CHAPTER 1

All writing is hard. Creative writing is intellectual drudgery of the hardest kind.
- Paul Johnson

CHAPTER 1

INTRODUCTION

The human population is expected to double in the next 40 years and it is thus important that in order to feed the multitude, crop yields should be increased concomitantly without harming the ecology and endangering human health (Caetano-Anollés, 1997). The symbiosis between legumes and rhizobia, which makes biological nitrogen fixation possible, may partially be the answer to the food problem facing man.

The plant family, Fabaceae consists of 16 000 to 19 000 species in approximately 750 genera. The family is divided into three subfamilies, namely the Mimosoideae (66 genera), Caesalpinoideae (177 genera) and Papilionoideae (505 genera) (Allen and Allen, 1981). The nodulation status of the subfamily Caesalpinoideae is low and many non-nodulating genera exist. However, the nodulation status of the other two subfamilies is high (Allen and Allen, 1981; Oyaizu, Matsumoto, Minamisawa and Gamou, 1993).

Recently several supposedly non-nodulating genera were found to be nodulated, i.e *Cassia* (Oyaizu *et al.*, 1993; Gao, Sun, Li, Wang and Chen, 1994; Dagutat, 1995), *Bauhinia* (Dagutat, 1995), *Schizolobium* (Dagutat, 1995), *Senna* (Kruger, 1998) and *Ceratonia* (El Idrissi, Aujjar, Belabed, Dessaux and Filali-Maltouf, 1996). Till recently only one non-legume genus, *Parasponia*, was known to be nodulated by a specific *Bradyrhizobium* strain (Jordan, 1984), but recently Basu, Ghosh and Dangar (1997) reported the nodulation of the royal palm (*Roystonea regia*) by a specific *Rhizobium* strain.

The rhizobia form part of the family *Rhizobiaceae* in the α-2-subgroup of the *Proteobacteria* (Jordan, 1984). Currently the nodulating bacteria are grouped into six distinct genera, namely *Allorhizobium* (De Lajudie, Laurent-Fulele, Willems, Torck, Coopman, Collins, Kersters, Dreyfus and Gillis, 1998a), *Azorhizobium* (Dreyfus, Garcia and Gillis, 1988), *Bradyrhizobium* (Jordan, 1984), *Mesorhizobium* (Jarvis, Van Berkum, Chen, Nour, Fernandez, Cleyet-Marel, and Gillis, 1997), *Rhizobium* (Jordan, 1984) and *Sinorhizobium* (Chen, Yan and Li, 1988).

The extensive use of legumes and the economic gain that can be achieved by inoculating legumes and in the future non-legumes as well, is well known (Strijdom, 1998). In order to discover better performing and better adapted inoculants, the wealth of indigenous rhizobia has to be studied and exploited (Dagutat, 1995).

Till now only two South African scientists have studied the diversity of indigenous rhizobia originating from geographically different regions. Dagutat (1995) isolated rhizobia from 147 nodulated legumes. The results of whole-cell protein (WCP) analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) indicated that some of the isolates are related to commercial inoculant strains. The possibility of new species, even a new genus in the South African rhizobia goldmine is clear from the results. Kruger (1998) used SDS-PAGE of WCP, Biolog and RFLP of 16S rDNA as part of a polyphasic approach to identify and determine the diversity of the indigenous rhizobia.

The rRNA molecules are present in all living organisms (except viruses), have a conserved function and the molecules contain both variable and conserved regions (Woese, 1987). The molecules are consequently suitable for use as molecular chronometers enabling phylogenetic lines of descent to be inferred from the rRNA sequences (Woese, 1987; Priest and Austin, 1993). The method can be used to differentiate between genera, species and, more distantly related strains of a species but not between recently diverged strains or species (Stackebrandt and Goebel, 1994). The partial sequence data can be used to identify or assign isolates to stable phylogenetic groups (Ludwig, Strunk, Klugbauer, Klugbauer, Weizenegger, Neumaier, Bachleitner and Schleifer, 1998). Graham, Sadowsky, Keyser, Barnet, Bradley, Cooper, De Ley, Jarvis, Roslycky, Strijdom and Young (1991) recommended the use of either rDNA:DNA hybridisation or 16S rRNA analysis as part of the polyphasic approach to describe a new genus or species in the stem- and root-nodulating bacteria.

Organisms which could not be characterised within the known rhizobia taxa, were isolated by Dagutat (1995). Dagutat (1995) and Kruger (1998) isolated putative rhizobia from legumes assumed to be non-nodulating. However, the identity of the isolates was not determined unequivocally by the techniques they used. Since analysis of the 16S rDNA sequence can give a definite answer concerning the identity of the isolates, the purpose of this investigation was to characterise and possibly identify selected indigenous and putative rhizobial isolates by 16S rDNA sequence analysis.

CHAPTER 2

CHAPTER 2

LITERATURE REVIEW

1. INTRODUCTION

The use of nitrogenous fertilisers has increased after World War II to increase crop yield and its use in developed countries is higher 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. The possible harmful effects make the use of nitrogenous fertiliser questionable (Bohlool, Ladha, Garrity and George, 1992; Caetano-Anollés, 1997).

More than 20% of the applied fertiliser is lost due to nitrification in the soil, leaching of toxic 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 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 show 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. The human population is expected to double in the next 40 years and it is thus important that the crop yields should be increased without harming the ecology and endangering human health (Caetano-Anollés, 1997).

An environmentally friendly alternative is the use of biologically fixed nitrogen. Worldwide BNF is 17.2 x 10^7 T / year, three times the amount fixed industrially. BNF is due to free-living nitrogen-fixers (spp. of *Azotobacter*, *Azospirillum*, *Klebsiella*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Rhodospirillum*, *Nostoc* and *Anabaena*) and symbiotic nitrogen-fixing systems (rhizobia and legumes, *Frankia* and *Alnus* and *Myrica*, *Nostoc* and *Collema* and *Anabaena* and *Azolla*). The amount contributed by the free-living nitrogen-fixers is low (Ishizuka, 1992).

In rice paddies, BNF sustains the fertility of the fields. BNF also 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 to 120 kg nitrogen / ha / year as urea in a rice ecosytem (Danso, Bowen and Sanginga, 1992).

In tree ecosystems, leguminous trees and scrubs contribute to the nitrogen levels in the soils of tropical regions. 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). About 200 plant species covering eight plant families and 17 genera in the tropics and sub-tropics fix nitrogen symbiotically with nitrogen-fixing actinomycetes (*Frankia*) (Peoples and Craswell, 1992).

It is not yet possible to replace nitrogen fertilisation with associative BNF on grasses and cereal crops. The amount of BNF by the associations is low and depends on several factors: the host, the bacteria and environmental factors. BNF enhances growth of the host by certain growth-regulating substances rather than by increased nitrogen fixation (Ishizuka, 1992). Studies have shown that some forage grasses, sugar cane and wetland rice derive considerable amounts of nitrogen from associated nitrogen-fixing bacteria under ideal conditions. It is possible to improve the nitrogen-fixing ability of sugar cane through breeding (Peoples and Craswell, 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).

Leguminous plants are used as primary sources of food, fuel, fibre and fertiliser. The plants can be used to enrich the soil, preserve moisture, to prevent soil erosion, act as windbreaks, ground cover, as well as providing a source of resins, gums, dyes and oils and ornamental plants (Bohlool *et al.*, 1992).

Legumes feature in cropping systems (intercropping or rotation cropping), grazing systems, plantation systems (legumes in inter-row spaces of tree crops such as cocoa, coffee, tea, rubber and oil palm) and agroforestry systems (tree and scrub legumes in combination with animals and crops). Legumes can improve ruminant productivity by increasing annual live weight gains and potential stocking rates (Peoples and Craswell, 1992).

In mixed legume/grass pastures the amount of fixed nitrogen throughout the world is in the range of 13-682 kg nitrogen / ha / year. BNF in the mixed pastures leads to the accumulation of soil nitrogen resulting in grass dominance (due to the inhibitory effect of higher nitrogen levels) and reduced BNF. Grass dominance reduces the soil nitrogen content leading to increased BNF. The relationship in the mixed legume/grass pastures is thus a dynamic one (Ledgard and Steele, 1992). Some nitrogen is also transferred to associated grasses by the decomposition of legume roots and nodules and animal excretions (Ledgard and Steele, 1992; Peoples and Craswell, 1992).

