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).

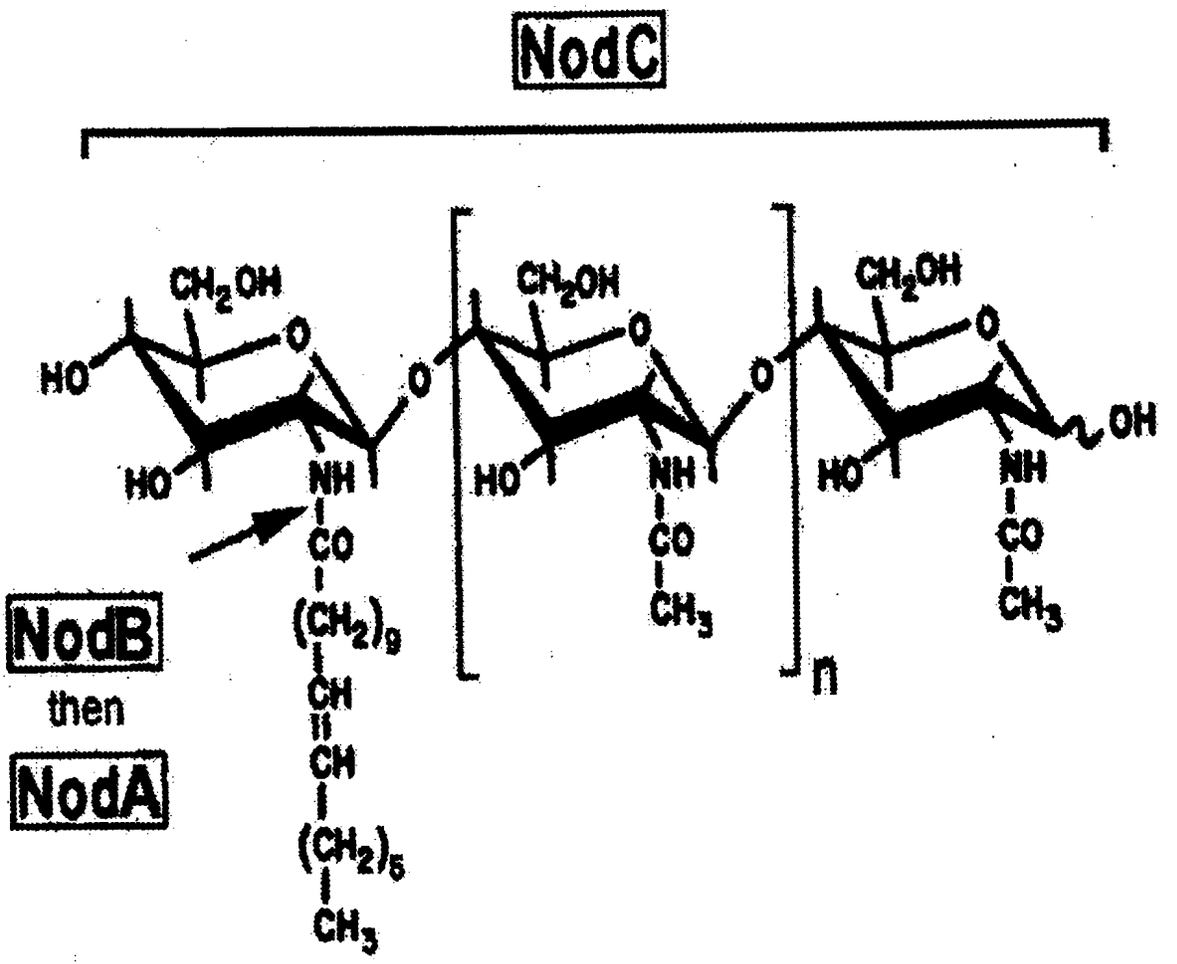

Figure 2.1: **General structure of a Nod-factor. NodC links the glucosamine monomers to form a chitin-like backbone. NodB removes an acetyl group from the non-reducing terminal residue of the oligosaccharide backbone. NodA transfers a fatty acyl chain to the acetyl-free C-2 carbon of the non-reducing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

