. Isolate CI 1 (*C. intermedia*) formed the third lineage, without a reference strain. The isolate shared 97.6% and 98.7% sequence similarity with *B. caledonica* and *B. fungorum* respectively. The closest neighbour of isolate UCT 30 (*C. sessiliflora*) was *B. caledonica* sharing 98.8% sequence similarity. Isolates collected from nine *Cyclopia* species (*C. buxifolia*, *C. dregeana*, *C. genistoides*, *C. glabra*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. meyeriana* and *C. subternata*) belonged to the *B. fungorum* lineage. These isolates shared high sequence similarity values ranging from 99.7%-100%.

Cluster IV

Burkholderia phenazinium LMG 2247^T, an isolate obtained from soil enriched with threonine and isolate CS 2 (*C. subternata*) grouped in cluster IV sharing 99.7% sequence similarity. The closest neighbours of these strains were the two *Cyclopia* isolates of cluster V.

Cluster V

In cluster V the two *Cyclopia* isolates CS 6 (*C. subternata*) and UCT 71 (*C. glabra*) grouped. These isolates shared 99.3% sequence similarity.

Cluster VI

Isolate CD 9 (*C. dregeana*) belonged to cluster VI. The reference strains *B. sordicola* SNU 020123 and *B. glathei* LMG 14190^T clustered in this group. *B. sordicola* and *B. glathei* shared 98.9% and 98.2% sequence similarity with isolate CD 9 respectively.

DISCUSSION

The root-nodulating rhizobial reference species belonged to several distinct lineages in the α-*Proteobacteria*. The genera *Azorhizobium* and *Bradyrhizobium* were more related to each other than to the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* as was also found by other researchers (De Lajudie *et al.*, 1998a; Velázquez *et al.*, 1998; Wang *et al.*, 1999b). *Methylobacterium nodulans* and *Devosia neptuniae*, both new α-*Proteobacteria* root-nodulating species formed separate lineages unrelated to the other root-nodulating genera. The inclusion of reference strains from other genera of the α-*Proteobacteria*, as well as the use of full-length or near full-length sequences, in the comparative sequence analysis would enhance the resolution of the relationships of the α-*Proteobacteria* rhizobial species and the other α-*Proteobacteria* species.

Only seven *Cyclopia* isolates belonged to the α-*Proteobacteria*, one of which (Cpub 4) could not be identified yet, but according to BLAST results (Altschul *et al.*, 1990) and comparative sequence analysis, the closest relative was "*Kaistia adipata*". Two isolates UCT 50 and Cplic 1 were found related to the *Rhizobium tropici-Agrobacterium rhizogenes* branch based on sequence data. Several researchers found this close association of *R. tropici* and *A. rhizogenes* (Chen *et al.*, 1997; Terefework *et al.*, 1998). In the IGS-RFLP study, UCT 50 grouped in the same cluster as *Azorhizobium caulinodans* and *B. elkanii*. This might reflect the inability of IGS-RFLP to differentiate between genera. Willems *et al.* (2001b) reported the inability of the IGS sequence analysis to distinguish between genera, since the strains of the genera *Nitrobacter* and *Blastobacter* grouped in the genus *Bradyrhizobium*. Based on the comparative sequence analysis UCT 50 and isolate Cplic 1 (*C. plicata*) is most probably *R. tropici* strains. Isolate UCT 53 is most related to *Bradyrhizobium japonicum*, while the identity of UCT 42 and Cmey 1 is not that clear from the 16S rDNA tree. However, these strains clearly belonged to the genus *Bradyrhizobium*.

The soil from which the *Cyclopia* isolates have been collected was very acidic (J. Bloem, personal communication). The finding of mainly *Bradyrhizobium* strains would be expected, since the slow-growing strains are better adapted to these environmental conditions (Graham *et al.*, 1994). *Rhizobium tropici* strains are also more acid-tolerant than other fast-growing rhizobial species (Martínez-Romero *et al.*, 1991; Graham *et al.*, 1994). The higher acid

tolerance of *Bradyrhizobium* strains and *Rhizobium tropici* strains would thus explain the finding of these bacteria in the root-nodules of some *Cyclopia* plants. Lafay and Burdon (1998) also reported the isolation of these acid-tolerant rhizobia from plants growing in areas with low pH soils in Australia. It can thus be assumed that the *Burkholderia* isolates also have high acid-tolerance.

Based on the results of both techniques the identity of the *Cyclopia* isolates of 16S rDNA cluster I could be proposed as strains of the species *Burkholderia tuberum* (Moulin *et al*, 2001; Vandamme *et al*, 2002). This would also include all the isolates, which grouped in IGS cluster 2A. It is clear from the IGS-RFLP dendrogram that this collection of strains forms a highly homogeneous group. However, isolates CD 10, CD 12a, UCT 28 and UCT 52 might be members of two additional *Burkholderia* species closely related to *B. tuberum* based on the separate branches that they formed in cluster I.

The identity of the isolates in cluster II is not clear from the tree reconstructed from the 16S sequence data. The significance of the branching in the same lineage as *Burkholderia graminis*, a species described by Viallard *et al.* (1998), was not supported with a bootstrap value higher than 50%. The addition of more similar sequences or the use of longer sequences might change the association.

All the isolates in cluster III, except isolates UCT 30 and CI 1 could be strains belonging to the species *Burkholderia fungorum* described by Coenye *et al.* (2001). However, the *B. fungorum* branch was supported by only 55% of the 1000 replicates generated with the bootstrap analysis. The use of partial sequence data might hinder the differentiation between closely related isolates, since it does not reflect true relationships. Thus to draw conclusions on the species affiliation of these isolates full-length 16S sequence analysis should be done.

The significance of the branching of clusters IV and V, as determined with bootstrap is 97% and 87% respectively. The high significance level and sequence similarity would support the identity of isolate CS 2 as a possible strain of *Burkholderia phenazinium* (Viallard *et al.*, 1998). No definite conclusions can however be made on the identity of isolates CS 6 and UCT 71.

The branching pattern of cluster VI was found in 64% of the 1000 bootstrap generated replicates, but the branch leading to CD 9 and *B. sordicola* was found highly significant (86%). The use of full-length sequence analysis would be able to unequivocally determine the correct species affiliation of isolate CD 9.

None of the isolates was related to the other root-nodulating *Burkholderia* species, *Burkholderia phymatum* (Moulin *et al.*, 2001 and Vandamme *et al.*, 2002). Based on the 16S rDNA sequence data, the closest neighbour of *B. phymatum* was *B. caribiensis*, as previously found by Vandamme *et al.* (2002). Vandamme *et al.* (2002) also identified root-nodulating isolates from tropical legumes as members of the species *B. caribiensis* and *B. cepacia* genomovar VI.

Other researchers have shown that IGS-RFLP analysis is a useful method for determining the diversity of bacterial populations (Laguerre *et al.*, 1996; Vinuesa *et al.*, 1998; Diouf *et al.*, 2000, Doignon-Bourcier *et al.*, 2000). In this study, it has been found that IGS-RFLP analysis was an easy and reproducible method for the diversity determination of the *Cyclopia* isolates, even showing intraspecific differences between the strains. The partial 16S rDNA sequencing analysis corroborated the results of the IGS-RFLP analysis. In a study conducted by Willems *et al.* (2001a), the researchers found an agreement between the clustering of the same *Bradyrhizobium* strains obtained with IGS sequence analysis and the clustering obtained from IGS-RFLP analysis as done by Doignon-Bourcier *et al.* (2000). Willems *et al.* (2001a) proved in the study of *Bradyrhizobium* strains that the groupings obtained with IGS sequence analysis and AFLP (amplified fragment length polymorphism) analysis correlated with data generated with DNA homology analysis. It might thus be possible to draw conclusions on different genomic species from the IGS-RFLP dendrogram if a threshold value for species delineation could be determined.

The different size ranges of the amplified IGS products were expected, since the IGS products of several bacteria vary in length due to the insertion of tRNA genes (Gürtler and Stanisich, 1996). The rRNA operon is also present in multiple copies and the insertion of tRNA genes could explain the length differences between IGS products of the same strain (Gürtler and Stanisich, 1996; Laguerre *et al.*, 1996; LeBlond-Bourget *et al.*, 1996).

CHAPTER 7

PHYLOGENETIC, SYMBIOTIC AND PHENOTYPIC CHARACTERISATION OF SOME *BURKHOLDERIA* SPP. ISOLATES

ABSTRACT

The phylogenetic position of some *Burkholderia* strains isolated from different *Cyclopia* species was determined using near full-length 16S rDNA sequencing. The data showed the identity of several isolates as *B. tuberum*. Several possible novel *Burkholderia* species were found. However, DNA homology studies remain to be done to confirm and delineate the novel species. All the *nodA* sequences of the isolates displayed high sequence similarity. The *nodA* sequence of *B. tuberum* isolated from *Aspalathus carnosa* shared highest sequence similarity with the *Cyclopia* isolates. The phenotypic study confirmed the isolates as members of the genus *Burkholderia* and thus clearly different from the rhizobial genera included in the analysis.

Keywords: *Burkholderia*, 16S rDNA sequencing, *nodA* sequencing, substrate utilisation patterns

INTRODUCTION

The study of more legume hosts from diverse environmental conditions opened the door for new discoveries. The understanding of the rhizobium-legume symbiosis changed significantly in recent years. The description of β-*Proteobacteria* (Chen *et al.*, 2001; Moulin *et al.*, 2001 and Vandamme *et al.*, 2002), methylotrophic bacteria (Sy *et al.*, 2001; Jaftha *et al.*, 2002), *Devosia neptuniae* (Rivas *et al.*, 2002; Rivas *et al.*, 2003) and the budding bacteria *Blastobacter denitrificans* capable of root-nodulation changed the rhizobium taxonomy.

The genus *Burkholderia* contains plant and animal pathogens (Brett *et al.*, 1998), obligate endosymbionts of Rubiaceae and Myrsinaceae hosts (Van Oevelen *et al.*, 2002), an endosymbiont of *Gigaspora margarita* (arbuscular mycorrhizal fungus) [Minerdi *et al.*, 2001] strains capable of bioremediation (Fain and Haddock, 2001), biocontrol (Trân Van *et al.*, 2000; Peix *et al.*, 2001) and plant growth promotion (Trân Van *et al.*, 2000; Peix *et al.*, 2001; Ciccillo *et al.*, 2002). Strains of the genus can fix nitrogen (Gillis *et al.*, 1995; Minerdi *et al.*, 2001). *B. vietnamensis* was described for nitrogen-fixing isolates from rice in Vietnam (Gillis *et al.*, 1995). *B. brasilensis*, *B. kururiensis*, *B. tropicalis* (Marin *et al.*, 2003) in addition to *B. phymatum* and *B. tuberum* (Moulin *et al.*, 2001; Vandamme *et al.*, 2002) are nitrogen-fixers.

The 16S rDNA molecule can be used to identify and determine the phylogenetic position of isolates. The use of full-length sequences is essential for phylogenetic conclusions (Ludwig *et al.*, 1998). The limitation of 16S sequence data is the relatively conserved nature of the molecule, since closely related species cannot be differentiated with 16S data (Vandamme *et al.*, 1996; LeBlond-Bourget *et al.*, 1996; Rosselló-Mora and Amann, 2001; Stackebrandt *et al.*, 2002). However, this technique has been widely used to identify and determine the phylogenetic position of isolates. Lafay and Burdon (1998) used the technique to rapidly identify novel rhizobial isolates from scrubby legumes in Southeastern Australia, while Terefework *et al.* (1998) used the technique to determine the phylogenetic position of *Rhizobium galegae* in the *Rhizobiaceae*.

Rhizobia recognise specific signals from legumes, which activate the regulatory NodD proteins and induce the *nod* gene expression. The common genes (*nodABC*) are involved in the formation of the backbone of the Nod-factor (lipo-chitooligosaccharide signal), which induces specific infection and nodulation in legumes. NodA is an acyltransferase, which

transfers a fatty acyl chain to the acetyl-free C-2 carbon of the non-reducing end of the oligosaccharide molecule (Perret *et al.*, 2000; Hirsch *et al.*, 2001). NodA is also host-specific, since it transfers specific acyl chains (Ritsema *et al.*, 1996). The *nodA* gene is found as a single copy in rhizobia. The phylogenetic trees reconstructed from the nod genes, *nodA*, *nodB*, *nodC* and *nodD*, agree with each other, but differ from that of the 16S rRNA (Haukka *et al.*, 1998). The study of the *nodA* gene is a rapid technique to determine the host range of isolates (Haukka *et al.*, 1998; Ba *et al.*, 2002).

Numerical taxonomy provides descriptive phenotypic information about strains (Vandamme *et al.*, 1996). Several authors used this technique to characterise strains and differentiate between different phenotypes of a strain. McInroy *et al.* (1999) used Biolog™ and partial 16S rRNA sequencing to characterise rhizobia isolated from African acacias and other tropical woody legumes. In a study on the diversification of *Pseudomonas corrugata* 2140 Barnett *et al.* (1999) used Biolog™ GN microplates (Biolog Inc., Hayward, California, USA) to identify new phenotypes.

MATERIALS AND METHODS

Bacterial strains used

The strains used in this study were received from the Agricultural Research Council's-Plant Protection Research Institute (Private Bag X134, Pretoria, 0001, South Africa) and the Botany Department of the University of Cape Town (Rondebosch, 7701, Cape Town, South Africa) [Table 7.1]. All the *Cyclopia* isolates included in this study were analysed with IGS-RFLP analysis and partial 16S rDNA sequence analysis. The reference strains of *Burkholderia tuberum* and *B. phymatum* used in this study were obtained from the culture collection of the Laboratoire des Symbioses Tropicales et Méditerranéennes (STM), Montpellier, France. All the sequences of the reference strains used in the 16S rDNA and *nodA* sequence analysis were obtained from the Genbank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi.nlm.nih.gov/Genbank/].

Table 7.1: List of isolates from *Cyclopia* spp. included in the 16S rDNA and partial *nodA* sequence analysis

Isolate	Host species	Locality	16S rDNA accession number[1]	*nodA* accession number[1]
CB 2	*C. buxifolia*	Helderberg, Somerset-West	AY178059	AY189248
CD 9	*C. dregeana*	Du Toitskloof, Paarl	AY178076	AY189250
CI 1	*C. intermedia*	Dennehoek, Joubertina	AY178060	AY189253
CI 2	*C. intermedia*	Dennehoek, Joubertina	AY178069	AY189229
CI 3	*C. intermedia*	Dennehoek, Joubertina	AY178072	AY189254
Clong 1	*C. longifolia*	Thornhill, Humansdorp	AY178061	AY189228
Clong 3	*C. longifolia*	Thornhill, Humansdorp	AY178070	AY189273
CM 1	*C. maculata*	Paarlberg, Paarl	AY178058	AY189256
Cpub 6	*C. pubescens*	Next to N1, Port Elizabeth	AY178071	AY189274
CS 2	*C. subternata*	Dennehoek, Joubertina	AY178065	AY189259
CS 6	*C. subternata*	Dennehoek, Joubertina	AY178066	AY189261
Cses 4	*C. sessiliflora*	Plattekloof, Heidelberg	AY178063	AY189230
UCT 15	*C. genistoides*	Constantiaberg	AY178068	AY189275
UCT 2	*C. genistoides*	Rein's Farms	AY178073	AY189267
UCT 21	*C. genistoides*	Paardeberg	AY178057	AY189276
UCT 29	*C. sessiliflora*	Callie's farm, Heidelberg	AY178062	AY189266
UCT 30	*C. sessiliflora*	Callie's farm, Heidelberg	AY178067	AY189268
UCT 31	*C. sessiliflora*	Grootvadersbosch	AY178074	AY189240
UCT 34	*C. glabra*	Matroosberg	AY178056	AY189241
UCT 43	*C. meyeriana*	Hottentots Holland mountains	AY178055	AY189271
UCT 56	*C. meyeriana*	Hottentots Holland mountains	AY178054	AY189245
UCT 70	*C. maculata*	Jonkershoek	AY178075	AY189277
UCT 71	*C. glabra*	unknown	AY178064	AY189278

[1] Genbank (www.ncbi.nlm.nih.gov/Genbank/)

Maintenance of bacterial cultures

The isolates were maintained on yeast mannitol agar (YMA) [1% (m/v) mannitol (UniVar), 0.5% (m/v) K_2HPO_4 (Merck), 0.02% (m/v) $MgSO_4.7H_2O$ (Merck), 0.01% (m/v) NaCl (NT Chemicals), 0.04% (m/v) yeast extract (Biolab) and 1.5% (m/v) bacteriological agar (Biolab)] slants and the long-term storage of the isolates was done in glycerol. The isolates were grown in yeast mannitol broth (YMB) for 5-7 d at 25-28°C with vigorous shaking. The broth cultures were mixed 1:1 with sterile 50% (v/v) glycerol (Merck) in sterile cryotubes and stored in duplicate at -20°C and -70°C.