Cereal/legume intercropping increases the dry matter production and grain yield of the crops compared to the cultivation of single crops. In a nitrogen deficient soil, BNF is responsible for the available nitrogen. The transfer of nitrogen from legume to cereal increases the cropping system's yield and efficiency of nitrogen use (Fujita, Ofoso-Budu and Ogata, 1992).

The considerable use of legumes and the economic gain that can be achieved by inoculating legumes and in the future non-legumes as well, is well established. In South Africa Grobbelaar and his 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).

Recently Dagutat (1995) isolated rhizobia from 147 nodulated legumes. The results of the WCP analysis by SDS-PAGE indicated that some of the isolates corresponded to commercial inoculant strains. It is important to study the diversity of the indigenous strains, since more inoculant strains may be found.

2. THE PLANT PARTNER

The plant family, Fabaceae consists of 16 000 to 19 000 species in approximately 750 genera. The family is divided into three subfamilies, namely the Mimosoideae (66 genera), Caesalpinoideae (177 genera) and Papilionoideae (505 genera) (Allen and Allen, 1981).

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 nodulation status of the subfamily Caesalpinoideae is low and the ratio of nodulated species is approximately 30%. The nodulation status of the phylogenetic younger subfamilies, Mimoisoideae and Caesalpinoideae is about 90-98% (Allen and Allen, 1981; Oyaizu, *et al.*, 1993).

The fact that some legumes are capable of forming nodules and other legumes not, can be explained by the ploidy of the plant, that is the number of chromosome sets. In the subfamily, Caesalpinoideae where non-nodulating species occur at a high rate, positive correlation between the ploidy and nodulation status was found. In the plant tribes where the number of chromosomal sets is equal to eight (x = 8), nodulation occurs. In the tribes where the chromosomal sets are equal to seven, no nodulation was observed (Allen and Allen, 1981).

The genus *Cassia* (subfamily Caesalpinoideae) was divided into *Cassia*, *Senna* and *Chamaecrista* by Irwin and Barneby in 1982 as quoted by Sprent (1994). The known nodulating species occur in *Chamaecrista* (250 species), while there is no evidence for the formation of nodules in the genera *Cassia* (30 species) and *Senna* (240 species) (Sprent, 1994).

2.1 Nodulation of supposedly non-nodulating legumes

2.1.1 *Cassia*

Dagutat (1995) isolated rhizobia from the nodules of *Cassia didymobotrya*, *C. floribunda* and a *Cassia* species. The *Cassia* isolates showed relationships with members isolated from *Acacia*, *Chamaecrista* and *Bolusanthus*.

Oyaizu *et al.* (1993) isolated *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. from the root nodules of *Cassia nomame*. The relationship of the isolates was determined by sequencing of the 16S rRNA gene.

Gao *et al.* (1994) isolated rhizobia from the nodules of *Cassia mimosoides* in the Hainan province, People's Republic of China.

2.1.2 *Bauhinia variegata*

In the study by Dagutat (1995) the formation of nodules on *Bauhinia variegata* was reported. The isolates were slow-growing rhizobia. According to WCP analysis by SDS-PAGE the isolates clustered in a separate cluster from the reference strains of the genus *Bradyrhizobium*.

2.1.3 *Schizolobium parahybum*

Until recently the genus *Schizolobium* was known as non-nodulating, but Dagutat (1995) isolated rhizobia from nodules on the roots of *Schizolobium parahybum*. The isolates clustered according to WCP analysis by SDS-PAGE in a group containing *R. tropici* IIA USDA 9030 as the reference strain.

2.1.4 *Senna petersiana*

In the study by Kruger (1998) rhizobial isolates from nodules on the roots of *S. petersiana* were reported to belong to the genus *Bradyrhizobium*, based on Biolog results and restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene.

2.1.5 *Ceratonia siliqua*

Ceratonia siliqua (Carob tree) is a leguminous tree endemic to the Mediterranean region. The tree is of use in the pharmaceutical industry, as an ornamental plant and fuel wood. The tree is capable of growth in semi-arid and arid zones where the mean annual rainfall varies from 200 to 400 mm and the mean temperature ranges from 5° to 40°C. The authors isolated rhizobia from nodules on the roots of the plants that were considered non-nodulating. The characteristics of the isolates corresponded to the characteristics of the genus *Rhizobium*. The isolates were more tolerant to high salinity (5% KCl) and higher temperatures (some strains as high as 45°C) than other *Rhizobium* strains (El Idrissi *et al.*, 1996).

2.2 Nodulation of non-legumes

2.2.1 *Parasponia*

Parasponia is a genus of the Ulmaceae and represents the only non-legumes for which root nodule symbiosis with rhizobia has been confirmed. *Parasponia* is a genus of plants endemic to the islands of South-East Asia (Baker, Dodd and Parsons, 1996). As quoted by Baker *et al.* (1996) Ham described the *Parasponia* symbiosis in 1909. The rhizobia are sensitive to nitrogen levels in the soil and the bacteria enter the plant roots via cracks. The rhizobia are not released from the infection threads within the nodule (Baker *et al.*, 1996).

2.2.2 *Roystonea regia*

Roystonea regia (royal palm) is an ornamental monocotyledonous tree of the family Arecaceae. Recently Basu *et al.* (1997) reported rhizobial root nodules on the plants. The mature nodules are creamy in colour and the shape varies from oval to cylindrical. The bacteroids display the typical shape of bacteroids of leguminous plants; that is they are club-shaped, V- or Y-shaped. The bacteroid remains within the infection thread. The bacteria showed the characteristics of a fast-growing *Rhizobium* species. The bacteria nodulated their original host effectively (nitrogen fixed in nodules) and none of the legumes tested (Basu *et al.*, 1997). It can be concluded that the association between the *Rhizobium* sp. and *R. regia* is a specific interaction.

3. THE RHIZOBIA

The rhizobia form part of the family *Rhizobiaceae* in the α-2-subgroup of the *Proteobacteria* (Jordan, 1984). Currently the nodule-forming organisms are grouped into six distinct genera (Table 1), namely *Allorhizobium* (De Lajudie *et al.*, 1998a), *Azorhizobium* (Dreyfus *et al.*, 1988), *Bradyrhizobium* (Jordan, 1984), *Mesorhizobium* (Jarvis *et al.*, 1997), *Rhizobium* (Jordan, 1984) and *Sinorhizobium* (Chen *et al.*, 1988). A detailed discussion of each genus will be given.

The rhizobia do not belong to a single phylogenetic lineage. The genera *Bradyrhizobium* and *Azorhizobium* are more related to other non-nodulating bacteria than to the other nodulating genera. *Bradyrhizobium* is highly related to *Blastobacter denitrificans* and to the genera *Afipia*, *Nitrobacter* and *Rhodopseudomonas*. *Azorhizobium* is related to the genus *Xanthobacter* (Willems and Collins, 1993).