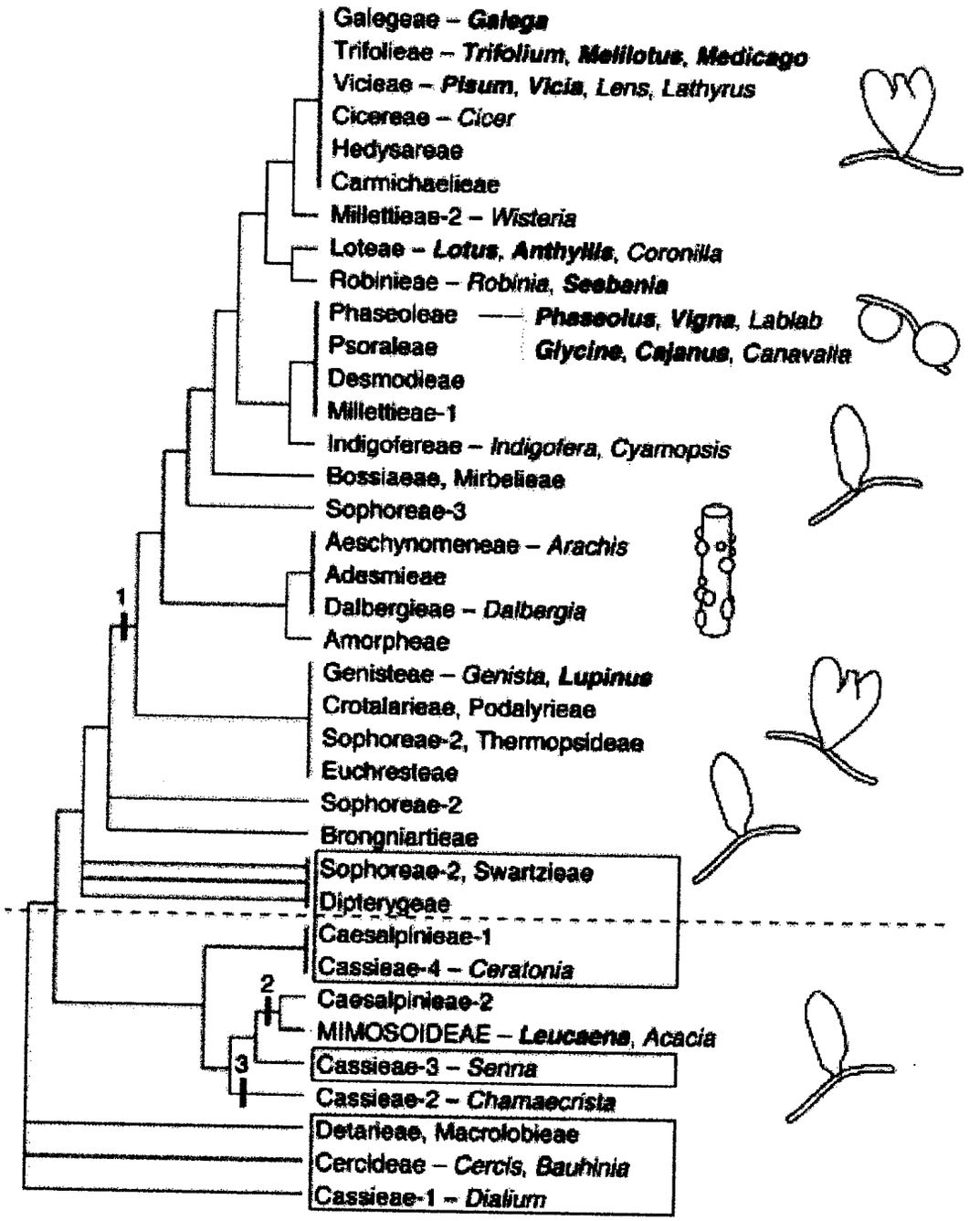

Figure 3.1: **Phylogeny of Fabaceae as obtained from chloroplast gene ribulose-1-5-bisphosphate carboxylase / oxygenase (*rbc*L) sequence data. All the tribes above the line belong to the Papilionoideae. Tribes in boxes do not nodulate. The numbers indicate possible origins of nodulation based on parsiminious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