Extraction of genomic DNA

A modified method for proteinase-K (Roche Molecular Biochemicals) treated cells as described by Laguerre *et al.* (1997) was used. A pure fresh culture of each strain was streaked

on a tryptone yeast (TY) agar slant [0.5% (m/v) tryptone (Difco), 0.3% (m/v) yeast extract (Biolab), 0.13% (m/v) $CaCl_2.6H_2O$ (UniLab), 1.5% (m/v) bacteriological agar] in a screw-cap tube. The strains were incubated at 28 °C and checked for sufficient growth. Sterile 4.5 ml dH_2O was added to the slant growth to harvest the cells. An inoculation loop was used to aid the release of cells clinging to the agar. The volume of the water added was adjusted according to the amount of growth. Less water was used if the growth was poor and *vice versa*. The cell-suspension was collected in a clear plastic tube and vortexed to ensure a uniform suspension. The absorbancy of the suspension was measured with dH_2O as the spectrophotometric blank at 620 nm. A formula was used to determine the volume of the cells to be treated further. The volume to be used in ml was equal to 0.2 divided by the abosorbancy at 620 nm. Two tubes of the same strain were filled with the appropriate volume of cells and centrifuged at 13 000 *g* for 5 min at 4 °C. The supernatant was discarded and the excess media blotted dry. One of the tubes was stored at -20 °C for future use. In the second tube, 100 µl ddH_2O, 100 µl Tris-HCl (10 mM, pH 8.2) and 10 µl proteinase-K (15 mg/ml) (Roche Molecular Biochemicals) were added to the cell pellet. The mixture was incubated at 55 °C overnight. In order to inactivate the proteinase-K the mixture was boiled for 10 minutes. The cell lysates were stored at -20 °C until needed.

Amplification of the 16S rDNA and the partial *nodA* gene

Amplification of the 16S rDNA gene of selected strains (Table 7.1) was performed with the primers fD1 and rP2 (Table 7.2) as described by Weisburg *et al.* (1991). The linker sequences of the primers were not included in the primer synthesis, since no cloning reactions were anticipated. These shorter primers were thus designated fD1SHRT and rP2SHRT. The PCR mixture of each strain contained: 5 µl of the cell lysate, 50 pmol of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Gold Taq DNA polymerase (Southern Cross Biotechnology) in a 50 µl reaction volume. The amplification reactions were performed on a Perkin Elmer GeneAmp PCR System 2400 thermocycler using the following thermal profile: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 1 minute followed by a final extension step at 72 °C for 5 minutes. Aliquots (5 µl) of the amplified products were evaluated with horizontal agarose gel electrophoresis (Sambrook *et al.*, 1989) using 0.9% (m/v) agarose gels (Promega) in a 1X TAE buffer (40 mM Tris-HCl, 20 mM NaOAc

and 1 mM EDTA pH 8.5) stained with ethidium bromide (10 mg/ml) [results not shown]. Molecular marker VI (Roche Molecular Biochemicals) was included on each gel as a standard lane.

The 16S PCR product of each strain was purified to remove any traces of unincorporated dNTPs, primers, etc. which could negatively influence the 16S sequencing reaction. The products were purified using a Qiagen QIAquick PCR Purification kit (Southern Cross Biotechnology). Purification reactions were done as prescribed by the manufacturer. The concentration and purity of each purification reaction was verified visually. An aliquot (1 µl) of each purified 16S PCR product was run on 0.9% (m/v) horizontal agarose gels (Promega) [results not shown]. On each gel, a standard marker, molecular marker VI (Roche Molecular Biochemicals) was included.

Table 7.2: Primers used in the amplification and/or sequencing of the 16S rDNA and the nodA.

Primer name*	Primer sequence (5'-3')#	Target gene	Reference
fD1SHRT	5'-AGAGTTTGATCCTGGCTCAG-3'	16S rDNA	Weisburg *et al.*, 1991
rP2SHRT	5'-ACGGCTACCTTGTTACGACTT-3'	16S rDNA	Weisburg *et al.*, 1991
16SRNAII-S	5'-GTGTAGCGGTGAAATGCGTAG-3'	16S rDNA	Kuhnert *et al.*, 1996
16SRNAVI-S	5'-CTACGCATTTCACCGCTACAC-3'	16S rDNA	Kuhnert *et al.*, 1996
NodAunivF145u	5'-TGGGCSGGNGCNAGRCCBGA-3'	nodA	Moulin *et al.*, 2001
NodAR.brad	5'-TCACARCTCKGGCCCGTTCCG-3'	nodA	Moulin *et al.*, 2001

* All the primers were synthesised by Roche Molecular Biochemicals, Mannheim, Germany

\# Abbreviations: B= G/C/T, K= G/T, N= A/G/C/T, R= A/G, S= G/C

The amplification of the *nodA* gene was performed with primer set NodAunivF145u and NodAR.brad (Table 7.2) as used by Moulin *et al.* (2001) in the first report of *Burkholderia* strains capable of root-nodulation. In each 50 µl amplification reaction the following was added: 5 µl of the cell lysate, 50 pmol of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Southern Cross Biotechnology). The following thermal profile was used: a hot start at 95 °C for 3 minutes, then 35 cycles of denaturation (94 °C for 30 seconds), annealing (55 °C for 45 seconds) and extension (72 °C for 1 minute), followed by a final extension step (72 °C for 5 minutes). The reactions were performed on a Perkin Elmer GeneAmp PCR System 2400 thermocycler. The success of the amplification reactions was checked with horizontal agarose gel electrophoresis (Sambrook *et al.*, 1989) using the method described for 16S rDNA amplification.

The *nodA* amplification products were purified to remove all traces of inhibitors of the sequencing reaction, as well as to ensure the presence of a single product for sequencing. The products were purified with a combined method using the binding buffer of the High Pure Purification PCR kit (Roche Molecular Biochemicals) and the columns and chemicals from the Qiagen QIAquick PCR Purification kit (Southern Cross Biotechnologies). The total volume of the amplification product of each isolate was run on a 0.9% (m/v) agarose gel (Promega) in a 1X TAE buffer (40 mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA pH 8.5) stained with ethidium bromide (10 mg/ml) (Sambrook *et al.*, 1989). The molecular marker VI was included on each gel. The fragment of the correct size was excised from the gel and the weight of the agarose was determined for each isolate. To each fragment, 300 µl binding buffer (Roche Molecular Biochemicals) for each 100 mg of agarose gel was added. The mixture was vortexed to aid in dissolving the agarose. The tubes were incubated at 50-60 °C for 10 minutes, while vortexing the tubes every 2-3 min. After the incubation period, 150 µl of isopropanol (Merck) per 100 mg of agarose was added to each tube. This mixture was added to the Qiagen QIAquick PCR Purification kit columns. The rest of the purification procedure was done as prescribed by the manufacturer. The success of the purification reactions was verified using the method as described for 16S rDNA (results not shown).

Sequence analysis of the 16S rDNA and the partial *nodA* gene

The near full-length sequence of each purified amplified 16S rDNA product of the chosen isolates were determined with the internal forward primer 16SRNAII-S (Kuhnert *et al.*, 1996), the reverse primer rP2SHRT (Weisburg *et al.*, 1991), the internal reverse primer 16SRNAVI-S (Kuhnert *et al.*, 1996) and the forward primer fD1SHRT (Weisburg *et al.*, 1991) using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (AmpliTaq® DNA Polymerase, FS) (PE Applied Biosystems). In the sequencing reactions of the *nodA* gene the forward primer NodAunivF145u was used. Each 5 µl sequencing reaction contained the following: 2 µl of the ready reaction mix supplied with the kit which contains the dye terminators, dNTP's, AmpliTaq® DNA polymerase, $MgCl_2$ and Tris-HCl buffer pH 9.0; 12.5 pmol primer and approximately 100 ng template DNA. The sequencing reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler with the following thermal profile: an initial denaturation at 96 °C for 5 seconds followed by 25 cycles of denaturation (96 °C for 10 seconds), annealing (50 °C for 5 seconds) and extension (60 °C for

4 minutes). The products were precipitated using the protocol as suggested by the manufacturer. For analysis, the purified products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer (Perkin Elmer Applied Biosystems). The loading buffer was prepared by combining de-ionised formamide and 25 mM EDTA (pH 8.0) containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran. The resuspended products were denatured for 2 min at 90°C and loaded onto the ABI Prism model 377 DNA sequencer gel.

Phylogenetic analysis of the 16S rDNA and *nodA* sequences

The sequencing gels were analysed and sequences edited with the ABI Prism Sequencing Analysis 3.1 and the ABI Prism Sequencing Navigator 1.0.1 computer programmes (Perkin Elmer Applied Biosystems). Both strands were sequenced with the primers used and the strands could be aligned to correct ambiguous positions. The resulting two unambiguous strands were overlapped to give a continuous near full-length sequence for each isolate. The *nodA* sequences were checked visually to see that the peaks and the corresponding nucleotides were correct. The ClustalX programme (Thompson *et al.*, 1997) was used to analyse the edited sequences as well as the reference sequences obtained from GenBank (Table 7.3 and Table 7.4). A distance matrix was constructed by pair-wise alignment of the sequences. The neighbour-joining method (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the distance matrix. Branch lengths were proportional to the estimated divergence along each branch. A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the tree topologies (Felsenstein, 1985). The phylogenetic trees were visualised with NJplot (Perrière and Gouy, 1996).

Table 7.3 Reference strains obtained from GenBank included in the comparative 16S sequence analysis

Reference strain	Strain number	Host plant or relevant characteristics	GenBank[1] Accession number
Burkholderia ambifaria	MVPC 1/4	*B. cepacia* complex	AY028444
Burkholderia andropogonis	ATCC 23061^T	Sorghum (*Sorghum bicolor)*	X67037
Burkholderia anthina	R-4183^T	Rhizosphere soil, *B. cepacia* complex	AJ420880
Burkholderia brasilensis	M130	Plant-associated N_2-fixer	AJ238360
Burkholderia caledonica	LMG 19076^T	Rhizosphere soil	AF215704
Burkholderia caribiensis	LMG 18531^T	Vertisol microaggregates	Y17009
Burkholderia caryophylli	ATCC 25418^T	Carnation (*Dianthus caryophyllus)*	AB021423
Burkholderia cenocepacia	LMG 16656^T	Cystic fibrosis patients, plant associated *B. cepacia* genomovar III	AF148556
Burkholderia cepacia	ATCC 25416^T	Cystic fibrosis patients, B. cepacia genomovar I	AF097530
Burkholderia fungorum	LMG 16225^T	*Phanerochaete chrysosporium*	AF215705
Burkholderia gladioli	ATCC 10248^T	*Gladiolus* sp.	X67038
Burkholderia glathei	LMG 14190^T	Fossil lateritic soil	U96935
Burkholderia glumae	LMG 2196^T	Rice (*Oryza sativa)*	U96931
Burkholderia graminis	AUS 35	Rhizosphere	U96941
Burkholderia hospita	LMG 20598^T	Agricultural soil	AY040365
Burkholderia kururiensis	LMG 19447^T	Trichloroethylene degrader	AB024310
Burkholderia mallei	NCTC 10260	NS	AF110187
Burkholderia multivorans	LMG 13010^T	*B. cepacia* complex	Y18703
Burkholderia phenazinium	LMG 2247^T	Soil enriched with threonine	U96936
Burkholderia phymatum	STM 815^T	*Machaerium lunatum*	AJ302312
Burkholderia plantarii	LMG 9035^T	*Oryza sativa* pathogen	U96933
Burkholderia pseudomallei	V686	Soil	AF093052
Burkholderia pyrrocinia	LMG 14191^T	soil	U96930
Burkholderia sacchari	LMG 19450^T	Soil from sugarcane plantation	AF263278
Burkholderia sordicola	SNU 020123	Associated with white rot fungus *Phanerochaete sordicola*	AF512827
Burkholderia stabilis	LMG 14294^T	Formerly *B. cepacia* complex IV	AF148554
Burkholderia terricola	LMG 20594^T	Agricultural soil	AY040362
Burkholderia thailandensis	E264^T	*Pseudomallei* group	U91838
Burkholderia tropicalis	Ppe8	Plant-associated N_2-fixer	AJ420332
Burkholderia tuberum	STM 678^T	*Aspalathus carnosa*	AJ302311
Burkholderia ubonensis	GTC-P3-415	NS	AB030584
'Burkholderia unamae'	MT1-641^T	maize	AY221956
Burkholderia vietnamensis	LMG 10929^T	N_2-fixer from rice rhizophere	AF097534
Candidatus Burkholderia kirkii	Strain19536779	*Psychotria kirkii* var. *tarambassica*	AF475063
Pandoraea norimbergensis	NS*	Alkaliphilic sulphur oxidiser	Y09879
Ralstonia picketti	MSP 3	Rhizosphere, soil	AB004790
Ralstonia solanacearum	ATCC 11696	*Lycopersicon lycopersicum*	X67036
Ralstonia taiwanensis	LMG 19424^T	*Mimosa pudica*	AF300324

* See footnotes of Table 7.4

Table 7.4 ***NodA*** **sequences obtained from GenBank included in the comparative** ***nodA*** **sequence analysis**