3.1 *Allorhizobium*

Strains from this genus form effective nodules on *Neptunia natans*. The legume is an aquatic legume indigenous to waterlogged areas of Senegal. The legume is currently evaluated as green manure for rice cultivation in India and the legume is consumed in South-East Asia. The genus contains one species, *Allorhizobium undicola*. The G + C content of the DNA is 60,1 mol%. The strains form effective nodules on their original host and induce infective

TABLE 1 **Current taxonomy of rhizobia and their common host plants.**

Current classification	Common host plants	Reference
The genus *Allorhizobium*		De Lajudie *et al.*, 1998a
Allorhizobium undicola	*Neptunia natans*	De Lajudie *et al.*, 1998a
The genus *Azorhizobium*		Dreyfus *et al.*, 1988
Azorhizobium caulinodans	*Sesbania rostrata*	Dreyfus *et al.*, 1988
The genus *Bradyrhizobium*		Jordan, 1984
Bradyrhizobium elkanii	*Glycine* spp.	Kuykendall *et al.*, 1992
Bradyrhizobium japonicum	*Glycine* spp.	Jordan, 1984
Bradyrhizobium liaoningense	*Glycine max* and *Glycine soja*	Xu *et al.*, 1995
The genus *Mesorhizobium*		Jarvis *et al.*, 1997
Mesorhizobium loti	*Lotus corniculatus, Lotus tenuis, Lupinus densiflorus* and *Anthyllis vulneria*	Jarvis *et al.*, 1982 Jarvis *et al.*, 1997
Mesorhizobium huakuii	*Astragalus sinicus*	Chen *et al.*, 1991 Jarvis *et al.*, 1997
Mesorhizobium ciceri	*Cicer arietinum*	Nour *et al.*, 1994 Jarvis *et al.*, 1997
Mesorhizobium mediterraneum	*Cicer arietinum*	Nour *et al.*, 1995 Jarvis *et al.*, 1997
Mesorhizobium tianshanense	*Glycyrrhiza uralensis, G. pallidiflora, Sophora alopeucuroides, Halimodendron holodendron, Caragana polourensis* and Glycine *max*	Chen et al., 1995 Jarvis et al., 1997
Mesorhizobium plurifarium	*Acacia senegal, A. tortilis* subsp. *raddiana, A. nilotica, A. seyal, Leucaena leucocephala* and *Neptunia oleracea*	De Lajudie *et al.*, 1998
Mesorhizobium amorphae	*Amorpha fruticosa*	Wang *et al.*, 1999
The genus *Rhizobium*		Jordan, 1984
Rhizobium leguminosarum	*Pisum* spp., *Lathyrus* spp., *Vicia* spp., *Lens* spp., *Phaseolus* spp. and *Trifolium* spp.	Jordan, 1984
Rhizobium galegae	*Galega orientalis* and *Galega officinalis*	Lindström, 1989
Rhizobium tropici	*Phaseolus vulgaris, Leucaena leucocephala* and *L. esculenta*	Martínez-Romero *et al.*, 1991
Rhizobium etli	*Phaseolus vulgaris*	Segovia *et al.*, 1993
Rhizobium hainanense	*Stylosanthes guyanansis, Centrosema pubescens, Desmodium triquctrum, D. gyroides, D. heterophyllum, Tephrosia candida, Acacia sinuata, Zornia diphylla* and *Macroptilium lathyroides*	Chen *et al.*, 1997
Rhizobium gallicum	*Phaseolus vulgaris*	Amarger *et al.*, 1997
Rhizobium giardinii	*Phaseolus vulgaris*	Amarger *et al.*, 1997
Rhizobium mongolense	*Medicago ruthenica*	Van Berkum *et al.*, 1998
Rhizobium huautlense	*Sesbania herbacea* and *Leucaena leucocephala*	Wang *et al.*, 1998
The genus *Sinorhizobium*		Chen *et al.*, 1988
Sinorhizobium fredii	*Glycine soja, Glycine max, Vigna unguiculata* and *Cajanus cajan*	Scholla and Elkan, 1984 Chen *et al.*, 1988
Sinorhizobium meliloti	*Melilotus* spp., *Medicago* spp. and *Trigonella* spp.	Jordan, 1984 Chen *et al.*, 1988
Sinorhizobium xinjiangensis	*Glycine soja, Glycine max, Vigna unguiculata* and *Cajanus cajan*	Chen *et al.*, 1988
Sinorhizobium saheli	*Sesbania* spp., *Acacia seyal, Leucaena leucocephala* and *Neptunia oleracea*	De Lajudie *et al.*, 1994
Sinorhizobium teranga	*Sesbania* spp., *Acacia* spp., *Leucaena leucocephala* and *Neptunia oleracea*	De Lajudie *et al.*, 1994
Sinorhizobium medicae	*Medicago polymorpha, M. truncatula, M. sativa, M. minima, M. orbicularis* and *M. rigidula*	Rome *et al.*, 1996b

nodules on *Medicago sativa*, *Acacia senegal*, *A. seyal*, *A. tortilis* subsp. *raddiana*, *Lotus arabicus* and *Faidherbia albida* (De Lajudie *et al.*, 1998a).

3.2 *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. The G + C content of the strains is 65 to 70 mol%. The strains nodulate their original host effectively. Dreyfus *et al.* (1988) described only one species based on identical protein electrophoregrams, high levels of DNA-DNA binding and phenotypic similarities.

Rinaudo, Orenga, Fernandez, Meugnier and Bardin (1991) found two distinct genomic species in the *Azorhizobium* genus. Based on DNA reassociation studies the genus contains two species. Strains of genomic species 2 showed less than 60% reassociation (44-53%) with the type strain of *Azorhizobium caulinodans* (LMG 6465). Phenotypically the genomic species showed no difference and this hindered the authors in describing a new species.

Adebayo, Watanabe and Ladha (1989) showed that *Azorhizobium* strains are present as epiphytic bacteria on their host plants (10^5 to 10^7 bacteria per gram dry weight of leaves and flowers). Their epiphytic survival and the 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, Ndoye, Lortet, Ndiaye, De Lajudie and Dreyfus, 1997a).

3.3 *Bradyrhizobium*

Jordan (1984) described *Bradyrhizobium* as slow-growing bacteria that nodulate some tropical-zone and temperate-zone leguminous plants. The strains exhibit host range specificity.

The G + C content of the DNA of the genus is 61-65 mol%. Only one species, *B. japonicum*, was included in the genus described by Jordan (1984). The author concluded that the genus *Bradyrhizobium* represents an extremely heterogeneous collection of strains. Two other species were later described namely, *B. elkanii* (Kuykendall, Saxena, Devine and Udell, 1992) and *B. liaoningense* (Xu, Ge, Cui, Li and Fan, 1995). After describing *B. elkanii*, Kuykendall *et al.* (1992) stated the possibility of two or three more species of *Bradyrhizobium*. The *Bradyrhizobium* strains nodulate *Glycine* (soybean), *Vigna* (cowpea), *Macroptilium* (siratro), *Lotus* spp. and *Acacia* spp. A non-legume host, *Parasponia* is nodulated very specifically by a *Bradyrhizobium* sp. (Jordan, 1984).

A phototrophic symbiotic strain (BTAil) isolated from the stem nodules of *Aeschynomene indica* belongs to *Bradyrhizobium* based on 16S rRNA and fatty acid methyl ester analyses (FAME) (Young, Downer and Eardly, 1991; So, Ladha and Young, 1994). Later more phototrophic strains were isolated from the stem nodules of *Aeschynomene*. All the phototrophic strains fall into a homogenous group in the *Bradyrhizobium* genus (Boivin, Ndoye, Molouba, De Lajudie, Dupuy and Dreyfus, 1997b).

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*.

Willems and Collins (1992) showed a close phylogenetic relationship between the type strains of *Blastobacter denitrificans*, *Afipia* (causative agent of cat scratch disease) and *Bradyrhizobium japonicum*, based on the similarity of the 16S rRNA sequences. The 16S rRNA sequences of *B. japonicum* and *Blastobacter denitrificans* are 98,5% similar and showed 96,8-98,5% relatedness to *Afipia* species. The closest relative of the lineage is *Rhodopseudomonas acidophila*.

3.3.2 *Bradyrhizobium elkanii*

Two distinct groups in the soybean nodulating *Bradyrhizobium* group were described by Hollis, Kloos and Elkan (1981), based on DNA homology analysis. Kuykendall *et al.* (1992) described *B. elkanii* for the group II strains. The creation of the species is justified on the basis of DNA homology, RFLP analysis, fatty acid and antibiotic resistance profiles, extracellular polysaccharide (EPS) and cytochrome composition and 16S rRNA sequence data.

3.3.3. *Bradyrhizobium liaoningense*

The species, *B. liaoningense* was described for the extra slow growing rhizobia isolated from soybean (Xu *et al.*, 1995). Strains of this species nodulate *Glycine max* and *G. soja* effectively (nitrogen fixation). No nodules are formed on *Pisum sativum*, *Lotus* sp., *Astragalus sinicus* and *Melilotus* spp.

