CHARACTERIZATION AND IDENTIFICATION OF SOME INDIGENOUS RHIZOBIA USING 16S rDNA SEQUENCE ANALYSIS

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

Signature: _____

Date: ___6/3/2000___

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SUMMARY

The use of different characteristics (the polyphasic approach) to describe bacterial taxa is a prerequisite for a stable classification. The taxonomy of root- and stem-nodulating rhizobia is in a state of transition. As more legumes are studied, new species and genera of rhizobia are described. It is important to study the indigenous South African rhizobia, as without them a complete rhizobial taxonomy is not possible. Furthermore, strains with superior nitrogen fixation abilities may be discovered. Indigenous strains better adapted to the harsh South African environment are possible candidates for commercial inoculants for cropped legumes.

Only two local studies have been done on the diversity of the indigenous rhizobia. These studies revealed the diversity of rhizobia existing in the South African context. As part of a polyphasic approach used to identify and determine the diversity of the indigenous rhizobia, 16S rDNA sequencing analysis was performed on some selected rhizobial and putative rhizobial isolates.

The aim of the study was to characterise and identify the indigenous isolates by 16S rDNA sequencing analysis and compare our data with those available in the GenBank database.

Results showed that most of the indigenous isolates were slow-growers belonging to the genus *Bradyrhizobium*. Two isolates from supposedly non-nodulating legume genera (*Cassia* and *Senna*) were found to belong to the genus *Bradyrhizobium*. Some of the isolates were shown to belong to the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. The identity of five isolates was not clear and further studies need to be performed to unequivocally determine their taxonomic position. Partial sequence analysis of 16S rDNA proved a valuable tool to characterise and identify the indigenous isolates. However, the method was unable to clearly distinguish between closely related species and strains.

KARAKTERISERING EN IDENTIFIKASIE VAN SOMMIGE INHEEMSE RHIZOBIUMS MET BEHULP VAN 16S rDNS VOLGORDE-BEPALING

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OPSOMMING

'n Stabiele klassifikasiesisteem vir die beskrywing van bakteriese taksa is slegs moontlik deur verskillende eienskappe (die poli-fasiese benadering) te gebruik. Die taksonomie van die wortel- en stamnodulerende rhizobiums verander gedurig. 'n Volledige rhizobiumtaksonomie is slegs moontlik indien die inheemse Suid-Afrikaanse rhizobiums bestudeer word. Geharde inheemse rasse met voortreflike stikstofbindende vermoëns kan ontdek word. Hierdie rasse is kandidate vir kommersiële inokulums vir verboude peulplante.

Net twee plaaslike studies is gedoen om die diversiteit van die inheemse rhizobiums te bepaal. Die studies het bewys dat die inheemse rhizobiums baie divers is. As deel van die polifasiese benadering om die diversiteit van die inheemse rhizobiums te identifiseer en te bepaal, is 16S rDNS volgordebepaling gedoen op uitgesoekte rhizobia en sogenaamde rhizobia isolate.

Die doel van die studie was die karakterisering en identifisering van die inheemse isolate deur 16S rDNS volgordebepaling en die vergelyking van die data met dié beskikbaar in die GenBank databasis. Die resultate wys dat die meeste inheemse isolate stadige groeiers is en

dus behoort aan die genus *Bradyrhizobium*. Twee isolate vanaf sogenaamde nie-nodulerende peulplantgenusse (*Cassia* en *Senna*) behoort ook tot die genus *Bradyrhizobium*. Sommige isolate behoort tot die genusse *Mesorhizobium*, *Rhizobium* en *Sinorhizobium*. Die identiteit van vyf isolate was nie duidelik nie en verdere studies is nodig om hul taksonomiese posisie ondubbelsinnig te bepaal. Die gedeeltelike volgordebepaling van die 16S rDNS was 'n waardevolle hulpmiddel om die inheemse isolate mee te karakteriseer en te identifiseer, alhoewel die metode nie tussen nabyverwante spesies en rasse kon onderskei nie.