Reference strain	Strain number	Host plant	Accession number GenBank[1]
Azorhizobium caulinodans	ORS 571[T]	*Sesbania rostrata*	L18897
Bradyrhizobium elkanii	USDA 94	NS	U04609
Bradyrhizobium japonicum	110spc4	NS	AF322013
Bradyrhizobium **sp.**	NC92	*Arachis hypogaea*	U33192
Bradyrhizobium **sp.**	WM9	*Lupinus* sp.	AF222753
Bradyrhizobium **sp.**	ANU289	*Parasponia* sp.	X03720
Bradyrhizobium **sp.**	ORS 285	Photosynthetic	AF284858
Bradyrhizobium **sp.**	ORS 287	*Aeschynomene afraspera*	AJ437607
Bradyrhizobium **sp.**	ORS 301	*Aeschynomene americana*	AJ437608
Bradyrhizobium **sp.**	ORS 302	*Aeschynomene pfundii*	AJ437609
Bradyrhizobium **sp.**	ORS 304	*Aeschynomene elaphroxylon*	AJ437610
Bradyrhizobium **sp.**	ORS 309	*Aeschynomene uniflora*	AJ437611
Bradyrhizobium **sp.**	ORS 336	*Aeschynomene afraspera*	AJ437612
Bradyrhizobium **sp.**	ORS 364	*Aeschynomene nilotica*	AJ437613
Burkholderia tuberum	STM 678[T]	*Aspalathus carnosa*	AJ302321
Mesorhizobium ciceri	USDA 3383	*Hedysarum boreale*	AJ250140
Mesorhizobium loti	NS	NS	L06241
Mesorhizobium mediterraneum	USDA 3392	NS	AJ250141
Mesorhizobium plurifarium	ORS 1096	*Acacia tortilis* subsp. *raddiana*	AJ302678
Mesorhizobium **sp.**	BR3804	*Chamaecrista ensiformis*	Z95249
Mesorhizobium **sp.**	DW0366	*Acacia polycantha*	Z95248
Mesorhizobium **sp.**	7653R	*Astragalus sinicus*	AJ249353
Mesorhizobium **sp.**	N33	*Oxytropis arctobia*	U53327
Mesorhizobium tianshanense	USDA 3592	NS	AJ250142
Methylobacterium nodulans	ORS 2060[T]	*Crotalaria podocarpa*	AF266748
Rhizobium etli	CFN 42[T]	*Phaseolus vulgaris*	NC_004041
Rhizobium galegae	HAMBI 1174	*Galega orientalis*	X87578
Rhizobium leguminosarum **bv.** ***phaseoli***	NS	NS	M58625
Rhizobium leguminosarum **bv.** ***trifolii***	ANU843	NS	X03721
Rhizobium leguminosarum **bv.** ***viciae***	NS	NS	Y00548
Rhizobium tropici	CFN 299	*Phaseolus* sp.	X98514
Sinorhizobium arboris	HAMBI 1700	*Acacia senegal*	Z95235
Sinorhizobium fredii	USDA 257	NS	M73699
Sinorhizobium kostiense	HAMBI 1489[T]	*Acacia senegal*	Z95236
Sinorhizobium meliloti	NS	NS	X01649
Sinorhizobium saheli	ORS 609	*Sesbania cannabina*	Z95241
Sinorhizobium **sp.**	NGR234	Broad host range	AE000076
Sinorhizobium **sp.**	BR827	*Leucaena leucocephala*	Z95232
Sinorhizobium **sp.**	BR4007	*Prosopis juliflora*	Z95240
Sinorhizobium **sp.**	M6	*Prosopis* sp.	Z95233
Sinorhizobium **sp.**	ORS 1085	*Acacia tortilis* subsp. *raddiana*	AJ302677
Sinorhizobium terangae	ORS 1009	*Acacia laeta*	Z95237

[1] GenBank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi.nlm.nih.gov/Genbank/]

[T] Type strain

NS Not stated

ATCC American Type Culture Collection, Rockville, Maryland, USA

CFN	Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico
HAMBI	Culture Collection of the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland
LMG	BCCM™/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium
NCTC	National Collection of Type Cultures, Central Public Health Laboratory, London, UK
ORS	ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal
STM	Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France
USDA	United States Department of Agriculture, *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, MD, USA

Numerical taxonomy

The substrate utilisation patterns of selected *Cyclopia* isolates (Table 7.5) and the two root-nodulating *Burkholderia* species were determined with Biolog GN Microplates™ (Biolog Inc., Hayward, USA). Each plate has 96 wells, comprising a negative control and 95 preselected carbon sources (Appendix A). The method as prescribed by the manufacturer was used to grow the cultures and inoculate the microplates. A positive reaction depends on the reduction of tetrazolium violet to form a purple dye in wells where oxidation of the carbon source takes place. A positive reaction was scored as one (1), while a negative reaction was scored as zero (0). These data were analysed with the simple matching coefficient in the Bionum programme (Applied Maths, Kortrijk, Belgium). A dendrogram was constructed from the distance values with the unweighted pair group method with arithmetic mean (UPGMA) in Gelcompar 4.0 (Applied Maths, Kortrijk, Belgium). Substrate utilisation profiles of the rhizobial reference strains included in the analysis were obtained from a previous study (Kruger, 1998).

Table 7.5 Isolates included in the substrate utilisation determination

Isolate	Host plant	Isolate	Host plant
Burkholderia tuberum STM 678^T	*Aspalathus carnosa*	CS 6	*Cyclopia subternata*
Burkholderia phymatum STM 815^T	*Machaerium lunatum*	Cses 4	*Cyclopia sessiliflora*
CB 2	*Cyclopia buxifolia*	UCT 30	*Cyclopia sessiliflora*
CD 9	*Cyclopia dregeana*	UCT 34	*Cyclopia glabra*
CI 3	*Cyclopia intermedia*	UCT 70	*Cyclopia maculata*
CS 2	*Cyclopia subternata*	UCT 71	*Cyclopia glabra*

RESULTS

Amplification of the 16S rDNA

The primers fD1 and rP2 (Weisburg *et al.*, 1991) were able to amplify the 16S rDNA gene of the selected isolates. The size of the amplification products of the 16S rDNA gene was in the range of approximately 1500 bp (results not shown).

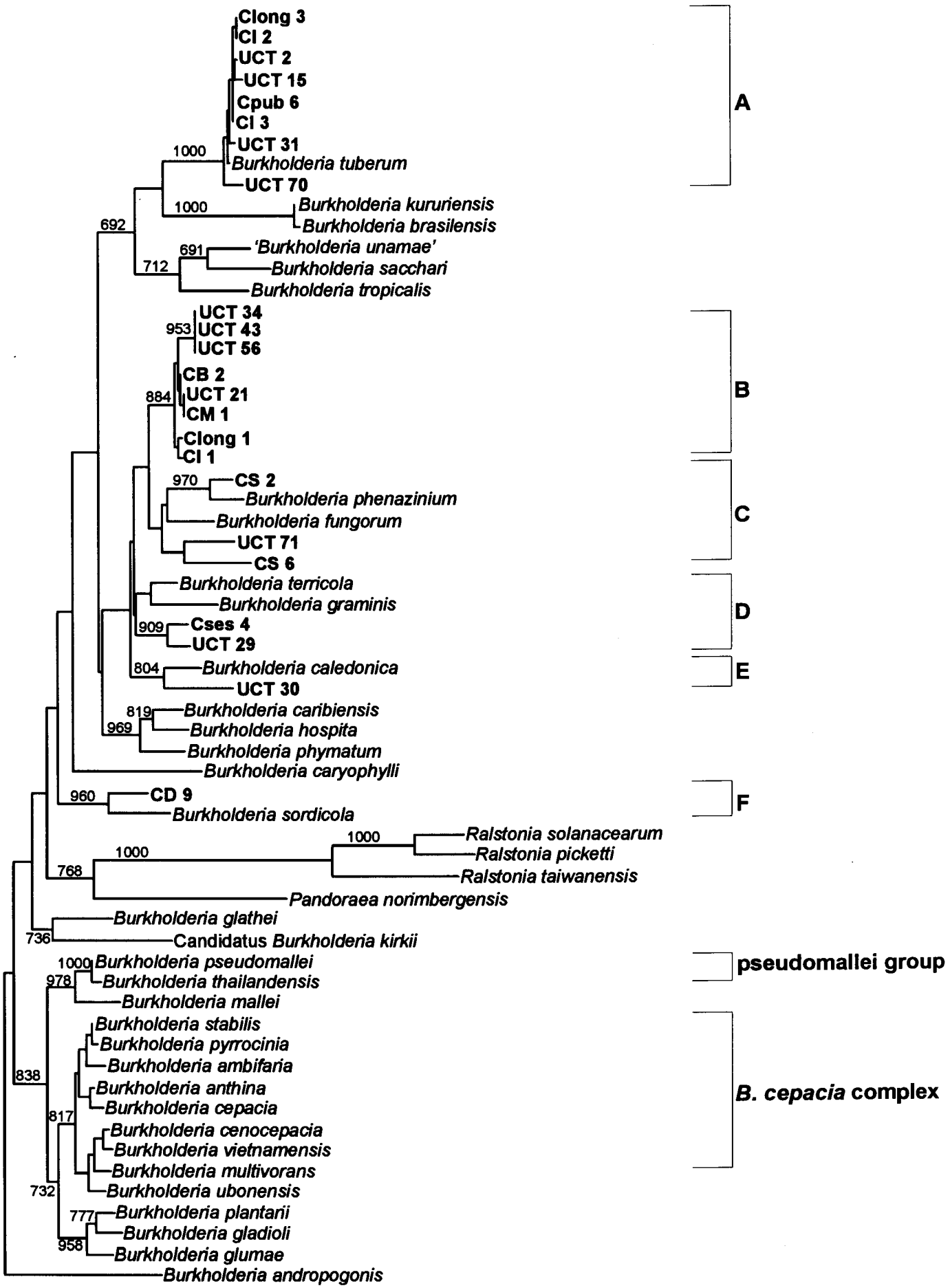
16S rDNA sequence analysis

The sequencing reactions conducted with the two primer sets, namely the internal forward primer 16SRNAII-S (Kuhnert *et al.*, 1996) and the reverse primer rP2 (Weisburg *et al.*, 1991), the forward primer fD1 (Weisburg *et al.*, 1991) and the internal reverse primer 16SRNAVI-S (Kuhnert *et al.*, 1996) were able to give two unambiguous DNA sequences for each isolate of approximately 700 bp. The last part of each strand had ambiguous positions, since the sequencer had problems to distinguish the correct signal. However, the ambiguous positions could be resolved using the other strand. It was possible to overlap the two strands, since the end and beginning of the respective strands were the same sequence. The sequences were deposited in the GenBank database (see Table 7.1 for accession numbers).

Phylogenetic relationship of *Cyclopia* isolates in the β-*Proteobacteria* determined with near full-length 16S rDNA sequence analysis

The *Cyclopia* isolates clustered in six different groups based on the comparative 16S sequence analysis (see Fig. 7.1). Three lineages could be recognised to which the *Cyclopia* isolates belong. Clusters B, C, D and E belonged to one lineage, while cluster A belonged to a lineage, which shared a common ancestor with the *Burkholderia* spp.: *B. kururiensis*, *B. brasilensis*, *B. sacchari* and *B. tropicalis*. The undescribed possibly new species from maize roots, *B. unamae* was the closest neighbour of *B. sacchari* showing sequence similarity values of 98.5%. Cluster F formed a separate lineage.

Figure 7.1 (next page): **Unrooted phylogenetic tree reconstructed with the neighbour-joining method from the comparative 16S rDNA sequence analysis of the *Cyclopia* isolates and reference strains of the genus *Burkholderia*. Reference strains of the genera *Pandoraea* and *Ralstonia* were included for clarity. Branch lenghts are proportional to the phylogenetic distances, while the vertical branches are non-informative. The scale bar shows 1% nucleotide difference. Bootstrap values higher than 600 are indicated.**


Clong 3
CI 2
UCT 2
UCT 15
Cpub 6
CI 3
UCT 31
Burkholderia tuberum
UCT 70
Burkholderia kururiensis
Burkholderia brasilensis
'Burkholderia unamae'
Burkholderia sacchari
Burkholderia tropicalis
UCT 34
UCT 43
UCT 56
CB 2
UCT 21
CM 1
Clong 1
CI 1
CS 2
Burkholderia phenazinium
Burkholderia fungorum
UCT 71
CS 6
Burkholderia terricola
Burkholderia graminis
Cses 4
UCT 29
Burkholderia caledonica
UCT 30
Burkholderia caribiensis
Burkholderia hospita
Burkholderia phymatum
Burkholderia caryophylli
CD 9
Burkholderia sordicola
Ralstonia solanacearum
Ralstonia picketti
Ralstonia taiwanensis
Pandoraea norimbergensis
Burkholderia glathei
Candidatus Burkholderia kirkii
Burkholderia pseudomallei
Burkholderia thailandensis
Burkholderia mallei
Burkholderia stabilis
Burkholderia pyrrocinia
Burkholderia ambifaria
Burkholderia anthina
Burkholderia cepacia
Burkholderia cenocepacia
Burkholderia vietnamensis
Burkholderia multivorans
Burkholderia ubonensis
Burkholderia plantarii
Burkholderia gladioli
Burkholderia glumae
Burkholderia andropogonis
1000
1000
692
691
712
953
884
970
909
804
819
969
960
1000
1000
768
736
1000
978
838
817
732
777
958
A
B
C
D
E
F
pseudomallei group
B. cepacia complex

Cluster A

In cluster A, *Cyclopia* isolates from six different *Cyclopia* species (*C. genistoides*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. pubescens* and *C. sessiliflora*) grouped with the root-nodulating isolate from *Aspalathus carnosa*, *Burkholderia tuberum*. The *Cyclopia* isolates shared high sequence similarities with the reference strain *B. tuberum*, ranging from 99.6-99.9%. Cluster A was found well resolved with a bootstrap value of 100%. *B. kururiensis* was the closest neighbour of the clade sharing 97% sequence similarity with *B. tuberum*.

Cluster B

Cluster B is a highly related cluster in which no reference strain clustered. This cluster was supported by a significant bootstrap value of 95.3%. Isolates from seven different *Cyclopia* species (*C. buxifolia*, *C. genistoides*, *C. glabra*, *C. intermedia*, *C. longifolia*, *C. maculata*, *C. meyeriana*) belonged to this cluster. Isolates UCT 43 and UCT 56 (both from *C. meyeriana*) and UCT 34 (*C. glabra*) shared 100% sequence similarity with each other and differed most from the rest of the cluster.

Cluster C

In cluster C, three groups could be distinguished. On one branch isolate CS 2 (*C. subternata*) and the soil organism *B. phenazinium* grouped, while *B. fungorum* formed another branch and the two *Cyclopia* isolates UCT 71 (*C. glabra*) and CS 6 (*C. subternata*) formed the other branch. Unlike cluster A and cluster B, cluster C was not so closely related. Isolate CS 2 shared the highest similarity with *B. phenazinium* (99.1%). The two isolates UCT 71 and CS 6 shared 98.2% sequence similarity, while sharing 98.1% and 97.9% sequence similarity with *B. fungorum* respectively. Cluster C was not a significant and repeatable cluster, though the branch leading to the CS 2 and *B. phenazinium* was significant (97%). The branch leading to isolates UCT 71 and CS 6 was supported with a low bootstrap value (52.4%).

Cluster D

The reference strains *B. terricola* and *B. graminis* grouped together with the isolates Cses 4 and UCT 29 (both from *C. sessiliflora*) in cluster D. The two reference strains formed a separate branch, while the two *Cyclopia* isolates formed another branch. The clustering of the two *Cyclopia* isolates was supported with a highly significant bootstrap value of 90.9%. These two isolates shared 99.3% sequence similarity. The reference strain *B. terricola* shared 98.6% and 98.5% with Cses 4 and UCT 29 respectively.

Cluster E

The rhizosphere isolate, *B. caledonica* and isolate UCT 30 (*C. sessiliflora*) belonged to cluster E, sharing 98.4% sequence similarity. Cluster E was a well-resolved grouping, since it was supported with a bootstrap value of 80.4%.

Cluster F

In cluster F, the reference strain *B. sordicola* and isolate CD 9 (*C. dregeana*) clustered. Isolate CD 9 shared 98.5% sequence similarity with *B. sordicola*. Cluster F formed a well-resolved clade with a high bootstrap value (96%).