Velázquez, Cruz-Sánchez, Mateos and Martínez-Molina (1998) proposed that the assignment of *B. liaoningense* to the genus *Bradyrhizobium* should be revised, based on the results of low molecular weight RNA profiles. The 5S rRNA profile of the species differs significantly from that of the other bradyrhizobia. It is believed that the 5S rRNA profile can be used to differentiate between genera.

3.4. *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. The 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.

The G + C content of the DNA is 59 to 64 mol%. Jarvis *et al.* (1997) included the species *M.loti*, *M. huakuii*, *M. ciceri*, *M. mediterraneum*, *M. tianshanense* in the genus. Wang, Van Berkum, Sui, Beyene, Chen and Martínez-Romero (1999) described *M. amorphae* and *M. plurifarium* was described by De Lajudie, Willems, Nick, Moreira, Molouba, Hoste, Torck, Neyra, Collins, Lindström, Dreyfus and Gillis (1998b). The type species of the genus is *Mesorhizobium loti* (Jarvis *et al.*, 1997).

3.4.1 *Mesorhizobium loti*

Jarvis, Pankhurst and Patel (1982) described *Rhizobium loti* for the fast-growing *Lotus* rhizobia and related strains. Originally the species was a phylogenetically diverse collection of strains. Two more species were later described. The strains of the species form nitrogen-fixing nodules on the roots of *Lotus corniculatus* (bird's-foot trefoil), *Lotus tenuis* (slender bird's-foot trefoil), *Lupinus densiflorus* (lupin) and *Anthyllis vulneria* (kidney vetch).

3.4.2 *Mesorhizobium huakuii*

Rhizobium huakuii was described by Chen, Li, Qi, Wang, Yuan and Li (1991) for rhizobia that nodulate the roots of *Astragalus sinicus*. *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. The nodulation of this legume increases the host plant yields by 15 to 30% and increases the nitrogen content in the plants by 2,0 to 2,5% compared with uninoculated plants. The strains of the species form effective nodules on *Vicia villosa, Phaseolus vulgaris* and *Sesbania* sp.

3.4.3 *Mesorhizobium ciceri*

Nour, Fernandez, Normand and Cleyet-Marel described *Rhizobium ciceri* (1994b). The strains of the species nodulate the legume *Cicer arietinum* L. (chickpea). *Cicer arietinum* L. is the third most widely grown grain legume in the world (Nour, Cleyet-Marel, Beck, Elfosse and Fernandez, 1994a). In their study Nour *et al.* (1994a) used multilocus enzyme electrophoresis (MLEE), RFLP of the 16S-23S intergenic spacer region (IGS) of the rRNA gene and more than 150 phenotypic characteristics to study the chickpea rhizobia. Their study showed two distinct phylogenetically distant groups, group A and group B. Group A contained the slow-growing rhizobia. The group A isolates were always thought to be *Bradyrhizobium* strains due to their growth rate. However, in the polyphasic study done by Nour *et al.* (1994b) the authors showed that the group A strains are *Rhizobium* isolates. Group B includes the fast-growing rhizobia and all the strains are all included in the species, *R. ciceri*, based on a polyphasic approach.

3.4.4 *Mesorhizobium mediterraneum*

Nour, Cleyet-Marel, Normand and Fernandez (1995) described *Rhizobium mediterraneum*. The group A isolates from *C. arietinum* L. (chickpea) as described by Nour *et al.* (1994a) form a genomic diverse group. Four genomic species can be distinguished in the group. One of the genomic species differs significantly from *R. ciceri* and the name *R. mediterraneum* was subsequently given to the genomic species (Nour *et al.*, 1995).

3.4.5 *Mesorhizobium tianshanense*

Isolates from *Glycyrrhiza uralensis*, *G. pallidiflora*, *Sophora alopecuroides*, *Swainsonia salsula*, *Halimodendron holodendron*, *Caragana polourensis* and *Glycine max* were included in the species, *Rhizobium tianshanense* (Chen, Wang, Wang, Li, Chen and Li, 1995). The strains were isolated from a region with low rainfall and a wide variation in temperature. The soil of the region (Xinjiang region of northwestern People's Republic of China) was saline alkaline desert soil. The strains of the species formed a single cross-inoculation group. They formed nodules on the plants they were isolated from, but did not nodulate other legumes (Chen *et al.*, 1995).

3.4.6 *Mesorhizobium plurifarium*

De Lajudie *et al.* (1998b) described *Mesorhizobium plurifarium* for tropical rhizobia previously referred to as cluster U (De Lajudie, Willems, Pot, Dewettinck, Maestrojuan, Neyra, Collins, Dreyfus, Kersters and Gillis, 1994). Based on SDS-PAGE of WCP and genotypic characterization by repetitive extragenic palindromic-polymerase chain reaction (REP-PCR), 16S rRNA gene sequencing and DNA-DNA hybridisation, cluster U was clearly a separate species within the *Mesorhizobium* genus (De Lajudie *et al.*, 1998b).

The isolates nodulate *Acacia senegal*, *A. tortilis* subsp. *raddiana*, *A. nilotica*, *A. seyal*, *Leucaena leucocephala* and *Neptunia oleracea* effectively. The *M. plurifarium* isolates were isolated from several places in East Africa, West Africa and South America (De Lajudie *et al.*, 1998b).

3.4.7 *Mesorhizobium amorphae*

Wang *et al.* (1999) described *Mesorhizobium amorphae* for rhizobia isolated from Chinese soils associated with the legume, *Amorpha fruticosa*. *Amorpha fruticosa* is a leguminous scrub native to South-Eastern and Mid-Western United States. The scrub is useful as a windbreak, as a soil cover for erosion control and the scrub provides food for wildlife. In China, the leaves are used as green manure and the seeds are used as a source of oil for glycerol production.

The polyphasic approach showed two distinct groups within the *M. amorphae* group, but only the most distinct group was described as the new species within the genus (Wang *et al.*, 1999).

3.5 *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 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 were recently included in the genus: *R. galegae* (Lindström, 1989), *R. tropici* (Martínez-Romero, Segovia, Mercante, Franco, Graham and Pardo, 1991), *R. etli* (Segovia, Young and Martínez-Romero, 1993), *R. hainanense* (Chen, Tan, Gao, Li and Wang, 1997), *R. gallicum* (Amarger, Macheret and Laguerre, 1997), *R. giardinii* (Amarger *et al.*, 1997), *R. mongolense* (Van Berkum, Beyene, Bao, Campbell and Eardly, 1998) and *R. huautlense* (Wang, Van Berkum, Beyene, Sui, Dorado, Chen and Martínez-Romero, 1998).

3.5.1 *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. 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 *Pisum*, *Lathyrus*, *Vicia*, *Lens*, *Phaseolus* and *Trifolium*.

The biovar, *R. leguminosarum* biovar phaseoli is however not a genetically homogeneous collection of strains. Different criteria (MLEE, DNA-DNA hybridization, plasmid profiles, protein profiles, etc.) clearly showed that two distinct types could be differentiated. The type I strains have multiple copies of nitrogenase *nifH* genes and a narrow host range whereas the type II strains have single copies of *nifH* genes and can form nodules on *Leucaena* spp. (Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1993).

3.5.2 *Rhizobium galegae*

Lindström (1989) described *R. galegae* for fast-growing root nodule bacteria isolated from *Galega orientalis* and *G. officinalis*. The strains form effective nodules only on their original host plant. Other rhizobia form ineffective nodules occasionally on *Galega* spp.

In their study Wang *et al.* (1999) found that *R. galegae* showed common ancestry with *R. leguminosarum* rather than with *Agrobacterium vitis*. The same result was also found in a previous study by Wang *et al.* (1998).

3.5.3 *Rhizobium tropici*

Martínez-Romero *et al.* (1991) described *Rhizobium tropici* for the strains previously known as *R. leguminosarum* biovar phaseoli Type II. Two distinct subgroups, Type IIA and Type IIB were reported by the authors. The subgroups differ in phenotypic and genotypic characteristics. Recently a specific megaplasmid was found in each subgroup (Geniaux, Flores, Palacios and Martinez, 1995).