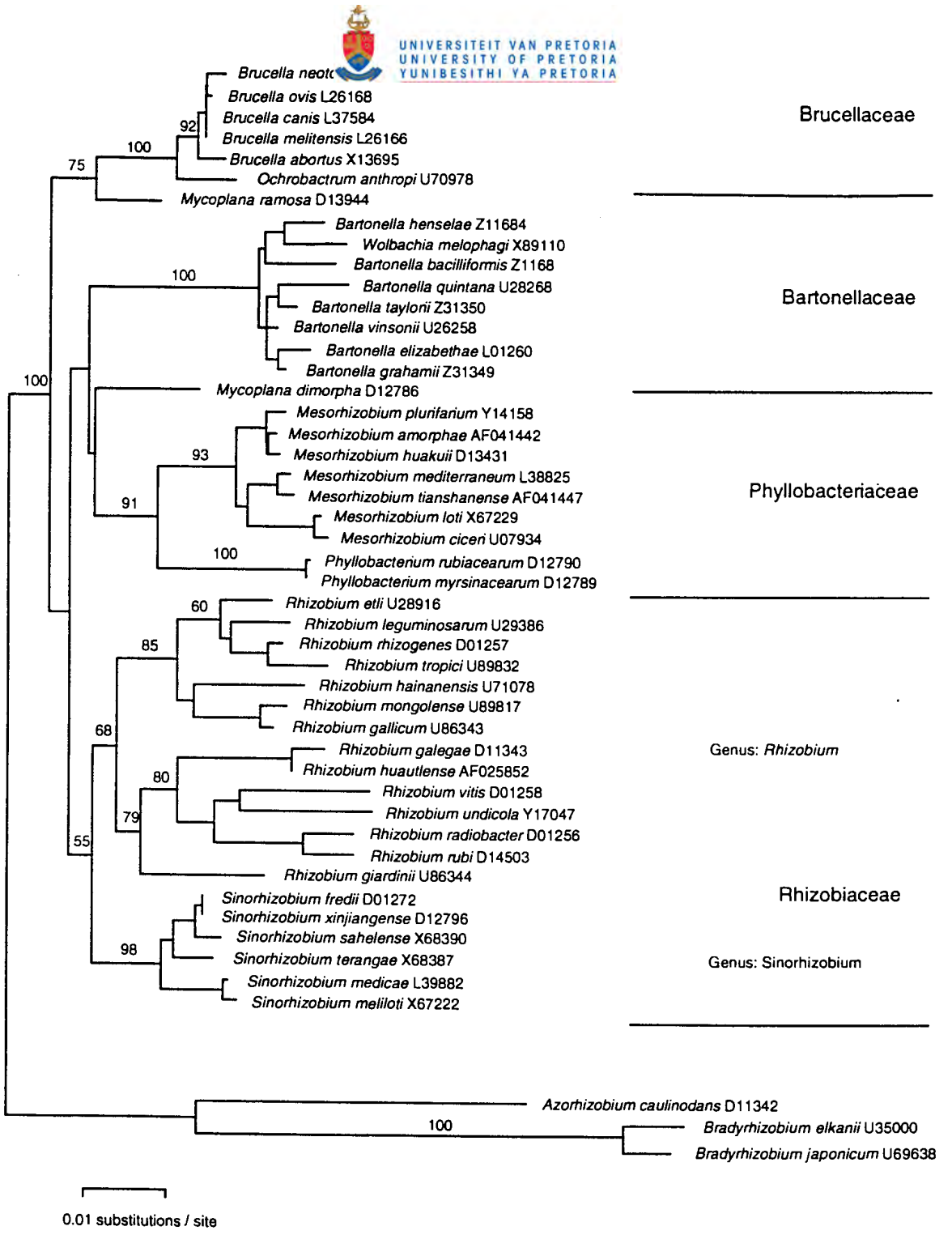

Figure 5.1: **Neighbour-joining tree showing the relationships of the *Rhizobiaceae* and closely-related families in the order Rhizobiales reconstructed from 16S rDNA sequence data. The branch lengths are proportional to the estimated number of nucleotide substitutions and bootstrap probabilities (percentages)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