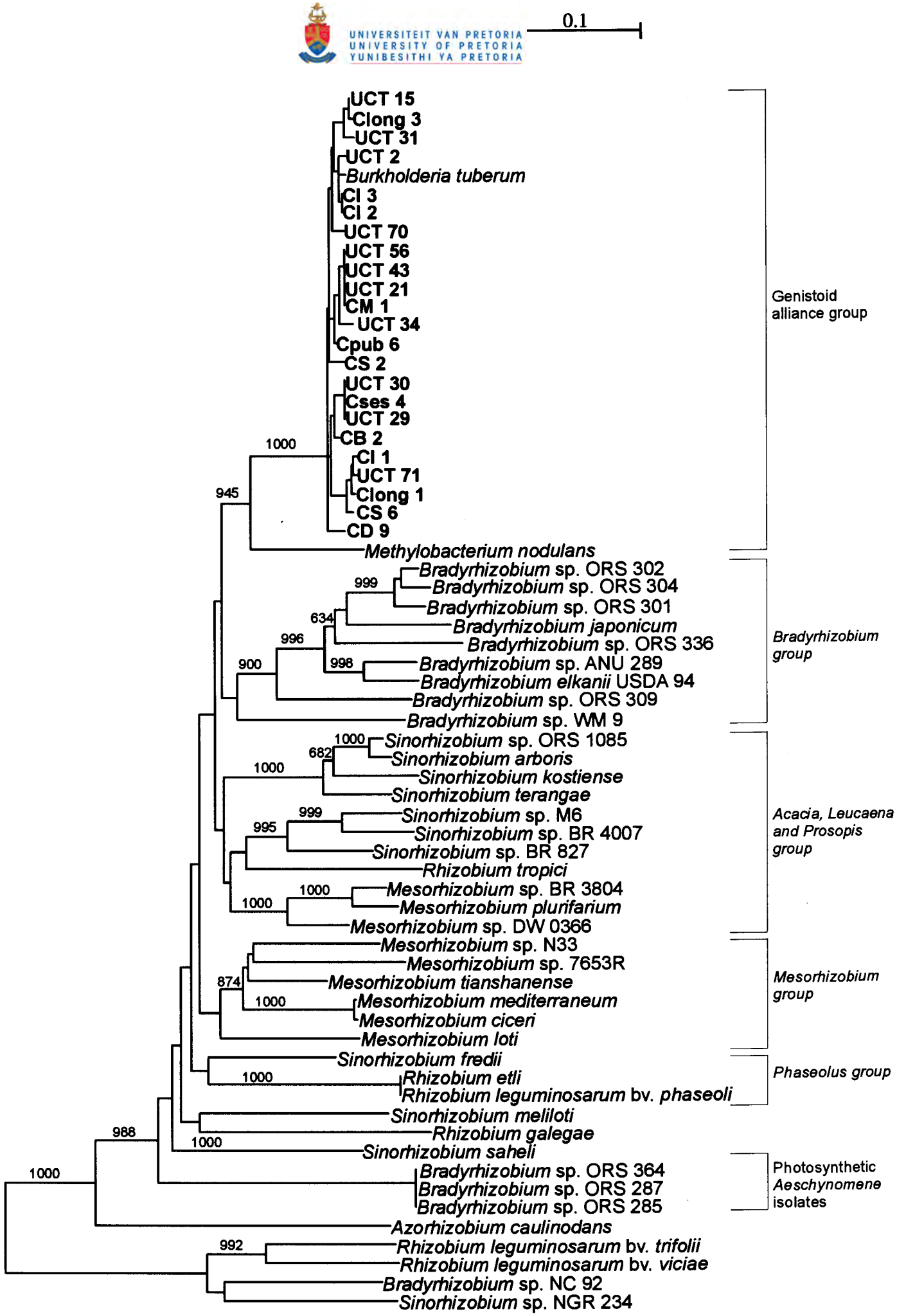
***NodA* PCR**

The primer set (NodAunivF145u and NodAR.brad) used was able to amplify the *nodA* gene resulting in a fragment size of 455 bp as was expected from the results of Moulin *et al.* (2001). In some of the isolates, non-specific fragments were also amplified, since the primers are degenerate.

***NodA* sequence analysis**

The forward primer used was able to determine the sequence of the *nodA* gene. The sequencing results were checked visually by comparing the peaks and the called nucleotides, since the sequencer sometimes calls two nucleotides for a single peak. The sequences were also edited using the *nodA* sequence of *Burkholderia tuberum* as a reference sequence. Any

Figure 7.2 (next page): **Unrooted neighbour-joining tree reconstructed from comparative partial *nodA* sequence analysis. Horizontal branch lengths reflect phylogenetic distances, while the vertical branch lengths are non-informative and set for clarity. The scale bar indicates 10% nucleotide difference. Bootstrap values found in more than 600 of the 1000 replications are shown.**



ambiguous positions were corrected. A strand of approximately 390 bp of unambiguous positions was obtained. The sequences were deposited in the GenBank database (see Table 7.1 for accession numbers).

All the *nodA* genes of the *Cyclopia* isolates formed a single well-resolved clade based on the comparative sequence analysis (see Fig. 7.2). This clade was supported with a 100% bootstrap value. In the clade, the *nodA* gene of *Burkholderia tuberum* also clustered. The *nodA* gene of the α-*Proteobacteria* species, *Methylobacterium nodulans* shared 83.5% sequence similarity with the *Burkholderia* spp. clade.

Numerical taxonomy

The substrate utilisation of 95 carbon sources of the selected isolates could be determined with the Biolog microplates. The 95 carbon sources were divided into the 11 groups as done by Garland and Mills (1991). In Table 7.6, the different oxidation patterns of these isolates are shown. None of the isolates could utilise 2,3-butanediol, phenylethylamine, putrescine, glycyl-L-aspartic acid, gentobiose, maltose, D-melibiose, turanose, glucose-1-phosphate or α-cyclodextrin. All the isolates could utilise glycerol (an alcohol), succinamic acid (an amide), eleven of the 20 amino acids, urocanic acid (an aromatic chemical), bromo-succinic acid (a brominated chemical), thirteen of the 28 carbohydrates, thirteen of the 24 carboxylic acids, both esters tested for and finally two of the five polymers.

The substrate utilisation data were used to construct a dendrogram (see Fig. 7.3) to show the phenotypic similarities of the isolates in a schematic format. The data of the rhizobial isolates were included in the analysis to show the phenotypic differences/similarities between the known rhizobial isolates and the *Burkholderia* isolates. Three main clusters could be distinguished in the dendrogram. The first cluster contained all the *Cyclopia* isolates as well as the *Burkholderia* reference strains. In the second cluster, species from the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* grouped, while the third cluster contained strains of the *Bradyrhizobium* genus. Isolates in cluster 1 shared 80% similarity, while the isolates of clusters 2 and 3 shared 70.5% and 85% similarity respectively.

In cluster 1a, UCT 34, CB 2 and UCT 71 grouped with *B. phymatum* (STM 815). Isolates UCT 34 and CB 2 displayed high phenotypic similarity (93.5%). Inspection of the substrate

utilisation pattern revealed that CB 2 and UCT 34 could not use D-raffinose, citric acid and sucrose, while *B. phymatum* and UCT 71 did. Isolates CB 2 and UCT 34 could utilise itaconic acid, while *B. phymatum* and UCT 71 could not. UCT 71 is the only *Burkholderia* isolate tested capable of growth on inosine and β-methyl D-glucoside. *B. phymatum* did not utilise glycyl-L-glutamic acid, uridine or α-keto-glutaric acid, while the other isolates in the cluster did. The phosphorylated chemical, D, L-α-glycerol phosphate was uniquely used by *B. phymatum*.

Isolates Cses 4 and UCT 30 formed cluster 1b and displayed 91.5% similarity. Isolate UCT 30 could uniquely utilise cellobiose. Isolate Cses 4 was unable to utilise L-ornithine, *N*-acetyl-D-galactosamine, D-trehalose, γ-hydroxybutyric acid, α-keto-valeric acid or D-saccharic acid, while UCT 30 could utilise all the previously named substrates.

Cluster 1c contained CD 9 and CS 2, which shared 88.5% phenotypic similarity. The isolates could be distinguished based on their substrate utilisation. CD 9 could utilise glucuronamide, D-serine, xylitol, D-glucoronic acid, glucose-6-phosphate, while CS 2 was not able to utilise these substrates, but could utlise N-acetyl-galactosamine, γ-hydroxybutyric acid and dextrin. The isolates CD 9 and CS 2 were able to uniquely utilise *i*-erythritol and α-D-lactose respectively.

In cluster 1d isolates UCT 70, CI 3, CS 6 and *Burkholderia tuberum* (STM 678) grouped at an overall similarity value of 89.5%. *B. tuberum*, UCT 70 and CI 3 shared 93.5% phenotypic similarity. These three strains could be distinguished from CS 6 as well as the other *Burkholderia* isolates included in the analysis based on their inability to utilise D, L-carnitine. CS 6 was unable to grow on L-alanyl-glycine and α-hydroxybutyric acid, while these substrates were utilised by all *Burkholderia* isolates studied.

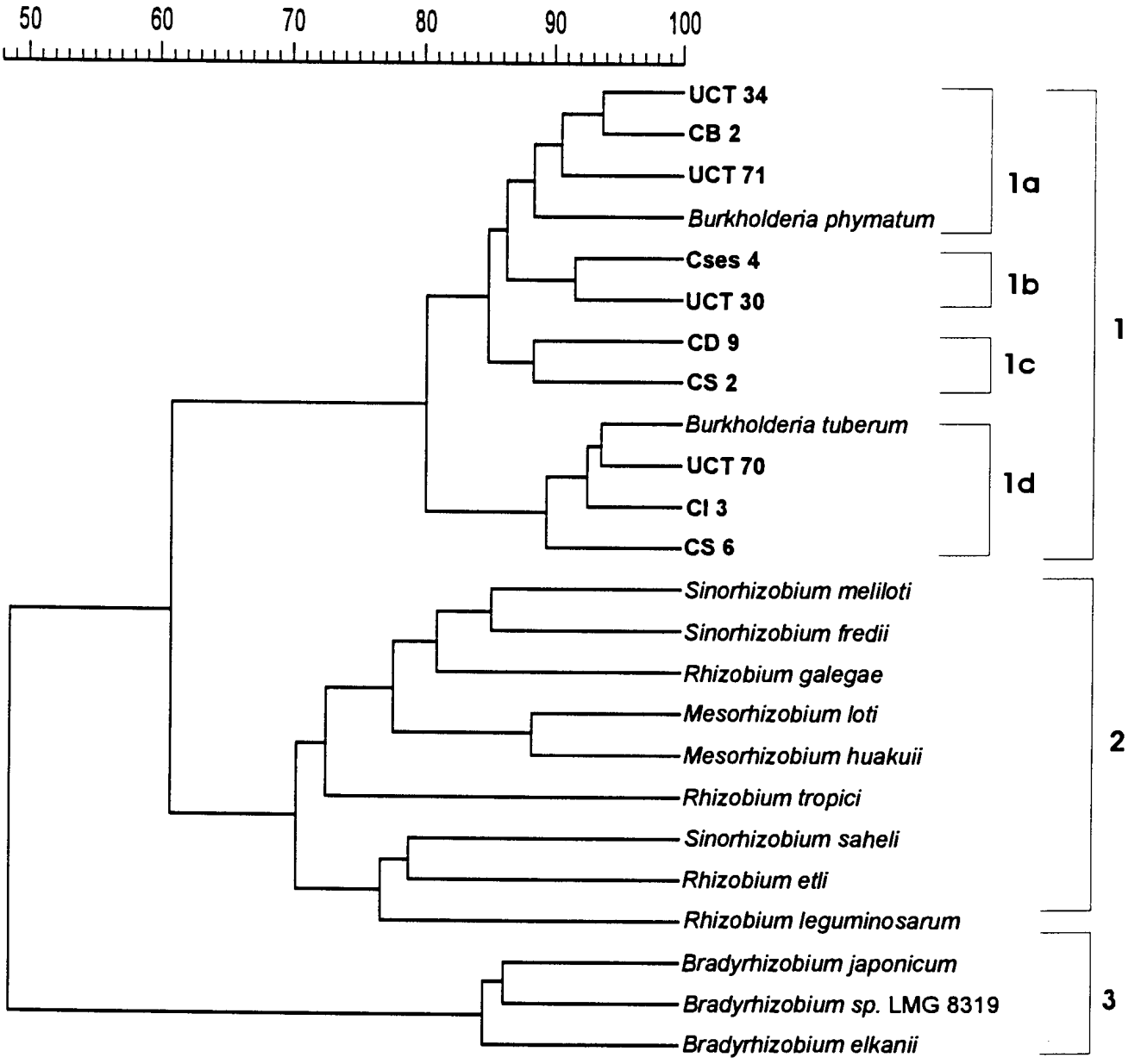


Figure 7.3: **Schematic representation of the substrate utilisation patterns of the *Cyclopia* isolates and the *Burkholderia* spp. included in the analysis. The rhizobial strains were included as references. The x-axis shows the correlation between the isolates and displays similarity values for convenience.**

Table 7.6: Oxidation patterns of the different carbon sources utilised by selected *Cyclopia* isolates and the *Burkholderia* sp. included in the study

Carbon sources	STM 678^T	STM 815^T	CB 2	CD 9	CI 3	CS 2	CS 6	Cses 4	UCT 30	UCT 34	UCT 70	UC 71
Alcohols												
2,3-butanediol	-	-	-	-	-	-	-	-	-	-	-	-
glycerol	+	+	+	+	+	+	+	+	+	+	+	+
Amides												
alaninamide	-	+	+	+	-	+	-	+	+	+	-	+
glucuronamide	-	-	+	+	+	-	-	-	-	-	+	-
succinamic acid	+	+	+	+	+	+	+	+	+	+	+	+
Amines												
2-amino-ethanol	-	+	+	+	-	+	-	+	+	+	-	+
phenylethylamine	-	-	-	-	-	-	-	-	-	-	-	-
putrescine	-	-	-	-	-	-	-	-	-	-	-	-
Amino acids												
D-alanine	+	+	+	+	+	+	+	+	+	+	+	+
L-alanine	+	+	+	+	+	+	+	+	+	+	+	+
L-alanyl-glycine	+	+	+	+	+	+	-	+	+	+	+	+
L-asparagine	+	+	+	+	+	+	+	+	+	+	+	+
L-aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+
L-glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+
glycyl-L-aspartic acid	-	-	-	-	-	-	-	-	-	-	-	-
glycyl-L-glutamic acid	+	-	+	+	-	+	-	-	-	+	+	+
L-histidine	+	+	+	+	+	+	+	+	+	+	+	+
hydroxy-L-proline	+	+	+	+	-	+	-	+	+	+	-	+
L-leucine	+	+	+	+	+	+	+	+	+	+	+	+
L-ornithine	-	+	+	-	-	-	-	-	+	+	-	+
L-phenylalanine	+	+	+	+	+	+	+	+	+	+	+	+
L-proline	+	+	+	+	+	+	+	+	+	+	+	+
L-pyroglutamic acid	+	+	+	+	+	+	+	+	+	+	+	+
D-serine	-	+	+	+	+	-	-	-	-	+	-	+
L-serine	+	+	+	+	+	+	+	+	+	+	+	+
L-threonine	+	+	+	+	+	+	-	+	+	+	+	+
D,L-carnitine	-	+	+	+	-	+	+	+	+	+	-	+
γ-amino butyric acid	-	+	+	+	-	+	-	+	+	+	-	+
Aromatic chemicals												
inosine	-	-	-	-	-	-	-	-	-	-	-	+
thymidine	-	-	-	-	-	+	-	-	-	-	-	-
uridine	-	-	+	+	-	+	-	-	-	+	-	+
urocanic acid	+	+	+	+	+	+	+	+	+	+	+	+
Brominated chemicals												
bromo-succinic acid	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrates												
N-acetyl-D-galactosamine	-	+	+	-	-	+	-	-	+	+	-	+
N-acetyl-D-glucosamine	+	+	+	+	+	+	+	+	+	+	+	+
adonitol	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+
L-arabitol	+	+	+	+	+	+	+	+	+	+	+	+
cellobiose	-	-	-	-	-	-	-	-	+	-	-	-
i-erythritol	-	-	-	+	-	-	-	-	-	-	-	-
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+
L-fucose	+	+	+	+	+	+	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+
gentobiose	-	-	-	-	-	-	-	-	-	-	-	-
α-D-glucose	+	+	+	+	+	+	+	+	+	+	+	+
m-inositol	+	+	+	+	+	+	+	+	+	+	+	+