The strains from the species form effective nodules on *Phaseolus vulgaris*, *Leucaena leucocephala* and *L. esculenta* (Martínez-Romero *et al.*, 1991). In her study, Dagutat (1995) isolated strains corresponding to *R. tropici* from nodules of *Bolusanthus* and *Spartium*. Van Berkum *et al.* (1998) isolated strains belonging to *R. tropici* from *Medicago ruthenica*.

3.5.4 *Rhizobium etli*

The species was described by Segovia *et al.* (1993) for the former *R. leguminosarum* bv. phaseoli Type I strains. According to Segovia *et al.* (1993), the strains only formed effective nodules on *Phaseolus vulgaris*. The authors included one biovar in the species, *R. etli* bv. phaseoli. Dagutat (1995) reported *R. etli* strains isolated from nodules of *Desmodium*, *Melolobium*, *Indigofera*, *Acacia melanoxylon* and *Chamaecrista stricta*.

3.5.5 *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. The strains of the species do not fall within a single cross-inoculation group. The type strain of the species can only nodulate its original host and not those of the other strains.

In the study of Chen *et al.* (1997), the strains within the species were isolated from 12 leguminous species classified into nine different genera. The strains were isolated from nodules of *Stylosanthes guyanansis*, *Centrosema pubescens*, *Desmodium triquctrum*, *D. gyroides*, *D. heterophyllum*, *Tephrosia candida*, *Acacia sinuata*, *Zornia diphylla* and *Macroptilium lathyroides*.

3.5.6 *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: *Phaseolus* spp., *Leucaena leucocephala*, *Macroptilium atropurpureum* 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* with a delay of one month. Nitrogen-fixing nodules are formed on *Phaseolus* *vulgaris*. The strains of *R. gallicum* bv. phaseoli contain three copies of the *nifH* gene (Amarger *et al.*, 1997).

Sessitsch, Ramírez-Saad, Hardarson, Akkermans and De Vos (1997) included Austrian isolates from *Phaseolus vulgaris* L. as well as the Mexican isolate FL27 in the species. The isolates were confirmed as *R. gallicum* strains based on phenotypic and phylogenetic analyses. The isolates belonged to the biovar, *R. gallicum* bv. gallicum. The isolates nodulated not only bean plants, but also cowpea plants, *Leucaena* and *Gliricidia*. The Austrian isolates and the Mexican isolate differ in their geographic origin, but are similar in their nodulation host range. This showed that the isolates contained a similar or related symbiotic plasmid (Sessitsch *et al.*, 1997).

3.5.7 *Rhizobium giardinii*

The species 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 can be divided into two biovars, *R. giardinii* bv. giardinii and *R. giardinii* bv. phaseoli.

The biovars differ according to their respective hosts and the presence or absence of *nifH* gene copies. *R. giardinii* bv. giardinii nodulate *Phaseolus* spp., *L. leucocephala* and *Macroptilium atropurpureum*. The strains are not able to form nitrogen-fixing nodules on *Phaseolus vulgaris*. There is no hybridisation with the *nifH* probe even under conditions of low stringency (Amarger *et al.*, 1997).

The strains of *R. giardinii* bv. phaseoli nodulate *Phaseolus* spp. and form nodules on *Macroptilium atropurpureum* after a delay of a month or more. The strains are weakly able to fix nitrogen with *Phaseolus vulgaris*. There are three copies of the *nifH* gene present in the genome of this biovar (Amarger *et al.*, 1997).

3.5.8 *Rhizobium mongolense*

Van Berkum *et al.* (1998) described *R. mongolense* for one of the three genotypes of rhizobia isolated from nodules of *Medicago ruthenica* [(L.) Ledebour]. The legume is a potential new forage crop, which is tolerant to stress since it is adapted to grow in dry locations with cold winters. Because *M. ruthenica* and *M. platycarpa* are the progenitors of other *Medicago* and *Trigonella* spp., they could provide a genetic source for the improvement of *M. sativa* to tolerate higher levels of stress (Van Berkum *et al.*, 1998).

The strains nodulate *M. ruthenica* and *Phaseolus vulgaris* effectively. The nodules formed on *M. alfalfa* are not effective (Van Berkum *et al.*, 1998).

3.5.9 *Rhizobium huautlense*

The species was described by Wang *et al.* (1998) for rhizobia isolated from *Sesbania herbacea* growing in Sierra de Huautla, Mexico. *Rhizobium huautlense* is closely related to *R. galegae*, based on 16S rRNA sequencing analysis (99,5% and 99,7% similarity). The species form effective nodules on *S. herbacea* and *Leucaena leucocephala* (Wang *et al.*, 1998).

3.6 *Sinorhizobium*

Chen *et al.* (1988) described the genus *Sinorhizobium* for fast-growing soybean bacteria from China. The results of numerical taxonomy, DNA-DNA hybridization, serological analysis data, G + C content, soluble protein patterns, bacteriophage typing and the composition of extracellular gum showed that the new genus differs adequately from *Bradyrhizobium*, *Rhizobium* and *Azorhizobium*. The authors included two species in the genus: *S. fredii*, previously *R. fredii* (Scholla and Elkan, 1984) and a new species, *S. xinjiangensis*. 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. teranga* 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. Rome, Fernandez, Brunel, Normand and Cleyet-Marel (1996b) described *S. medicae* isolated from annual *Medicago* spp.

Chen *et al.* (1988) described the G + C content of the genus as 59,9 to 63,8 mol%. The G + C content of the emended genus is 57 to 66 mol% (De Lajudie *et al.*, 1994). The G + C content still falls in the range of a well-defined genus, since they do not differ by more than 10% (Vandamme, Pot, Gillis, De Vos, Kersters and Swings, 1996). The strains of the genus do not nodulate a wide host range and exhibit host specificity (Chen *et al.*, 1988; De Lajudie *et al.*, 1994).

3.6.1 *Sinorhizobium fredii*

Rhizobium fredii was described by Scholla and Elkan (1984) for the fast-growing rhizobia isolated from mainland China that nodulate soybeans. The authors 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. The chemovars of *R. fredii* according to Scholla and Elkan (1984) were *R. fredii* chemovar fredii and *R. fredii* chemovar siensis.

Strains of this species nodulate *Glycine soja*, *Glycine max*, *Vigna unguiculata* and *Cajanus cajan* effectively (Chen *et al.*, 1988). The strains form nodules on genetically unimproved lines of 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 *Melilotus* (sweet clover), *Medicago* (alfalfa) and *Trigonella* (fenugreek) (Jordan, 1984). In a study done by Eardly, Materon, Smith, Johnson, Rumbaugh and Selander (1990) on isolates from various geographical regions and species of *Medicago*, the authors showed the presence of two phylogenetically different evolutionary lineages within *Rhizobium* (*Sinorhizobium*) *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, Brunel, Fernandez, Normand and Cleyet-Marel (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).

3.6.3 *Sinorhizobium xinjiangensis*

Chen *et al.* (1988) found two distinct 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. The type strains of each chemovar of *S. fredii* (Scholla and Elkan, 1984) cluster in the same group. The other group differs significantly from *S. fredii* and other known species and the strains in the group were assigned to a new species, *S. xinjiangensis* (Chen *et al.*, 1988).

The strains of this species were isolated from nodules in the suburbs of Xinjiang, People's Republic of China. Strains of this species nodulate *Glycine soja*, *Glycine max*, *Vigna unguiculata* and *Cajanus cajan* effectively (Chen *et al.*, 1988). According to De Lajudie *et al.* (1994), the taxonomic position of the species remains to be determined.

3.6.4 *Sinorhizobium saheli*

The strains were isolated from *Sesbania* species in the Sahel region in Africa. The 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*. When the roots of the plant are already nodulated, ineffective stem nodules are formed (Boivin *et al.*, 1997a).

Haukka, Lindström and Young (1998) proposed two biovars, *S. saheli* bv. acaciae and *S. saheli* bv. sesbaniae based on phylogenetic studies of the *nodA* gene.

3.6.5 *Sinorhizobium teranga*

The 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 *Sesbania* and *Acacia* spp., *Leucaena leucocephala* and *Neptunia oleracea* (De Lajudie *et al.*, 1994).