INDEX

CHAPTER 3
MATERIALS AND METHODS   **39**

CHAPTER 4
RESULTS   **46**

CHAPTER 5
DISCUSSION   **65**

CHAPTER 6
CONCLUSIONS   **70**

CHAPTER 7
REFERENCES

LIST OF ABBREVIATIONS

ACCC	Agricultural Culture Collection of China
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
BNF	biological nitrogen fixation
bp	base pair
bv	biovar
ClustalX	cluster analysis version X
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dITP	2'-deoxyinosine-5'-triphosphate
dTTP	2'-deoxytimidine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphospate
DNA	deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany
DTT	dithiotreitol
e^-	electron
EDTA	ethylenediaminotetra-acetic acid
EPS	extracellular polysaccharide
Fd	ferredoxin
IAM	Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan
IFO	Institute for Fermentation, Osaka, Yodogawa-ku, Osaka 532, Japan
IGS	intergenic spacer region
LMG	Laboratorium voor Mikrobiologie Gent Culture Collection, State University Gent, Belgium
MLEE	multilocus enzyme electrophoresis
ORS	ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
RAPD	random amplified polymorhic DNA
REP-PCR	repetitive extragenic palindromic - polymerase chain reaction
RFLP	restriction fragment length polymorphism
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	tons

T	type strain
TY	tryptone yeast medium
U	unit
UPGMA	unweighted pair group method using arithmetic mean
UPM	Universidad Politécnica Madrid, Spain
USDA	United States Department of Agriculture, Agriculture Research Service, Beltsville, USA
US$	USA dollar
VAM	vesicular arbuscular mycorrhizae
WCP	whole cell proteins
YMA	yeast mannitol agar
YMB	yeast mannitol broth

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).

1

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

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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 $92,1096 \times 10^6$ 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 23×10^6 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 17.2×10^7 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

7

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 alopecuroides, 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):

$$N_2 + 8H^+ + 8Fd^- + 16MgATP^{2-} + 18H_2O \rightarrow 2NH_4^+ + 2OH^- + H_2 + 8Fd + 16MgADP^- + 16H_2PO_4^-$$
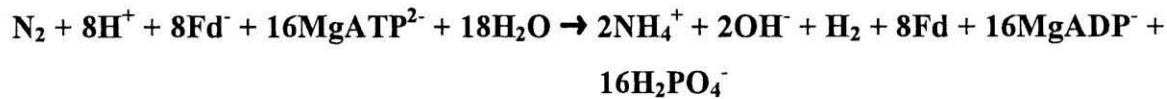
The energy requirement for nitrogen fixation is nearly the same as the energy required for nitrate assimilation. The nitrogenase enzyme is very sensitive to oxygen, but several protective mechanisms exist which protect the enzyme against too high oxygen levels (Kennedy and Tchan, 1992).