Table 7.6 continued

Carbon sources	STM 678^T	STM 815^T	CB 2	CD 9	CI 3	CS 2	CS 6	Cses 4	UCT 30	UCT 34	UCT 70	UC 71
α-D-lactose	-	-	-	-	-	+	-	-	-	-	-	-
lactulose	+	-	-	+	+	+	+	-	-	-	+	-
maltose	-	-	-	-	-	-	-	-	-	-	-	-
D-mannitol	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+	+
D-melibiose	-	-	-	-	-	-	-	-	-	-	-	-
β-methyl D-glucoside	-	-	-	-	-	-	-	-	-	-	-	+
psicose	+	+	+	+	-	+	-	+	+	+	+	+
D-raffinose	-	+	-	-	-	-	-	-	+	-	-	+
L-rhamnose	+	+	+	+	+	+	+	+	+	+	+	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	-	+	-	-	-	-	-	+	+	-	-	+
D-trehalose	-	+	+	-	-	-	-	-	+	+	-	+
turanose	-	-	-	-	-	-	-	-	-	-	-	-
xylitol	-	+	+	+	-	-	-	+	+	+	+	+
Carboxylic acids												
acetic acid	+	+	+	+	+	+	+	+	+	+	+	+
cis-aconitic acid	+	+	+	+	+	+	+	+	+	+	+	+
citric acid	+	+	-	+	+	+	+	-	-	-	+	+
formic acid	+	+	+	+	+	+	+	+	+	+	+	+
D-galactonic acid lactone	+	+	+	+	+	+	+	+	+	+	+	+
D-galacturonic acid	+	+	+	+	+	+	+	-	-	-	+	+
D-gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+
D-glucosaminic acid	+	+	+	+	+	+	+	+	+	+	+	+
D-glucoronic acid	+	+	+	+	+	-	+	-	-	-	+	+
α-hydroxybutyric acid	+	+	+	+	+	+	-	+	+	+	+	+
β-hydroxybutyric acid	+	+	+	+	+	+	+	+	+	+	+	+
γ-hydroxybutyric acid	-	-	-	-	-	+	-	-	+	+	-	-
p-hydroxyphenylacetic acid	+	+	+	+	+	+	+	+	+	+	+	+
itaconic acid	+	-	+	-	+	-	-	-	+	+	+	-
α-keto-butyric acid	+	+	+	+	-	+	-	+	+	+	+	+
α-keto-glutaric acid	+	-	+	-	-	-	-	+	+	+	-	+
α-keto-valeric acid	-	-	-	-	-	-	-	-	+	+	-	+
D,L-lactic acid	+	+	+	+	+	+	+	+	+	+	+	+
malonic acid	-	+	+	+	-	+	-	+	+	+	+	+
propionic acid	+	+	+	+	+	+	+	+	+	+	+	+
quinic acid	+	+	+	+	+	+	+	+	+	+	+	+
D-saccharic acid	+	+	+	+	+	+	+	-	+	+	+	+
sebacic acid	+	+	+	+	+	+	+	+	+	+	+	+
succinic acid	+	+	+	+	+	+	+	+	+	+	+	+
Esters												
mono-methylsuccinate	+	+	+	+	+	+	+	+	+	+	+	+
methylpyruvate	+	+	+	+	+	+	+	+	+	+	+	+
Phosphorylated chemicals												
D,L-α-glycerol phosphate	-	+	-	-	-	-	-	-	-	-	-	-
glucose-1-phosphate	-	-	-	-	-	-	-	-	-	-	-	-
glucose-6-phosphate	-	+	-	+	-	-	-	+	+	-	-	-
Polymers												
glycogen	-	+	+	+	-	+	-	-	-	+	+	+
α-cyclodextrin	-	-	-	-	-	-	-	-	-	-	-	-
dextrin	-	-	+	-	-	+	-	-	-	-	-	+
Tween-40	+	+	+	+	+	+	+	+	+	+	+	+
Tween-80	+	+	+	+	+	+	+	+	+	+	+	+

DISCUSSION

The nearly full-length 16S rDNA sequence data confirmed the identity of some of the *Cyclopia* isolates as strains of the species *Burkholderia tuberum* (Moulin *et al.*, 2001; Vandamme *et al.*, 2002). None of the *Cyclopia* isolates belonged to the species *B. phymatum*, which shared highest sequence similarity with *B. caribiensis* (Vandamme *et al.*, 2002). From the comparative sequence analysis, it is clear that the rest of the isolates included do not unequivocally belong to one of the *Burkholderia* species. Similar new isolates have to be included in the description of new species to refrain from describing one strain species.

Isolates of cluster B are clearly strains of a new *Burkholderia* species, possibly even two new *Burkholderia* species. These isolates shared high 16S sequence similarities with each other and belonged to a well-resolved clade. *B. fungorum* is the closest phylogenetic neighbour (98.6%) of the new species based on 16S sequence data. The two representative isolates used in the phenotypic utilisation profile analysis shared high similarity values (93.5%). The strains can also be differentiated based on the distinct restriction patterns obtained with IGS-RFLP analysis. A new species, *Burkholderia capensis* sp. nov. is provisionally proposed for the cluster B strains. A formal species proposal would be done after DNA-DNA hybridisation analysis and G + C content determinations have been performed (Vandamme *et al.*, 1996; Stackebrandt *et al.*, 2002). A more extensive phenotypic characterisation of several of the cluster B strains would also first have to be done to gather phenotypic information to aid the differentiation of this species from the other *Burkholderia* species.

The species of the genus *Burkholderia* show high 16S sequence similarity, which highlights the problem of distinguishing between closely-related species (Leblond-Bourget *et al.*, 1996). DNA-DNA hybridisation studies would have to be done to determine the taxonomic position of the isolates. DNA-DNA homology studies provide a consolidated measure to delineate bacterial species and the technique can be used to identify unknown isolates (Rosselló-Mora and Amann, 2001).

The basic topology of the phylogenetic tree agreed with that previously found in other studies (Brämer *et al.*, 2001; Fain and Haddock, 2001; Goris *et al.*, 2002; Van Oevelen *et al.*, 2002). The high sequence similarity between *B. kururiensis* and *B. brasilensis* has been reported by other researchers (Fain and Haddock, 2001; Marin *et al.*, 2003). *Pandoraea norimbergensis*

was previously described as a species of *Burkholderia*, but was transferred by Coenye *et al.* (2000) to the genus *Pandoraea*. From the comparative sequence data, it is clear that this transfer was warranted, since *P. norimbergensis* formed a separate lineage on the phylogenetic tree. *B. andropogonis* formed a distinct separate branch, which has been found by other researchers as well (Viallard *et al.*, 1998; Coenye *et al.*, 2001; Fain and Haddock, 2001; Van Oevelen *et al.*, 2002).

The numerical taxonomy study confirmed the 16S rDNA sequence analysis and showed the phenotype of the *Cyclopia* isolates as different from that of the known rhizobial genera as the dendrogram clearly separated the *Burkholderia* isolates and the rhizobial isolates. The dendrogram reflected the considerable difference in phenotype between the genus *Bradyrhizobium* and the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. All the *Cyclopia* isolates as well as the *Burkholderia* spp. included could utilise D-galactose, D-glucose, glycerol, inositol, mannitol, D-mannose, sorbitol, L-arabinose and D-fructose. These carbon sources can be utilised by all *Burkholderia* strains (Viallard *et al.*, 1998). The inability to utilise maltose or D-turanose also confirmed the identity of the strains as *Burkholderia* strains (Viallard *et al.*, 1998).

All the *Cyclopia* isolates have nearly the same *nodA* gene, which indicates that the gene is relatively conserved in these organisms. Chaintreuil *et al.* (2001) reported the clear distinction between the *nodA* genes of the photosynthetic *Bradyrhizobium* isolates (ORS 285, ORS 287 and ORS 364) and the non-photosynthetic *Bradyrhizobium* isolates (ORS 301, ORS 302 and ORS 304) from *Aeschynomene*. This distinction and the forming of a separate lineage by the photosynthetic isolates, while the non-photosynthetic isolates belonged to the *Bradyrhizobium* clade was clear from the *nodA* gene tree. The conserved nature (100% sequence similarity) of the *nodA* gene of the photosynthetic isolates was also clear from the *nodA* tree as previously found by Chaintreuil *et al.* (2001). Rhizobia isolated from *Acacia*, *Leucaena* and *Prosopis* clustered in a well-resolved clade. *Rhizobium tropici* and *Mesorhizobium* sp. BR3804 also belonged to this clade as was reported by Ba *et al.* (2002). Strains of *R. tropici* can also nodulate *Leaucaena* sp. (Martínez-Romero *et al.*, 1991). As was previously reported by Zhang *et al.* (2000), the high sequence similarity between the *nodA* sequences of *Mesorhizobium ciceri* and *M. mediterraneum*, both isolated from *Cicer arietinum*, was also evident in the *nodA* tree reconstructed in this study.

From the phylogenetic tree based on partial *nodA* sequence data it is clear that different chromosomal backgrounds harbour the same symbiotic profile. The different *Cyclopia* species are not nodulated by a specific symbiotic genotype. Silva *et al.* (2003) studied the genetic structure of *R. etli* and *R. gallicum* strains in Mexico and concluded that the plant host impose selective pressure on the rhizobia which favours the maintenance of specific chromosomal and symbiotic combinations.

In their study on *Astragalus sinicus* rhizobia, Zhang *et al.* (2000) found that some rhizobia with different chromosomal genotypes had identical *nodA* genes, which suggest horizontal gene transfer of the *nod* genes between diverse rhizobia. It is possible that the *Burkholderia* isolates acquired the symbiotic genes through horizontal gene transfer from either *Bradyrhizobium* or *R. tropici* strains, which shared the niche with the *Burkholderia* organisms. The *Cyclopia* plants grow in soils with very low pH values (personal communication, J. Bloem). Curtis *et al.* (2002) reported the isolation of aciduric *Burkholderia* isolates from acidic soil capable of growth at pH ranges of approximately 3.5-8. These isolates shared high sequence similarity with *B. stabilis* (97%) and *B. fungorum* (98%). The nitrogen-fixing species *B. vietnamensis* was also isolated from acidic soil (Gillis *et al.*, 1995).

Burkholderia tuberum and *Methylobacterium nodulans* were both isolated from members of the genistoid alliance in the Papillionoideae. Members of the alliance produce characteristic quinolizidine alkaloids (Van Wyk, 2003). The characterisation of other members of this alliance might lead to the discovery of more novel associations.

CHAPTER 8

PHYLOGENETIC AND SYMBIOTIC CHARACTERISATION OF THE α-*PROTEOBACTERIA CYCLOPIA* ISOLATES

ABSTRACT

The isolates had been previously characterised with 16S-23S IGS-RFLP and partial 16S-sequencing analyses. In order to further investigate the phylogenetic position of these isolates near full-length 16S sequencing analysis was used. The symbiotic genotype of the isolates was determined with *nodA* sequence analysis. The isolates belonged to two *Bradyrhizobium* genomic species, *Rhizobium tropici* and a possible new genus in the α-*Proteobacteria*. All the isolates had been collected from acidic soil and the finding of *Bradyrhizobium* and *Rhizobium tropici* was thus expected. The significance of the isolate, which might be a member of a new genus, needs to be further investigated. The symbiotic genotype of all the isolates was similar to that of *Burkholderia tuberum*.

Keywords: 16S rDNA sequencing, *nodA* sequencing, *Bradyrhizobium*, *Rhizobium tropici*, acid-tolerant strains

INTRODUCTION

Legumes form a symbiotic association with root-nodulating bacteria, collectively called rhizobia. The gram-negative rhizobia belong to several genera in the α-*Proteobacteria*, namely *Allorhizobium* (De Lajudie *et al.*, 1998a), *Azorhizobium* (Dreyfus *et al.*, 1988), *Bradyrhizobium* (Jordan, 1984; Kuykendall *et al.*, 1992; Xu *et al.*, 1995; Yao *et al.*, 2002), *Mesorhizobium* (Jarvis *et al.*, 1997; De Lajudie *et al.*, 1998b; Wang *et al.*, 1999b; Veláquez *et al.*, 2001), *Sinorhizobium* (Chen *et al.*, 1988; De Lajudie *et al.*, 1994; Rome *et al.*, 1996b; Nick *et al.*, 1999; Wang *et al.*, 2002; Wei *et al.*, 2002, Toledo *et al.*, 2003) and *Rhizobium* (Jordan, 1984; Lindström, 1989; Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1993; Amarger *et al.*, 1997; Chen *et al.*, 1997; Van Berkum *et al.*, 1998; Wang *et al.*, 1998; Tan *et al.*, 2001b; Wei *et al.*, 2002; Squartini *et al.*, 2002; Wei *et al.*, 2003). New species of genera unknown to possess the ability to nodulate were described in recent years. *Devosiae neptuniae* (Rivas *et al.*, 2003) and *Methylobacterium nodulans* (Sy *et al.*, 2001) are both capable of nodulation. Recently, the description of species in the β-*Proteobacteria* capable of root-nodulation (Chen *et al.*, 2001; Vandamme *et al.*, 2002) clearly showed that the ability to nodulate rather than the phylogenetic position in the α-*Proteobacteria* warranted the name rhizobia (Geiger and López-Lara, 2002).

The interaction between the plant and the bacteria is specific, since the specific Nod-factor, lipo-chitooligosaccharide or LCO, as well as the concentration is important for nodulation (Perret *et al.*, 2000). The flavonoids of the host plant are recognised by the nodD protein, which then activates the transcription of the *nod* genes. The common genes (*nodABC*) are found in all rhizobia, while the host-specific genes (*nodFE*, *nodH*, *nodSU* and *nodZ*) are found in different combinations in rhizobial species. The common genes are involved in the formation of the LCO backbone, while the host-specific genes are involved in the addition of specific substitutions (Perret *et al.*, 2000; Zhang *et al.*, 2000). However, it has been shown that *nodA* and *nodC* are also host-specific genes, since nodC determines the length of the Nod-factor, while nodA recognises and transfers different acyl chains to the lipo-chitooligosaccharide backbone (Perret *et al.*, 2000).

The phylogeny of the different symbiotic genes, *nodA*, *nodB*, *nodC* and *nodD* resemble each other (Ueda *et al.*, 1995; Haukka et al., 1998). There is a correlation between the phylogeny

of the *nod* genes and host plant range (Haukka *et al.*, 1998; Zhang *et al.*, 2000; Laguerre *et al.*, 2001). The *nodA* has been found to be a good symbiotic marker, since the gene is present in all rhizobia as a single copy and *nodA* analysis reflects Nod-factor features (Haukka *et al.*, 1998; Chaintreuil *et al.*, 2001; Ba *et al.*, 2002). Evidence shows that the *nodA* phylogeny is similar to that of the host plants, which suggests *nod* gene evolution under host constraint and thus possible coevolution of the symbiotic partners as quoted by Radeva *et al.* (2001). The use of 16S rDNA sequence analysis has been found to be an excellent way to determine the phylogenetic position of isolates down to the genus level and has been used extensively (De Lajudie *et al.*, 1998; Khbaya *et al.*, 1998; Terefework *et al.*, 1998; Tan *et al.*, 2001b). All the *Cyclopia* isolates included in this study have been previously characterised with 16S-23S IGS-RFLP and partial 16S sequence analysis. The aim of this study was to further investigate the phylogenetic position of these isolates. *NodA* sequence analysis was conducted to determine the symbiotic genotype of these isolates.