Lortet, Méar, Lorquin, Dreyfus, De Lajudie, Rosenberg and Boivin (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*. 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 is 42 to 60%.

The strains of *S. medicae* have different host ranges from that of *S. meliloti*. The strains are adapted to form symbiosis with certain *Medicago* species, especially the annual medics of the Mediterranean basin. The strains nodulate their host plant with more specificity than *S. meliloti*. The strains effectively nodulate *M. polymorpha* (selective nodulating plant species) and the more promiscuous species *M. truncatula, M. sativa, M. minima, M. orbicularis* and *M. rigidula* (Rome *et al.*, 1996b).

4. EVOLUTION OF THE LEGUME-RHIZOBIUM SYMBIOSIS

The first ancestors of the Fabaceae appeared about 60 to 70 million years ago in the humid tropics. The three subfamilies were present 50 million years ago. Sprent (1994) does not share the belief that the subfamily Caesalpinoideae was the ancestor of the two other subfamilies. Other authors describe the subfamily Caesalpinoideae as the most primitive subfamily. Phylogenetic data has indicated that the Caesalpinoideae is polyphyletic (more than one lineage), while the other two families are monophyletic (Hirsch and LaRue, 1997). In the family, the other two subfamilies 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). Since the legumes are so diverse in morphology and ecology, symbiosis is not an adaptation to a specialised ecological niche, but depends on a genetic peculiarity of legumes (Van Rhijn and Vanderleyden, 1995).

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. The early form of the nodules is believed to be more parasitic (only one organism benefits from interaction) than mutualistic (both organisms benefit from interaction).

Sprent (1994) argued that in the genus *Chamaecrista* some species show bacteroids retained in the infection threads throughout the process of nitrogen fixation, while in other species the bacteroids were released from the infection threads. The nodules in which the bacteroids were released from the infection threads showed higher rates of nitrogen fixation. The nodules of legumes evolved early in the evolution of the legumes and evolved into the more symbiotic state coupled with the evolution of legumes from a woody perennial to a herbaceous annual habit. Sprent (1994) hypothesised that an annual legume had a higher demand for nitrogen than a perennial legume.

Sprent (1994) proposes that the development of photosynthetic rhizobia could be an evolutionary event in flooded areas. When the plants colonised drier areas the bacteria moved down to the roots and lost their photosynthetic ability.

Sprent (1994) argued that the legume-rhizobium symbiosis did not co-evolve, but adapted to survive in a changing environment. The changing conditions of the environment (soil, heat, salinity, etc.) placed stress on the rhizobium to survive in the soil and the plant had to adapt to grow. A plant will form a symbiotic association with the rhizobia able to nodulate and fix nitrogen efficiently, even if they are not common partners (Sprent, 1994).

Although Sprent (1994) argued that co-evolution of legumes and rhizobia did not happen, other authors proposed evidence to the contrary. Lie, Göktan, Engin, Pijnenborg and Anlarsal (1987) describe the co-evolution of *Pisum sativum* and the symbiotic rhizobia of the pea plants. The primitive pea plants occur naturally in the Middle East and Central Asia. The plants have compatible rhizobia able to efficiently nodulate the plants. Rhizobia capable of nodulating the cultivated pea plants form ineffective nodules on the primitive pea plants.

The phylogenetic trees based on the *nodC* genes of rhizobia and leghemoglobin of the host plants have the same topology suggesting that the common *nod* genes and their host plants co-evolved (Ueda, Suga, Yahiro and Matsuguchi, 1995).

According to Soltis, Soltis, Morgan, Swenson, Mullin, Dowd and Martin (1995) it appears that there is a single origin for the ability to nodulate and fix nitrogen in plants. The symbiotic nitrogen-fixing clade (legumes and actinorhizal plants) is found in the Rosid I lineage, which is a dicotyledonous clade. The clade is far removed from the clade containing the monocotyledonous plants.

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 (Doyle, 1994; Hirsch and LaRue, 1997).

5. NODULATION

5.1 The nodulation process

During the pre-infection period, the rhizobia compete with other microorganisms for colonisation of the root surface. The rhizobia respond to the *nod* gene-inducing flavonoid compounds present in the seed and plant exudates. The rhizobia react by releasing Nod-factors (lipo-oligosaccharide signals) which consist of an oligosaccharide backbone of ß-1, 4-linked N-acetyl-D-glucosamine varying in length from three to five 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 (Caetano-Anollés, 1997; Van Rhijn and Vanderleyden, 1995). Many rhizobia are capable of synthesising more than one type of Nod-factor molecule (Hirsch and LaRue, 1997).

Typically, 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).

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.). At the same time, a nodule meristem is produced through cell division in the outer or inner root cortex. The position of the nodule meristem depends on the type of nodule that is formed by the plant. The infection thread grows toward the meristem. 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) (Caetano-Anollés, 1997; Van Rhijn and Vanderleyden, 1995). Not all bacteria are released from the infection thread (Hirsch and LaRue, 1997).

Whether the morphology of a nodule is spherical (determinate) or elongate (indeterminate) is determined by the plant host and not the 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 plant also controls infection and nodulation efficiency by unknown mechanisms. The legumes can sense the amount of external nitrogen and thus regulate the symbiotic process with the rhizobia (Caetano-Anollés, 1997).

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):

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

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 too high oxygen levels (Kennedy and Tchan, 1992).

5.2 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 *M. 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).

It is to the advantage of bacteria to retain megaplasmids rather than to have one very large chromosome with unnecessary information. The replication of a smaller chromosome and a megaplasmid can be achieved faster than the replication of a large chromosome. The growth rate of the cells can be faster than it would have been (Stouthamer and Kooijman, 1993).

The *nod* genes are not expressed in culture. The flavonoids of the plant host activate the transcription of the *nod* genes by changing the conformation of the NodD protein. The NodD protein is a DNA-binding protein and thus binds to conserved DNA sequences (*nod* boxes) upstream of the inducible *nod* operons and transcription of the *nod* genes start. The structural *nod* genes can be divided into the common *nod* genes (*nodDABC*) and the host-specific *nod* genes. Although the *nodD* gene is regulatory it is still part of the common genes. The host-specific genes are linked to the common *nod* genes (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).

When nitrogen-fixing bacteria are analysed, the phylogeny of the *nifH* gene is in good correlation with 16S rDNA phylogeny. This reflects a common evolutionary history. There is also a correlation between the phylogeny of *nod* genes and the host range. The different phylogenetic groups of the rhizobia can be due to geographical separation or adaptation to different environmental conditions. Lateral transfer of symbiotic genes explains some of the differences between the phylogenetic groups based on *nod* and *nif* genes (Haukka *et al.*, 1998).

6. PRACTICAL APPLICATION OF THE SYMBIOSIS

6.1 Inoculation of legumes

Barran, Bromfield, Rastogi, Whitwill and Wheatcroft (1991) stated that 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. Often the inoculated plant does not perform better than the uninoculated plant.

Gandee, Harrison and Davies (1999) found that there was no difference in the dry weight of inoculated and uninoculated *Medicago sativa* (lucerne). The uninoculated *M. sativa* was grown in plots where the crop was not cultivated previously and no introduced strain of *Sinorhizobium meliloti* was expected to be present in the soil.

The inoculum may occupy only a small percentage of the nodules when a substantial indigenous population is present. In a genetic analysis (random amplified polymorphic DNA fingerprinting) performed by Gandee *et al.* (1999) the percentage of nodules occupied by the inoculum strain was only 28%.

A further 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. However, some indigenous strains can be as effective as the inoculum strains (Henzell, 1988).

The characteristics of indigenous rhizobial populations that may affect the inoculation response are population density, effectiveness and competitive ability of strains. The available soil nitrogen must be less than the nitrogen requirement of the plant for nitrogen fixation to take place. For nodulation occupation to be 50%, the inoculum must be applied at rates 1000 times greater than the number of indigenous rhizobia. 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, Singleton and Bohlool, 1991). The authors further showed that nodule occupancy of 50% by the inoculum strain did not improve the plant yield significantly. A significant increase was observed with nodule occupancy of 66%.