MATERIALS AND METHODS

Bacterial strains used

The strains used in this study (Table 8.1) were received from the Agricultural Research Council-Plant Protection Research Institute (Private Bag X134, Pretoria, 0001, South Africa) and the Botany Department, University of Cape Town (Rondebosch, 7701, Cape Town, South Africa). All the sequences of the reference strains used in the 16S rDNA and *nodA* sequence analysis were obtained from the GenBank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi.nlm.nih.gov/Genbank/].

Maintenance of cultures

The isolates were maintained on yeast mannitol agar (YMA) [1% (m/v) mannitol (UniVar), 0.5% (m/v) K_2HPO_4 (Merck), 0.02% (m/v) $MgSO_4.7H_2O$ (Merck), 0.01% (m/v) NaCl (NT Chemicals), 0.04% (m/v) yeast extract (Biolab) and 1.5% (m/v) bacteriological agar (Biolab)] slants and the long-term storage of the isolates was done in glycerol. The isolates were grown in yeast mannitol broth (YMB) for 5-7 d at 25-28°C with vigorous shaking. The broth cultures were mixed 1:1 with sterile 50% (v/v) glycerol (Merck) in sterile cryotubes and stored in duplicate at -20°C and -70°C.

Table 8.1 Isolates included in the phylogenetic analysis of the 16S rDNA and *nodA*

Isolate	Host species	16S rDNA GenBank accession number	*NodA* GenBank accession number
UCT 42	*C. plicata*	AY178077	AY189242
UCT 50	*C. sessiliflora*	AY178082	AY189243
Cmey 1	*C. meyeriana*	AY178079	AY189257
Cplic 1	*C. plicata*	AY178081	AY189258
Cpub 4	*C. pubescens*	AY178080	AY189232

Extraction of genomic DNA

A modified method for proteinase-K (Roche Molecular Biochemicals) treated cells as described by Laguerre *et al.* (1997) was used. A fresh culture of each strain, which had been checked for purity, was streaked on a tryptone yeast (TY) agar slant [0.5% (m/v) tryptone (Difco), 0.3% (m/v) yeast extract (Biolab), 0.13% (m/v) $CaCl_2.6H_2O$ (UniLab), 1.5% (m/v) bacteriological agar] in a screw-cap tube. The strains were incubated at 28 °C and checked for sufficient growth. Sterile 4.5 ml dH_2O was added to the slant growth to harvest the cells. An inoculation loop was used to release cells clinging to the agar. The volume of the water added was adjusted according to the amount of growth. Less water was used if the growth was poor and *vice versa*. The cell-suspension was collected in a clear plastic tube and vortexed to ensure a uniform suspension. The absorbancy of the suspension was measured with dH_2O as the spectrophotometric blank at 620 nm. A formula was used to determine the volume of the suspension to be treated further. The volume to be used in ml is equal to 0.2 divided by the absorbancy at 620 nm. Two tubes of the same strain were filled with the appropriate volume of the suspension and centrifuged at 13 000 *g* for 5 minutes at 4 °C. The supernatant was discarded and the excess media blotted dry. One of the tubes was stored at -20 °C for future use. In the second tube, 100 µl ddH_2O, 100 µl Tris-HCl (10 mM, pH 8.2) and 10 µl proteinase-K (15 mg/ml) (Roche Molecular Biochemicals) were added to the cell pellet. The mixture was incubated at 55 °C overnight. In order to inactivate the proteinase-K the mixture was boiled for 10 minutes. The cell lysates were stored at -20 °C until needed.

Amplification of the 16S rDNA and the partial *nodA* genes

Amplification of the 16S rDNA gene of strains (Table 8.1) were performed with the primers fD1 and rP2 (Table 8.2) as described by Weisburg *et al.* (1991). The linker sequences of the

primers were not included in the primer synthesis. These shorter primers were designated fD1SHRT and rP2SHRT. The PCR mixture of each strain contained: 5 µl of the cell lysate, 50 pmol of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Gold Taq DNA polymerase (Southern Cross Biotechnology) in a 50 µl reaction volume. The PCR reactions were done on a Perkin Elmer GeneAmp PCR System 2400 thermocycler using the following thermal profile: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation (94 °C for 30 seconds), annealing (55 °C for 30 seconds) and extension (72 °C for 1 minute). This was followed by a final extension step at 72 °C for 5 minutes. The concentration, purity and size of the products were evaluated by running an aliquot (5 µl) of each reaction on 0.9% (m/v) horizontal agarose gels (Promega) (results not shown). The standard marker molecular marker VI (Roche Molecular Biochemicals) was included on each gel.

Table 8.2: Primers used in the amplification and/or sequencing of the 16S rDNA and the *nodA* genes

Primer name*	Primer sequence (5'-3')#	Target gene	Reference
fD1SHRT	5'-AGAGTTTGATCCTGGCTCAG-3'	16S rDNA	Weisburg *et al.*, 1991
rP2SHRT	5'-ACGGCTACCTTGTTACGACTT-3'	16S rDNA	Weisburg *et al.*, 1991
16SRNAII-S	5'-GTGTAGCGGTGAAATGCGTAG-3'	16S rDNA	Kuhnert *et al.*, 1996
16SRNAVI-S	5'-CTACGCATTTCACCGCTACAC-3'	16S rDNA	Kuhnert *et al.*, 1996
NodAunivF145u	5'-TGGGCSGGNGCNAGRCCBGA-3'	*nodA*	Moulin *et al.*, 2001
NodAR.brad	5'-TCACARCTCKGGCCCGTTCCG-3'	*nodA*	Moulin *et al.*, 2001

* All the primers were synthesised by Roche Molecular Biochemicals, Mannheim, Germany

\# Abbreviations: B= G/C/T, K= G/T, N= A/G/C/T, R= A/G, S= G/C

The partial *nodA* gene was amplified with the primers NodAunivF145u and NodAR.brad (Table 8.2) as used by Moulin *et al.* (2001) using the same PCR reaction mixture as described for 16S rDNA. The same thermal profile as used for the amplification of the 16S rDNA product was used, except that the annealing time was extended to 45 seconds. Analysis of the amplified product was done as described for the verification of the amplified 16S product (results not shown).

The amplification products of the 16S rDNA and *nodA* were purified, since any traces of unincorporated dNTPs, primers, etc. can negatively influence the sequencing reactions. The 16S products were purified using a Qiagen QIAquick PCR Purification kit (Southern Cross Biotechnology). Purification reactions were done as prescribed by the manufacturer. The

nodA primers are degenerate, resulting in additional amplification products. The products of the desired size were excised from the gel and then purified using the initial steps for gel extraction as prescribed by the manufacturers of the High Pure PCR purification kit (Roche Molecular Biochemicals). The gel solution (containing the desired fragment) was transferred to the columns of the Qiagen QIAquick PCR Purification kit (Southern Cross Biotechnologies). The protocol for the purification was then followed as prescribed by the manufacturers. The concentration and purity of each purification reaction was verified visually. An aliquot (1 µl) of each purified product was run on 0.9% (m/v) horizontal agarose gels (Promega) (results not shown). On each gel, a standard marker, molecular marker VI (Roche Molecular Biochemicals) was included.

16S rDNA and *nodA* sequencing

The sequences of the purified 16S rDNA and *nodA* products were determined using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (AmpliTaq^R DNA Polymerase, FS) (Perkin Elmer Applied Biosystems). The near full-length sequence of each 16S rDNA product was determined with the internal forward primer 16SRNAII-S (Kuhnert *et al.*, 1996), the forward primer fD1SHRT (Weisburg *et al.*, 1991), the internal reverse primer 16SRNAVI-S (Kuhnert *et al.*, 1996) and the reverse primer rP2SHRT (Weisburg *et al.*, 1991). The purified *nodA* products were sequenced with the forward primer NodAunivF145u (Moulin *et al.*, 2001). Each 5 µl sequencing reaction contained the following: 2 µl of the ready reaction mix supplied with the kit which contains the dye terminators, dNTP's, AmpliTaq^R DNA polymerase, $MgCl_2$ and Tris-HCl buffer pH 9.0; 12.5 pmol primer and approximately 100 ng template DNA. The sequencing reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler with the following thermal profile: an initial denaturation at 96 °C for 5 seconds followed by 25 cycles of denaturation (96 °C for 10 seconds), annealing (50 °C for 5 seconds) and extension (60 °C for 4 minutes). The products were precipitated using the protocol as suggested by the manufacturer. For analysis, the purified products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer (Perkin Elmer Applied Biosystems). The loading buffer was prepared by combining deionised formamide and 25 mM EDTA (pH 8.0) containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran. The resuspended products were denatured for 2 min at 90°C and loaded onto the ABI Prism model 377 DNA sequencer gel.

Phylogenetic analysis of the 16S rDNA and *nodA* sequences

The sequencing gels were analysed and sequences edited with the ABI Prism Sequencing Analysis 3.1 and the ABI Prism Sequencing Navigator 1.0.1 computer programmes (Perkin Elmer Applied Biosystems). The *nodA* sequences were edited visually comparing the nucleotides and their corresponding peaks. Both strands of the 16S rDNA products were sequenced with the primers used and the strands could be aligned to correct ambiguous positions. The final edited two strands were overlapped in the ABI Prism Sequencing Navigator 1.0.1 computer programme to form a continuos sequence reading. The ClustalX programme (Thompson *et al.*, 1997) was used to analyse the edited sequences as well as the reference sequences obtained from GenBank (Table 8.3 and Table 8.4), which were suitably edited. A distance matrix was constructed by pair-wise alignment of the sequences. The neighbour-joining method (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the distance matrix. Branch lengths were proportional to the estimated divergence along each branch. Confidence levels of the phylogenies were estimated with the bootstrap method (Felsenstein, 1985). The phylogenetic trees were visualised with NJplot (Perrière and Gouy, 1996).

Table 8.3: Reference sequences obtained from Genbank[1] included in the partial 16S rDNA sequence analysis.

Reference strain	Strain number	Host plant or relevant characteristics	Genbank[1] Accession number
Acetobacter diazotrophicus	LMG 7603^T	*Saccharum officinarum* root	X75618
Afipia clevelandensis	NS	NS	M69186
Afipia felis	NS	NS	M65248
Agrobacterium larrymoorei	NS	NS	Z30542
Agrobacterium radiobacter	ATCC 19358^T	NS	AJ389904
Agrobacterium rhizogenes	LMG 152	NS	X67224
Agrobacterium rubi	IFO 13261	NS	D14503
Agrobacterium tumefaciens	LMG 196	NS	X67223
Agrobacterium vitis	NCPPB 3554	*Vitis vinifera*	D14502
***α-Proteobacterium* strain**	LMG 20591	Agricultural soil	AY040361
Allorhizobium undicola	LMG 11875^T	*Neptunia natans*	Y17047
Aquaspirillum magnetotacticum	NS	NS	M58171
Azorhizobium caulinodans	LMG 6465^T	*Sesbania rostrata*	X67221
Azospirillum brasilense	DSM 2298	NS	X79734
Azospirillum lipoferum	NCIMB 11861	NS	Z29619
Bartonella bacilliformis	NS	NS	M65249
Beijerinckia indica	ATCC 9039^T	Acid soil	M59060
Blastobacter denitrificans	LMG 8443^T	Surface water	S46917
Bradyrhizobium elkanii	USDA 76^T	*Glycine max*	U35000
Bradyrhizobium japonicum	LMG 6138^T	*Glycine max*	X66024

Table 8.3: continued

Reference strain	Strain number	Host plant or relevant characteristics	Genbank[1] Accession number
Bradyrhizobium liaoningense	LMG 18230^T	*Glycine max*	AJ250813
***Bradyrhizobium* genosp. A**	BDV 5028	*Bossiaea ensata*	Z94811
***Bradyrhizobium* genosp. O**	BDV 5840	*Gompholobium huegelii*	Z94823
***Bradyrhizobium* sp.**	Ppau 3-41	*Phaseolus pauciflorus*	AF384137
Bradyrhizobium yuanmingense	CCBAU 10071^T	*Lespedeza cuneata*	AF193818
Brucella neotomae	ATCC 23459	NS	L26167
Devosia neptuniae	J1^T	*Neptunia natans*	AF469072
Ensifer adhaerens	LMG 20582	NS	AY040360
Kaistia adipata	Chj 404^T	Rhizobiaceae group	AY039817
Mesorhizobium amorphae	ACCC 19665	*Amorpha fruticosa*	AF041442
Mesorhizobium chacoense	PR-5^T	*Prosopis alba*	AJ278249
Mesorhizobium ciceri	UPM-Ca7^T	*Cicer arietinum*	U07934
Mesorhizobium huakuii	IAM 14158^T	*Astragalus sinicus*	D12797
Mesorhizobium loti	LMG 6125^T	*Lotus corniculatus*	X67229
Mesorhizobium mediterraneum	UPM-Ca36^T	*Cicer arietinum*	L38825
Mesorhizobium plurifarium	LMG 11892^T	*Acacia senegal*	Y14158
Mesorhizobium tianshanense	A-1BS^T	*Glycyrrhiza pallidiflora*	Y71079
Methylobacterium nodulans	ORS 2060^T	*Crotalaria podocarpa*	AF220763
Mycoplana dimorpha	IAM 13154^T	Soil	D12786
Nitrobacter winogradskyi	ATCC 14123	NS	L35507
Ochrobactrum anthropi	IAM 14119	NS	D12794
Paracoccus denitrificans	LMG 4218^T	Garden soil enriched with 5% K-Na-tartrate + 2% KNO_3	X69159
Phyllobacterium myrsinacearum	IAM 13584	NS	D12789
Phyllobacterium rubiacearum	IAM 13587	NS	D12790
Rhizobium etli	CFN 42^T	*Phaseolus vulgaris*	U28916
Rhizobium galegae	USDA 3394	*Galega officinalis*	AF025853
Rhizobium gallicum	R602sp^T	*Phaseolus vulgaris*	U86343
Rhizobium giardinii	H152^T	*Phaseolus vulgaris*	U86344
Rhizobium hainanensis	I66^T	*Desmodium sinuatum*	U71078
Rhizobium huautlense	USDA 4900^T	*Sesbania herbacae*	AF025852
Rhizobium indigoferae	CCBAU 71042^T	*Indigofera amblyantha*	AY034027
Rhizobium leguminosarum	LMG 8820	*Phaseolus vulgaris*	X67227
Rhizobium loessense	CCBAU 7190B^T	*Astragalus complanatus*	AF364069
Rhizobium mongolense	USDA 1844^T	*Medicago ruthenica*	U89817
Rhizobium sullae	IS123^T	*Hedysarum coronarium*	Y10170
Rhizobium tropici	CIAT 899^T	*Phaseolus vulgaris*	U89832
Rhizobium yanglingense	CCBAU 71462	*Coronilla varia*	AF195031
Rhodobacter sphaeroides	IF0 12203^T	NS	D16425
Rhodoplanes roseus	NS	NS	D25313
Rhodopseudomonas palustris	ATCC 17001	NS	D25312
Rickettsia rickettsii	ATCC VR 891	NS	M21293
Sinorhizobium arboris	HAMBI 1552^T	*Prosopis chilensis*	Z78204
Sinorhizobium fredii	LMG 6217^T	*Glycine max*	X67231
Sinorhizobium kostiense	HAMBI 1489^T	*Acacia senegal*	Z78203
Sinorhizobium kummerowiae	CCBAU 71714^T	*Kummerowia stipulacea*	AY034028
Sinorhizobium medicae	A321^T	*Medicago truncatula*	L39882
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*	X67222
Sinorhizobium morelense	Lc04^T	*Leucaena leucocephala*	AY024335
Sinorhizobium saheli	LMG 7837^T	*Sesbania pachycarpa*	X68390
Sinorhizobium terangae	LMG 6463	*Sesbania rostrata*	X68387
Sinorhizobium xinjiangensis	IAM 14142	*Glycine max*	D12796
Xanthobacter agilis	SA 35	NS	X94198
Xanthobacter autotrophicus	NS	NS	X94201
Xanthobacter flavus	NS*	NS	X94199

Table 8.4 ***NodA*** **sequences obtained from GenBank included in the comparative** ***nodA*** **sequence analysis**

Reference strain	Strain number	Host plant	Accession number GenBank[1]
Azorhizobium caulinodans	ORS 571[T]	*Sesbania rostrata*	L18897
Bradyrhizobium elkanii	USDA 94	NS	U04609
Bradyrhizobium japonicum	110spc4	NS	AF322013
Bradyrhizobium sp.	NC92	*Arachis hypogaea*	U33192
Bradyrhizobium sp.	WM9	*Lupinus* sp.	AF222753
Bradyrhizobium sp.	ANU289	*Parasponia* sp.	X03720
Bradyrhizobium sp.	ORS 285	Photosynthetic isolate	AF284858
Bradyrhizobium sp.	ORS 287	*Aeschynomene afraspera*	AJ437607
Bradyrhizobium sp.	ORS 301	*Aeschynomene americana*	AJ437608
Bradyrhizobium sp.	ORS 302	*Aeschynomene pfundii*	AJ437609
Bradyrhizobium sp.	ORS 304	*Aeschynomene elaphroxylon*	AJ437610
Bradyrhizobium sp.	ORS 309	*Aeschynomene uniflora*	AJ437611
Bradyrhizobium sp.	ORS 336	*Aeschynomene afraspera*	AJ437612
Bradyrhizobium sp.	ORS 364	*Aeschynomene nilotica*	AJ437613
Burkholderia tuberum	STM 678[T]	*Aspalathus carnosa*	AJ302321
Mesorhizobium ciceri	USDA 3383	*Hedysarum boreale*	AJ250140
Mesorhizobium loti	NZP 2213[T]	*Lotus corniculatus*	L06241
Mesorhizobium mediterraneum	USDA 3392	NS	AJ250141
Mesorhizobium plurifarium	ORS 1096	*Acacia tortilis* subsp. *raddiana*	AJ302678
Mesorhizobium sp.	BR3804	*Chamaecrista ensiformis*	Z95249
Mesorhizobium sp.	DW0366	*Acacia polycantha*	Z95248
Mesorhizobium sp.	7653R	*Astragalus sinicus*	AJ249353
Mesorhizobium sp.	N33	*Oxytropis arctobia*	U53327
Mesorhizobium tianshanense	USDA 3592	NS	AJ250142
Methylobacterium nodulans	ORS 2060[T]	*Crotalaria podocarpa*	AF266748
Rhizobium etli	CFN 42[T]	*Phaseolus vulgaris*	NC_004041
Rhizobium galegae	HAMBI 1174	*Galega orientalis*	X87578
Rhizobium leguminosarum bv. ***phaseoli***	NS	NS	M58625
Rhizobium leguminosarum bv. ***trifolii***	ANU843	NS	X03721
Rhizobium leguminosarum bv. ***viciae***	NS	NS	Y00548
Rhizobium tropici	CFN 299	*Phaseolus* sp.	X98514
Sinorhizobium arboris	HAMBI 1700	*Acacia senegal*	Z95235
Sinorhizobium fredii	USDA 257	NS	M73699
Sinorhizobium kostiense	HAMBI 1489[T]	*Acacia senegal*	Z95236
Sinorhizobium meliloti	NS	NS	X01649
Sinorhizobium saheli	ORS 609	*Sesbania cannabina*	Z95241
Sinorhizobium sp.	NGR234	Broad host range	AE000076
Sinorhizobium sp.	BR827	*Leucaena leucocephala*	Z95232
Sinorhizobium sp.	BR4007	*Prosopis juliflora*	Z95240
Sinorhizobium sp.	M6	*Prosopis* sp.	Z95233
Sinorhizobium sp.	ORS 1085	*Acacia tortilis* subsp. *raddiana*	AJ302677
Sinorhizobium terangae	ORS 1009	*Acacia laeta*	Z95237

1 Genbank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi.nlm.nih.gov/Genbank/]

T Type strain

ACCC Agricultural Center of Culture Collection, Chinese Academy of Agriculture, Beijing, China

ATCC American Type Culture Collection, Rockville, Maryland, USA

CCBAU Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China

CFN	Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico
CIAT	Rhizobium Collection, Centro International de Agricultura Tropical, Cali, Columbia
DSM	Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany
HAMBI	Culture Collection of the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland
IAM	Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan
IFO	Institute for Fermentation, Osaka, Japan
LMG	BCCM™/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium
NCIMB	National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, UK
NCPPB	National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Hertfordshire, UK
NCTC	National Collection of Type Cultures, Central Public Health Laboratory, London, UK
NZP	Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand
ORS	ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal
STM	Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France
UPM	Universidad Politécnica Madrid, Spain
USDA	United States Department of Agriculture, *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, MD, USA

RESULTS

Amplification of the 16S rDNA and the *nodA* gene

The primers fD1SHRT and rP2SHRT (Weisburg *et al.*, 1991) were able to amplify the 16S rDNA gene of the isolates and size of the products corresponded to the expected size of approximately 1500 bp. The partial *nodA* gene was amplified with the primers NodAunivF145u and NodAR.brad used by Moulin *et al.*, (2001) and a product of the expected size of 455 bp was obtained. Due to the degenerate nature of the primers, faint additional bands were visible on the horizontal agarose gel electrophoresis. These bands did not hinder the sequencing reaction since the correct fragment was excised and purified.

Sequence analysis of the 16S rDNA and the *nodA* gene

The sequencing reactions of the 16S rDNA products conducted with the four primers were able to give an unambiguous DNA sequence for each isolate of approximately 1250 bp. The last part of each strand had ambiguous positions. However, the ambiguous positions could be resolved using the other strand. The two edited strands of each isolate could be overlapped to give an uninterrupted sequence. The partial *nodA* sequence could be determined with the primer used. An unambiguous strand of approximately 390 bp was obtained for each isolate. All the sequences were deposited in the GenBank database. The relevant accession numbers can be seen in Table 8.1.

Phylogenetic analysis of the 16S rDNA and the *nodA* gene

The comparative sequence analysis based on the 16S rDNA sequences reflected the polyphyletic nature of the rhizobia (see Fig. 8.1). The rhizobial genera formed five distinct lineages; the *Sinorhizobium* lineage, *Mesorhizobium* lineage, *Rhizobium* lineage, *Bradyrhizobium* lineage and the *Azorhizobium* lineage. The root-nodulating species, *Devosia neptuniae* and *Methylobacterium nodulans* belonged to two additional lineages. The relatively close phylogenetic relationship between the genera *Allorhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* is evident from the tree. The significant phylogenetic separation between *Azorhizobium* and *Bradyrhizobium* and the other rhizobial genera is evident from the tree.

Isolates Cplic 1 and UCT 50 clustered on the *Rhizobium tropici-Agrobacterium rhizogenes* branch, which formed a well-resolved clade supported by a bootstrap value of 100%. Isolate Cplic 1 displayed sequence similarity values of 99.2% and 99.5% with *R. tropici* and *A. rhizogenes* respectively. *A. rhizogenes* and *R. tropici* shared 99.6% and 99.0% with UCT 50.

Isolates UCT 42, UCT 53 and Cmey 1 did not belong to any of the described *Bradyrhizobium* reference strains. These isolates belonged to two different *Bradyrhizobium* genospecies based on 16S rDNA sequence analysis. Cmey 1 shared 100% 16S sequence similarity with the Australian *Bradyrhizobium* genosp. O. The branch leading to the two strains was supported with a bootstrap value of 100%. Isolates UCT 42 and UCT 53 shared 100% sequence

Figure 8.1 (next page): **Phylogenetic tree reconstructed with the neighbour-joining algorithm from a distance matrix of the comparative 16S rDNA sequences analysis of the *Cyclopia* isolates and representative reference strains from the α-*Proteobacteria*. The branch lengths are proportional to the phylogenetic divergence between isolates. The vertical branches are set for clarity and are non-informative. The scale bar indicates 2% sequence divergence. Bootstrap values higher than 600 are indicated on the tree.**

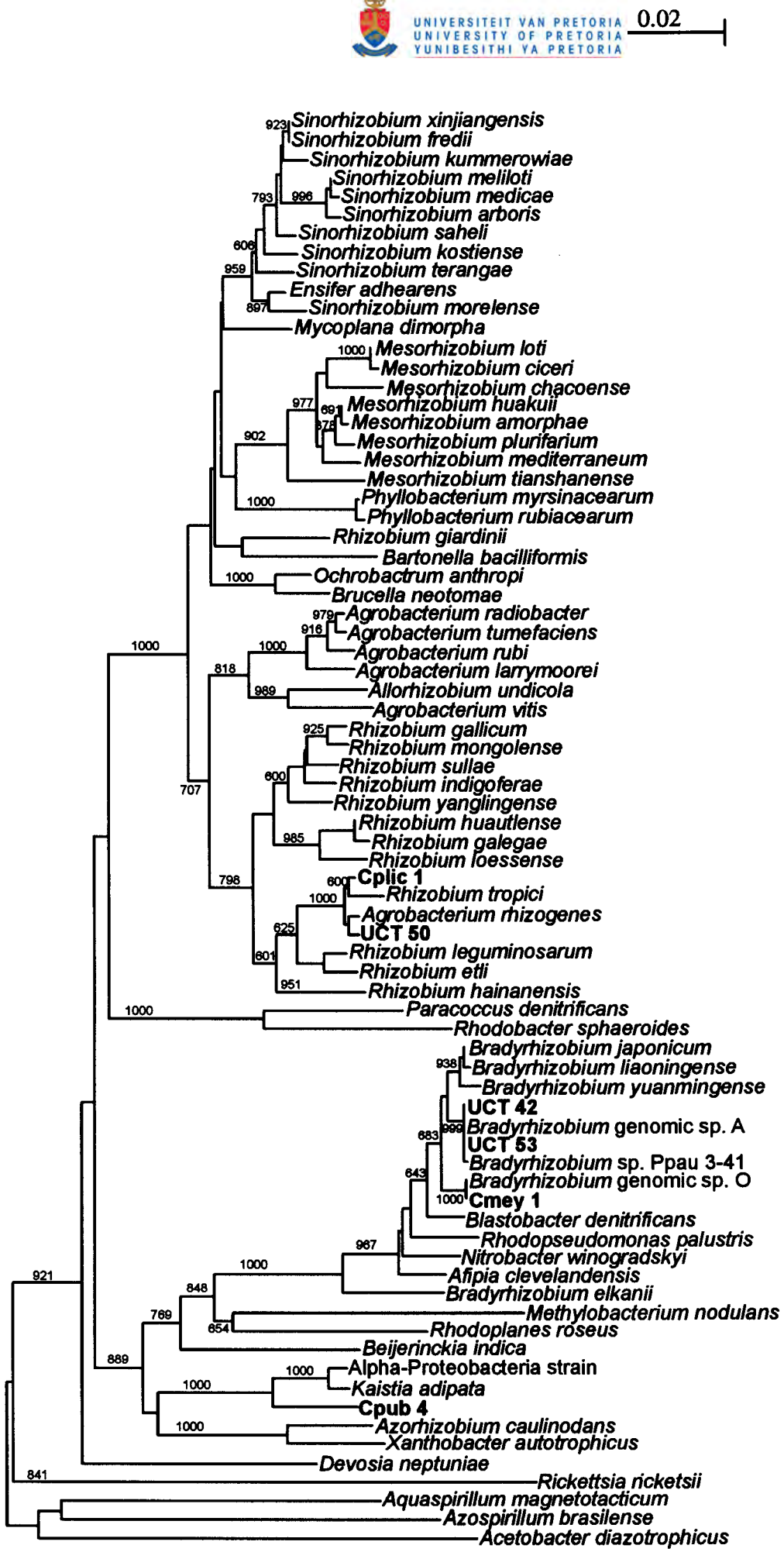
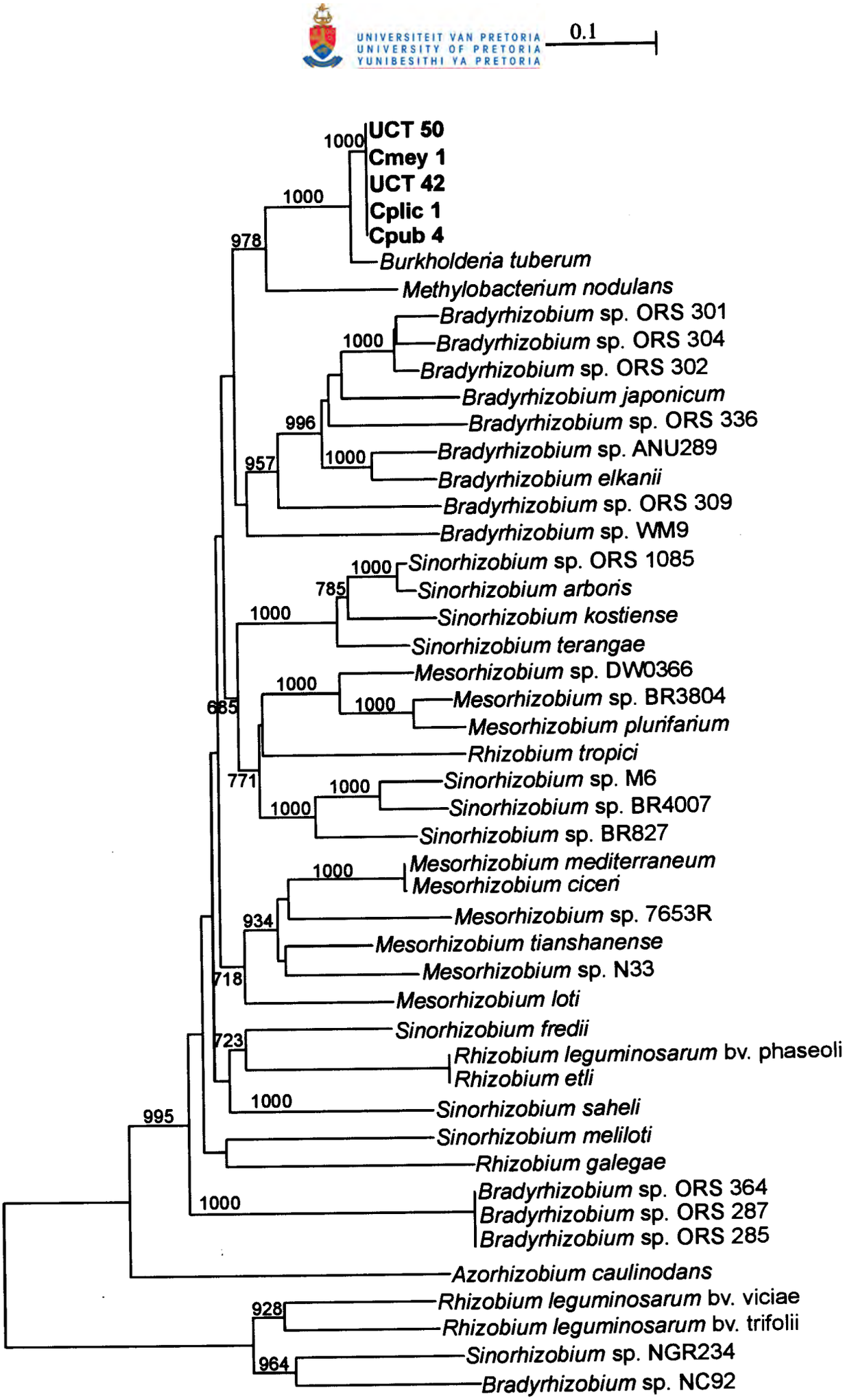