The response of the host to inoculation is determined by the presence and quality of indigenous rhizobial populations, soil nitrogen availability, physical and chemical constraints of the soil (acidity, toxicity and low fertility) and climatic conditions (low rainfall, inadequate soil temperatures and air temperatures, insufficient solar radiation, disease and insect predation) (Thies *et al.*, 1991; Dowling and Broughton, 1986).

6.2 Improvement of the inoculation response

A commercial inoculant should be more competitive than the indigenous rhizobia of the soil for nodulation (Gandee *et al.*, 1999). 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 5×10^8 rhizobial cells / g of peat. It was found that sterilisation by means of steam and Gamma irradiation resulted in excellent inoculants. According to Strijdom (1998), a new patented carrier developed by Dagutat appears to be superior to peat carriers. Survival of the bacterial cells, sticking ability and suspension characteristics of the carrier are better.

For an inoculation to be effective, the ratio of nodules occupied by inoculated rhizobia must be increased to more than 50%. An inoculum rate of at least a 1000 times the soil population must be used. The nodule formation also depends on the type of carrier used (clay or peat). It is important to note that certain host plant genotypes exist that have preferences for certain rhizobia, while preventing infection by other rhizobia. The inoculum strain and the legume host must be compatible (Ishizuka, 1992 and 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, breeding plants for improved plant yield and breeding plants for nitrate tolerance (Peoples and Craswell, 1992).

Extreme soil acidity has a negative effect on the host plants since low pH is linked with high aluminium levels, which inhibit the growth of the host plant. High aluminium levels do not necessarily affect the survival of the rhizobial population. Acid soils can be improved by liming, which will improve the nodulation and nitrogen fixation of the host plants. A rhizobial strain will compete best, at the temperature at which it grows best. Drought and salt stress go hand in hand. The rhizobial strains from arid regions are adapted to such adverse environmental conditions (the host plants are more sensitive than the rhizobial strains). Predatory protozoa can decrease the number of the rhizobia population in the soil. The rhizobia are also sensitive to low concentrations of fungicides. Sometimes treatment of seed with fungicides can negatively affect nodulation (Dowling and Broughton, 1986).

It is important to remember that rhizobia are organisms of the rhizosphere. When seed is inoculated, vegetative cells are introduced in a non-rhizosphere environment and must survive until the seedlings are established. The indigenous soil rhizobia have a competitive advantage since they are already adapted to their environment (Dowling and Broughton, 1986).

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).

Many nodules are the result of mixed infections. 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).

6.3 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 vesicular arbuscular mycorrhizae (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 phosphor, 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, Manalo, Aggangan and Tambalo, 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 also be improved by co-inoculation with several rhizobacteria. 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, Saxena, Murali and Tilak, 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.

7. POLYPHASIC TAXONOMY

Taxonomy is the science of classification, the purpose of which is to summarise and catalogue information about an organism. Classification also provides insights into the evolutionary pathways of organisms. A classification system should facilitate the identification of new isolates (Sneath, 1989; Priest and Austin, 1993).

Classification systems should have high information content, be stable and scientifically based (Priest and Austin, 1993). In many taxonomic studies, several of these requirements were not met. Many of the classifications were highly subjective and different classifications existed for the same organisms depending on the particular field of the taxonomist. The taxonomists realised that a system depending on several characters should be used (Krieg, 1988). In his article on bacterial evolution, Woese (1987) stressed that a small number of characters is an unreliable basis upon which to define taxa. The move to a polyphasic approach (using several different characters) was made to ensure that only a valid new species or a genus is created. A species is defined as a collection of strains with approximately 70% or more DNA-DNA relatedness and with T_m-values, which do not differ more than 5°C (Wayne, Brenner, Colwell, Grimont, Kandler, Krichevsky, Moore, Moore, Murray, Stackebrandt, Starr and Trüper, 1987).

In polyphasic taxonomy all genotypic (DNA-DNA hybridisation, G + C content, etc.), phenotypic (SDS-PAGE, physiological characteristics, etc.) and phylogenetic (rRNA gene sequencing, elongation factor Tu sequencing, etc.) information should be combined to describe taxa. The phenotype of an organism is the result of expression of the genetic information (genotype). Phenetic studies include the phenotype and the genotype. Depending on the method of analysis, trees reconstructed from sequence data can also be phenetic (Sneath, 1989). The classification of taxa based on a polyphasic approach is more stable than classification based on a single approach (Priest and Austin, 1993; Vandamme *et al.*, 1996).

The International Subcommittee for the Taxonomy of *Rhizobium* and *Agrobacterium* recommended that the publication of a new taxonomic unit should be the culmination of a great deal of research. When a new species or genus in the root- and stem-nodulating bacteria is to be described, it should be based on phylogenetic and phenotypic (symbiotic, cultural, morphological and physiological) traits. The studies should include a large number of strains, isolated from different geographical regions, emphasising the region or regions of origin of the host legume. Type strains of possibly related genera or species should be included (Graham *et al.*, 1991).

7.1 Ribosomal studies

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

a) They are present in all living organisms (except viruses).
b) They have a conserved function (protein synthesis) and subsequently have changed little during evolution.
c) They consist of variable regions, used for comparing or grouping of more closely related organisms, and more conserved regions, for comparing distantly related organisms.
d) Phylogenetic lines of descent can be inferred from rRNA sequences (Priest and Austin, 1993). Changes in their nucleic acid sequence 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).

Within the genomes of bacteria, the ribosomal operon can be repeated up to ten times. However, species of *Mycoplasma, Rickettsia prowazekii* and some mycobacteria contain a single ribosomal operon. In the stem- and root-nodulating bacteria, most of the rhizobia contain three copies. The slow-growing bacteria belonging to the genus *Bradyrhizobium* contain a single copy. It is argued that the number of ribosomal operons is directly proportional to the growth rate of the organisms (Kündig, Beck, Hennecke and Göttfert, 1995).

The 5S rRNA molecule is too small to be of any use to measure evolutionary relatedness between organisms. The molecule is however used in low molecular weight RNA profiles. Staircase electrophoresis (increase of voltage in 50V steps from 100 to 2300V) of low molecular weight RNA profiles (class 1 tRNA molecules, class 2 tRNA molecules and 5S rRNA molecules) makes it possible to assign strains to known species. This method was used to analyse strains belonging to the *Rhizobiaceae* and the results were consistent with the established taxonomic classification (Velázquez *et al.*, 1998). The dendrograms corresponded to data obtained by DNA-DNA hybridisation and 16S rRNA sequencing. The method may enable easier, less time consuming for the identification of new isolates.

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 database of 23S rRNA sequences is not as large as that of the 16S rRNA molecule. RFLP analysis of the amplified 23S rDNA gene and the amplified intergenic spacer region (IGS) between the 16S and 23S (very variable region) was used by several authors as part of a polyphasic study or a method of identification and typing of strains (Gürtler and Stanisich, 1996; Khbaya, Neyra, Normand, Zerhari and Filali-Maltouf, 1998; Terefework, Nick, Suomalainen, Paulin and Lindström, 1998; Vinuesa, Rademaker, De Bruijn and Werner, 1998). The 16S-23S IGS is more variable than the 16S rDNA gene and can be exploited in the identification of closely related strains. The RFLP analysis of the amplified IGS region can be used to verify the taxonomic position of an isolate (Selenska-Pobell, Evguenieva-Hackenberg, Radeva and Squartini, 1996). It is assumed that the evolutionary rate of the 16S-23S IGS region is ten times greater than that of the 16S rDNA. Analysis of this region makes it possible to distinguish between strains and recently diverged species (Aakra, Utåker and Nes, 1999).

The 16S rRNA molecule has nine variable regions (Figure 1) (Woese, Gutell, Gupta and Noller, 1983; Neefs, Van de Peer, De Rijk, Chapelle and De Wachter, 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, Pace, Olsen Stahl, Sogin and Pace, 1985; Weisburg, Barns, Pelletier and Lane, 1991). The use of sequencing made possible the classification of unculturable organisms (Amann, Ludwig and Schleifer, 1995).