0.02
Sinorhizobium xinjiangensis
Sinorhizobium fredii
Sinorhizobium kummerowiae
Sinorhizobium meliloti
Sinorhizobium medicae
Sinorhizobium arboris
Sinorhizobium saheli
Sinorhizobium kostiense
Sinorhizobium terangae
Ensifer adhearens
Sinorhizobium morelense
Mycoplana dimorpha
Mesorhizobium loti
Mesorhizobium ciceri
Mesorhizobium chacoense
Mesorhizobium huakuii
Mesorhizobium amorphae
Mesorhizobium plurifarium
Mesorhizobium mediterraneum
Mesorhizobium tianshanense
Phyllobacterium myrsinacearum
Phyllobacterium rubiacearum
Rhizobium giardinii
Bartonella bacilliformis
Ochrobactrum anthropi
Brucella neotomae
Agrobacterium radiobacter
Agrobacterium tumefaciens
Agrobacterium rubi
Agrobacterium larrymoorei
Allorhizobium undicola
Agrobacterium vitis
Rhizobium gallicum
Rhizobium mongolense
Rhizobium sullae
Rhizobium indigoferae
Rhizobium yanglingense
Rhizobium huautlense
Rhizobium galegae
Rhizobium loessense
Cplic 1
Rhizobium tropici
Agrobacterium rhizogenes
UCT 50
Rhizobium leguminosarum
Rhizobium etli
Rhizobium hainanensis
Paracoccus denitrificans
Rhodobacter sphaeroides
Bradyrhizobium japonicum
Bradyrhizobium liaoningense
Bradyrhizobium yuanmingense
UCT 42
Bradyrhizobium genomic sp. A
UCT 53
Bradyrhizobium sp. Ppau 3-41
Bradyrhizobium genomic sp. O
Cmey 1
Blastobacter denitrificans
Rhodopseudomonas palustris
Nitrobacter winogradskyi
Afipia clevelandensis
Bradyrhizobium elkanii
Methylobacterium nodulans
Rhodoplanes roseus
Beijerinckia indica
Alpha-Proteobacteria strain
Kaistia adipata
Cpub 4
Azorhizobium caulinodans
Xanthobacter autotrophicus
Devosia neptuniae
Rickettsia ricketsii
Aquaspirillum magnetotacticum
Azospirillum brasilense
Acetobacter diazotrophicus

Figure 8.2 (next page): **Unrooted neighbour-joining tree reconstructed from the comparative sequence analysis of the partial *nodA* sequences of the *Cyclopia* isolates and reference *nodA* sequences obtained from GenBank. Horizontal branch lengths are proportional to the phylogenetic divergence between isolates, while the vertical branch lengths are non-informative and set for clarity. The scale bar indicates 10% sequence divergence. Bootstrap probabilities higher than 600 are indicated at the respective nodes.**



similarity with the Austalian *Bradyrhizobium* genosp. A and nearly 100% similarity with *Bradyrhizobium* sp. Ppau3-41 from Mexico. This clade was also supported with a high bootstrap value (99.9%). These reference strains were included in the comparative analysis since BLAST (Altschul *et al.*, 1990) results revealed high sequence similarity with the *Cyclopia* isolates.

Isolate Cpub 4 showed a sequence similarity value of approximately 97.1% with both the α-*Proteobacteria* strain LMG 20591 isolated from soil and '*Kaistia adipata*'. The GenBank database was searched for similar sequences with the BLAST algorithm (Altschul *et al.*, 1990) and these strains displayed the highest sequence similarity with Cpub 4. Strain '*Kaistia adipata*' and the α-*Proteobacteria* strain LMG 20591 showed a sequence similarity value of 99.3%. The phylogenetic closest rhizobial reference strain, *Azorhizobium caulinodans* showed a sequence similarity value of 92.7% with Cpub 4.

The *nodA* gene of all the *Cyclopia* isolates was highly conserved. The *nodA* gene of *Burkholderia tuberum* was the closest phylogenetic neighbour of the *Cyclopia nodA* gene, sharing 97% sequence similarity. The branch leading to the *nodA* genes of the *Cyclopia* isolates was well resolved (100%), while the clade of the *nodA* genes of *B. tuberum* and the *Cyclopia* isolates were also supported with a bootstrap value of 100%.

DISCUSSION

The polyphyletic nature of the rhizobia (the root-nodulating organisms) was reflected in the phylogenetic tree based on 16S rDNA sequence data. Genera (such as *Agrobacterium*, *Bartonella*, *Brucella*, *Mycoplana*, etc.) unable to nodulate cluster among the root-nodulating bacteria (Willems and Collins, 1993). Most of the grouping is similar to that obtained by other researchers (Tan *et al.*, 2001b; Wei *et al.*, 2002; Yao *et al.*, 2002; Willems *et al.*, 2003), however with some differences.

It is clear that an extensive revision of the genus *Bradyrhizobium* is inevitable. Phylogenetic trees show the polyphyletic nature of the genus *Bradyrhizobium* and the tree constructed in this study supported this.

The phylogenetic relationships within the rhizobial genera could be resolved, though the distinction between the species *Sinorhizobium fredii* and *S. xinjiangense* could not be made. This problem was also experienced by other researchers (Tan *et al.*, 2001; Wei *et al.*, 2002; Yao *et al.*, 2002), which raised questions about the taxonomic validity of *S. xinjiangense*. Peng *et al.* (2002) included novel *S. xinjiangense* isolates in a genotypic (16S sequencing, IGS sequencing and DNA-DNA hybridisation) and phenotypic (SDS-PAGE) analyses and managed to differentiate between these closely related species.

In the *Sinorhizobium* clade which was supported with a high bootstrap value, the two non-nodulating species, *Ensifer adhaerens* and *S. morelense* are more related to each other than to the other *Sinorhizobium* species as previously found by Wang *et al.* (2002) and Willems *et al.* (2003). Willems *et al.* (2003) have requested an opinion for the transfer of *Ensifer adhearens* to the genus *Sinorhizobium*.

The polyphyletic nature of the genus *Rhizobum* prompted Young *et al.* (2001) to give an emended description for the genus *Rhizobium*. The emended description of the genus *Rhizobium* contained all the current *Rhizobium* species, as well as all the species from *Agrobacterium* and the single species genus, *Allorhizobium*. The tree constructed in this study supports the separate genus status of *Allorhizobium*. *Agrobacterium vitis* was more related to *Allorhizobium undicola* than to the *Agrobacterium* type species as found by De Lajudie *et al.* (1998). A need for the revision of the taxonomic position of *A. vitis* was pointed out by De Lajudie *et al.* (1998).

Rhizobium giardinii formed a separate lineage as found by other researchers (Amarger *et al.*, 1997). This species might constitute a possible novel genus (Laguerre *et al.*, 2001). *R. galegae*, *R. huautlense* and *R. loessense* formed a well-supported clade within the *Rhizobium* group. Several authors argued for the formation of a possible new genus for *R. galegae* and its closest neighbours. However, Wei *et al.* (2003) suggested the inclusion of more *R. galegae*-related bacteria before a decision is made on the taxonomic position of these strains. In the phylogenetic tree reconstructed in this study, it would seem that *R. galegae*, *R. huautlense* and *R. loessense* clearly belong to the *Rhizobium* genus. However, this association might change with the addition of more sequences, since the *Rhizobium* clade (including *R. gallicum*, *R. mongolense*, *R. sullae*, *R. indigoferae*, *R. yanglingense*) in which *R. galegae*, *R. huautlense* and *R. loessense* cluster, is not supported by a significant bootstrap value and thus

not stable. The topology of the tree could also be influenced by the algorithm used to construct the tree (Young *et al.*, 2001).

The two *Cyclopia* isolates UCT 50 and Cplic 1 clearly belong to the species *R. tropici*, which is phylogenetically closer related to *Agrobacterium rhizogenes* than to the other *Rhizobium* species. This high sequence similarity between *R. tropici* and *A. rhizogenes* has been well documented (Laguerre *et al.*, 1994; Khbaya *et al.*, 1998; Terefework *et al.*, 1998; Wei *et al.*, 2003).

Except for isolate Cpub 4, the rest of the *Cyclopia* isolates (UCT 42, UCT 53 and Cmey 1) belong to the genus *Bradyrhizobium*. It is clear that the isolates do not belong to one of the described species, but rather to two genomic species, *Bradyrhizobium* genomic sp. A and *Bradyrhizobium* genomic sp. O. The genomic species are related to *B. japonicum* (Lafay and Burdon, 1998). The *Bradyrhizobium* genomic sp. A strains were the dominant genotype isolated in the study of Lafay and Burdon (1998) in their extensive study to determine the rhizobia nodulating indigenous scrubby legumes in Southeastern Australia. Only six of the 745 rhizobial strains isolated in the study of Lafay and Burdon (1998) belonged to *Bradyrhizobium* genomic sp. O. The finding of the same *Bradyrhizobium* genospecies in South Africa and Australia again shows that a specific chromosomal genotype is not restricted to one continent.

The 16S rDNA sequences of the *Bradyrhizobium* genus display high similarity values, which makes it difficult to resolve the close relationships within the genus (Van Berkum and Fuhrmann, 2000). The use of DNA-DNA hybridisation will aid to clarify the position of the *Bradyrhizobium* isolates from *Cyclopia* spp., since this method is still an important criterion for species delineation (Stackebrandt and Goebel, 1994). It is clear that an extensive revision of the genus *Bradyrhizobium* is inevitable. Phylogenetic trees show the polyphyletic nature of the genus *Bradyrhizobium*, since the species *B. japonicum*, *B. liaoningense* and *B. yuanmingense* are more related to genera such as *Afipia* and *Blastobacter* than *B. elkanii*. The existence of several genomic species in the genus shows the huge scope for taxonomic revision of this genus.

Isolate Cpub 4, '*Kaistia adipata*' and the alpha-Proteobacteria strain isolated from agricultural soil might possibly belong to a new species, since they share more than 97% sequence

homology. However, sequence homology of 97% is not a guarantee for species identity, since species might share high sequence similarity, but differ significantly in their genotype, resulting in low DNA homology values (Stackebrandt and Goebel, 1994). DNA-DNA hybridisations and the determination of the G + C content would have to be done to determine the possible species status of these strains, since these techniques are required for species description (Wayne *et al.*, 1987).

The determination of the *nodA* sequence was an easy method to study host plant range. The *nodA* gene is a single copy gene (Haukka *et al.*, 1998) and direct PCR sequencing could thus be done. The topology of the *nodA* tree is in broad agreement with that obtained by other researchers (Haukka *et al.*, 1998). It is quite clear from the *nodA* tree that all the *Cyclopia* isolates contain the same conserved *nodA* gene. As was previously found by other authors, it is clear that different chromosomal genotypes harbour the same symbiotic genotype (Guo *et al.*, 1999; Zhang *et al.*, 2000; Laguerre *et al.*, 2001). The *Cyclopia* isolates probably acquired the symbiotic genes through horizontal gene transfer as proposed by other researchers (Haukka *et al.*, 1998). All of the five *Cyclopia* isolates have been collected from different geographical positions in the fynbos distribution pattern.

CHAPTER 9

CONCLUSIONS

In the study of the root-nodulating bacteria associated with the indigenous South African scrubby legume genus *Cyclopia*, several novel genotypes have been found. Approximately 42% of the Cyclopia isolates belong to one of the new root-nodulating species in the β-*Proteobacteria*, *Burkholderia tuberum* described by Vandamme *et al.* (2002). The collection of *B. tuberum* strains characterised in this study is the largest reported to date.

Members of the *Burkholderia* genus are highly related based on 16S rDNA sequence analysis, since several species share more than 97% sequence homology with each other. DNA-DNA hybridisation analysis is necessary to delineate species due to the inadequacy of 16S rDNA sequence analysis for this purpose (Stackebrandt and Goebel, 1994). Approximately 52% of the isolates are clearly new *Burkholderia* spp. Based on IGS-RFLP and partial 16S rDNA sequence analyses previously found similar strains will be included in the description of new species. This would be done after DNA-DNA hybridisation analysis. Unfortunately, our laboratory is not equipped to do this study. Substrate utilisation patterns of several representative strains of each possible novel species should also be done. The inclusion of several strains in the description of a new species is of utmost importance to refrain from describing species based on a single strain.

The finding that most of the root-nodulating isolates associated with the *Cyclopia* host plants belong to the β-*Proteobacteria* implies that co-evolution of the *Cyclopia* host plants and *Burkholderia* spp. has taken place for quite while.

All of the isolates, which belonged to the α-*Proteobacteria* with the exception of one strain (Cpub 4), belonged to the acid-tolerant *Bradyrhizobium* genus and the acid-tolerant *Rhizobium* species, *R. tropici*. The soils from the sites were mostly acidic and the finding of these rhizobia was thus not surprising.

One isolate (Cpub 4) and two other isolates, whose sequences were obtained from the GenBank database, might be members of a novel genus in the α-*Proteobacteria*. Future work should include phenotypic and more genotypic analyses of all three isolates and if possible the inclusion of similar strains to satisfy the requirements for the description of new species and genera.

The symbiotic genes of all the isolates in this study have been found to be highly conserved. Different chromosomal genotypes harbour the same symbiotic genotype, which suggests that horizontal gene transfer occurred between these root-nodulating organisms.

The focus shift from studying only rhizobia associated with agricultural crops to the characterisation of root-nodulating rhizobia of legumes indigenous to a given geographical region, led to the description of novel rhizobial and seemingly unrelated isolates. This would undoubtedly continue and would help to gain more information on the legume-rhizobium symbiosis and the evolutionary mechanisms involved.

CHAPTER 10

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APPENDIX

A1 water	A2 α-cyclodextrin	A3 dextrin	A4 glycogen	A5 tween-40	A6 tween-80	A7 *N*-acetyl-D-galactosamine	A8 *N*-acetyl-D-glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 *i*-erythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentobiose	B6 α-D-glucose	B7 *m*-inositol	B8 α-D-lactose	B9 lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-methyl D-glucoside	C3 D-psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turanose	C10 xylitol	C11 methyl pyruvate	C12 mono-methyl succinate
D1 acetic acid	D2 *cis*-aconitic acid	D3 citric acid	D4 formic acid	D5 D-galactonic acid lactone	D6 D-galacturonic acid	D7 D-gluconic acid	D8 D-glucosaminic acid	D9 D-glucoronic acid	D10 α-hydroxybutyric acid	D11 β-hydroxybutyric acid	D12 γ-hydroxybutyric acid
E1 *p*-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutaric acid	E5 α-keto valeric acid	E6 D,L-lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D-saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucuronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl-glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl-L-aspartic acid	F12 glycyl-L-glutamic acid
G1 L-histidine	G2 hydroxy-L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenylalanine	G6 L-proline	G7 L-pyroglutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 D,L-carnitine	G12 γ-amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3-butanediol	H9 glycerol	H10 D,L-α-glycerol phoshate	H11 glucose-1-phosphate	H12 glucose-6-phosphate