The amplified 16S rDNA gene can also be studied with RFLP as part of a polyphasic study. Variations in the 16S rRNA gene can be detected by RFLP of the amplified gene. The restriction enzymes recognize and cut specific base sequences. Laguerre, Allard, Revoy and Amarger (1994) obtained satisfactory results using four restriction enzymes. The resolution power of four enzymes was equivalent to that of nine restriction enzymes. The method could identify the closest relatives of new isolates and determine the genetic diversity of new strains. In her study, Kruger (1998) proposed a change in the combination of restriction enzymes used by Laguerre *et al.* (1994). One of the enzymes (*Nde* II) produced complex restriction patterns making the interpretation of the results difficult. By replacing the enzyme (*Nde* II) with *Rsa* I, the results were easier to interpret.

Fox, Wisotzkey and Jurtshuk (1992) concluded that 16S rRNA sequence analysis is not sufficient to define species that diverged recently. The sequences can, however, be used to determine relationships between genera and "older" species.

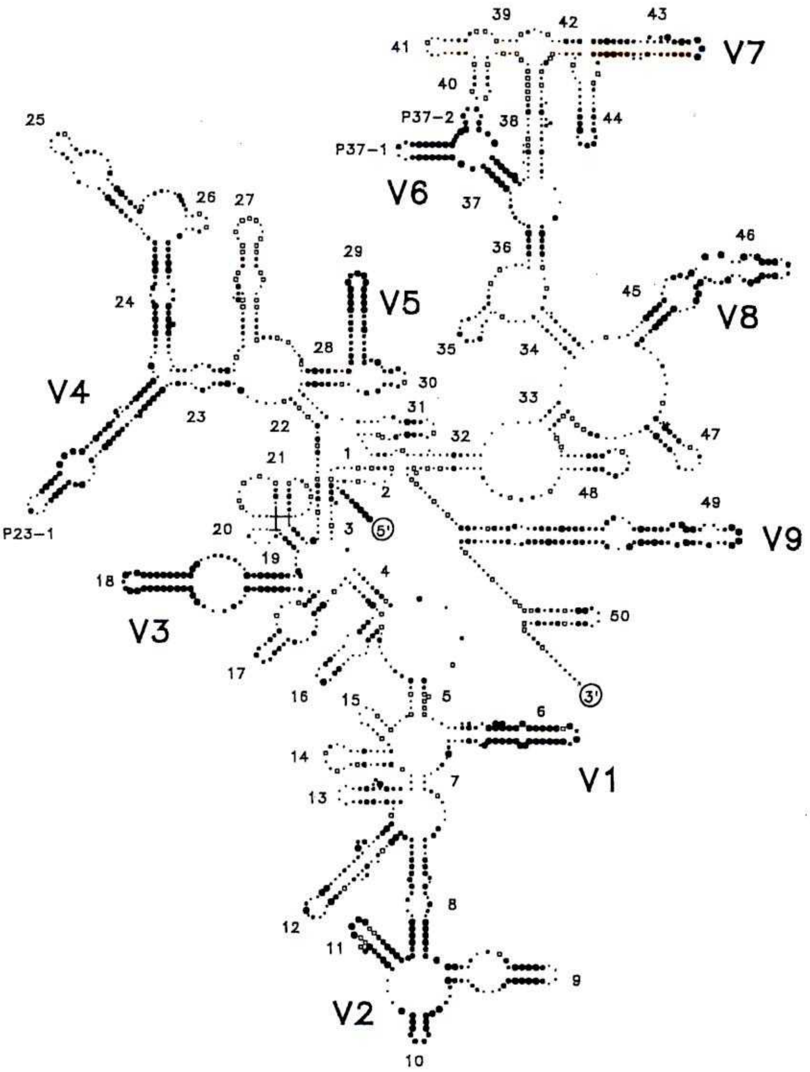

FIGURE 1 **Secondary structure model for prokaryotic 16S rRNA. The variable regions are labeled from V1 to V9. Variability is indicated by full circles of increasing diameter, conserved sites are indicated as hollow squares (Neefs *et al.*, 1993).**

7.2 Analysis of the sequence data

The sequences are aligned such that the maximum amount of homology is obtained. Gaps are inserted to allow for additions or deletions. The best alignment between two sequences is when the number of mismatches and gaps are kept to a minimum. The algorithms used for sequence alignment apply gap penalties to minimise the number of gaps, while achieving the fewest number of mismatches (Priest and Austin, 1993). The sequences can be aligned using the programme CLUSTAL, which is capable of multiple alignment of sequences (Higgins and Sharp, 1988).

7.3 Tree reconstruction

Different methods are used to reconstruct trees. They can be divided into distance and character-state approaches (Li and Graur, 1991).

7.3.1 Distance matrix method

A distance matrix is calculated using an algorithm such as the unweighted pair group method with arithmetic mean (UPGMA). The UPGMA method is a sequential clustering method. Initially, the two most similar operational taxonomic units (OTU) are identified and treated as a new single OTU. From the new group of OTUs, the most similar pair is identified until only two OTUs are left. If the rates of evolutionary change are constant in the different lineages, the UPGMA clustering will provide a reliable estimation of the true phylogenetic dendrogram. If not, it will only be a phenogram (dendrogram based on phenotypic similarities). Other distance matrix methods include the neighbour relation method and the transformed distance method (use outgroup to correct for unequal rates of evolution among lineages and then create distance matrix from new data with UPGMA) (Li and Graur, 1991; Priest and Austin, 1993).

7.3.2 Maximum parsimony method

A tree obtained by this method shows the shortest evolutionary pathway. The fewest number of mutations explain the differences between the gene sequences. The method is a character-state method, since it does not use estimates of distance or similarity, but raw data.

Informative sites (site is informative if it favours only some of all the possible trees) are identified. The minimum number of changes is determined for each possible tree and the tree with the fewest changes is calculated (Priest and Austin, 1993).

7.3.3 Maximum likelihood method

The tree reconstructed in this way is most likely to explain the evolution of the gene sequences used. In this method, the following are considered: the data, a possible evolutionary tree and a model of evolutionary change. The probability of obtaining the data with the given tree and model is computed. The Jukes-Cantor one-parameter model assumes that changes in the four nucleotides happen at the same rate. The Kimura two-parameter model sets independent rates for transitions (substitutions between two purines or two pyrimidines) and transversions (substitutions between a purine and pyrimidine or *vice versa*) (Priest and Austin, 1993).

7.3.4 Confidence limits

A statistical method, bootstrap, can be used to place confidence intervals on phylogenies. The bootstrap method randomly resamples the data and draws different trees for the same data. The number of the times that the same tree is created is the confidence value of that tree (Felsenstein, 1985). Bootstrap results do not state the probability that a particular clade is a real historical group. The results are only an indication of the degree of support of a particular technique for a particular clade. Internal branches with bootstrap proportions above 70% represent true clades (true phylogenetic groups) over 95% of the time (Hillis and Bull, 1993).

8. CONCLUDING REMARKS

In order to study the diversity of South African rhizobia a polyphasic approach was adopted by the Department of Microbiology and Plant Pathology. Dagutat (1995) used SDS-PAGE of WCP to determine the diversity and identity of indigenous rhizobial isolates. This technique can distinguish between species and closely related strains, but is ineffective above species level (Vandamme *et al.*, 1996).

Kruger (1998) used SDS-PAGE of WCP, RFLP analysis of the 16S rRNA gene and Biolog to determine the taxonomic position of indigenous isolates. The RFLP analysis could distinguish isolates at species and higher levels. Biolog can be used as an easy way of screening a large number of isolates. However, the method is not cost-effective since Biolog MicroPlates have to be imported from the United States of America and the Rand/dollar exchange rate is at present highly unfavourable.

The results of the different methods used by Dagutat (1995) and Kruger (1998) largely concur. The sequence analysis of 16S rDNA can be used to infer relationships between different species and genera, but not closely related, recently diverged, strains. Graham *et al.* (1991) recommended the use of either rDNA:DNA hybridisation or 16S rDNA sequence analysis as part of the polyphasic approach to describe a new genus or species in the stem- and root-nodulating bacteria. The latter method was evaluated in the present study.