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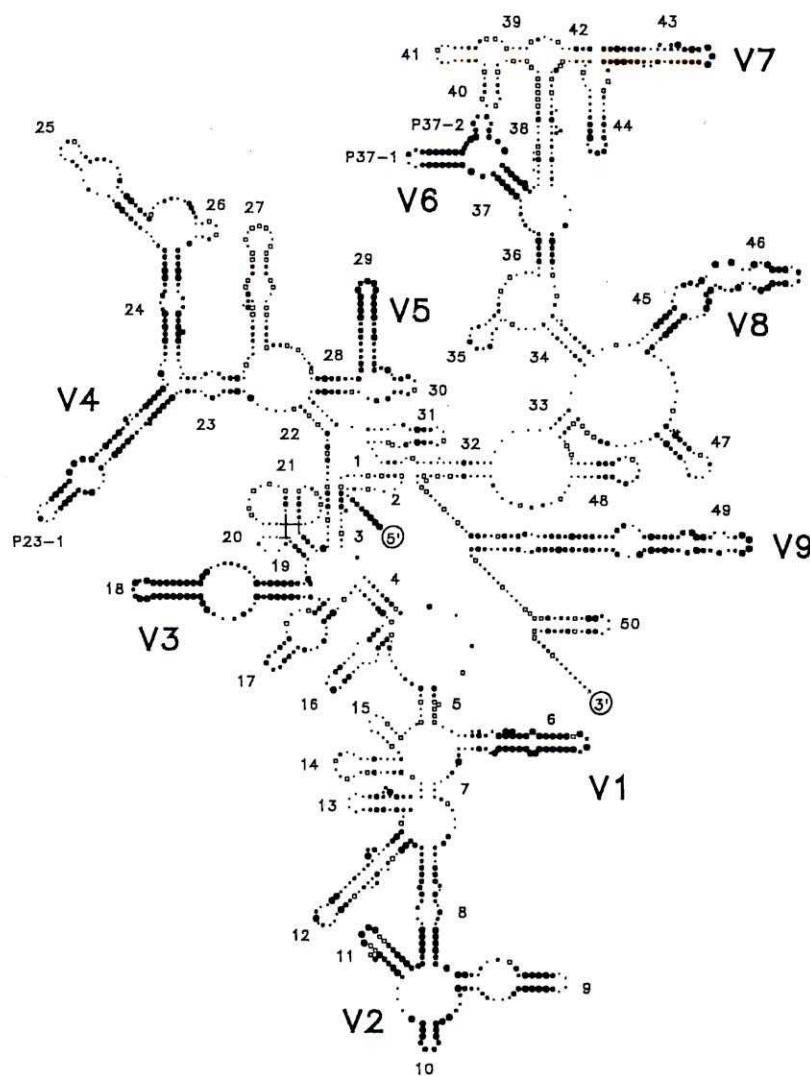
FIGURE 1   Secondary structure model for prokaryotic 16S rRNA. The variable regions are labeled from V1 to V9. Variability is indicated by full circles of increasing diameter, conserved sites are indicated as hollow squares (Neefs *et al.*, 1993).

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.

CHAPTER 3

CHAPTER 3

MATERIALS AND METHODS

3.1 STRAINS USED

Nineteen indigenous rhizobia isolates previously characterised by Dagutat (1995), using SDS-PAGE of WCP, were included in the study together with nine strains isolated by Kruger (1998) and two isolates received from the Agricultural Research Council (Table 2).

TABLE 2   List of indigenous rhizobia isolated by Dagutat (1995), isolates received from the Agricultural Research Council and rhizobia isolated by Kruger (1998) used in 16S rDNA analysis.

Isolate	Host legume	Subfamily of Fabaceae
13b	*Lotononis bainesii*	Papilionoideae
13c sterre	*Lotononis bainesii*	Papilionoideae
15c	*Desmodium tortuosum*	Papilionoideae
24 slym	*Desmodium tortuosum*	Papilionoideae
26c	*Strongylodon macrobotrys*	Papilionoideae
27d	*Teramnus labialis*	Papilionoideae
46c2	*Acacia sieberana* var. *woodii*	Mimosoideae
47c3a	*Cassia floribunda*	Caesalpinoideae
48a	*Tephrosia purpurea*	Papilionoideae
48b	*Tephrosia purpurea*	Papilionoideae
49b	*Indigofera melanadenia*	Papilionoideae
68a onseker	*Mucuna coriacea*	Papilionoideae
68d	*Mucuna coriacea*	Papilionoideae
70a	*Crotalaria brachycarpa*	Papilionoideae
79c	*Acacia caffra*	Mimosoideae
85a1t1 onseker	*Acacia xanthophloea*	Mimosoideae
94	*Vigna subterranea*	Papilionoideae
102a	*Chamaecrista biensis*	Caesalpinoideae
103b	*Indigofera hilaris*	Papilionoideae
M3b[#]	*Lotononis falcata*	Papilionoideae
M3c[#]	*Lotononis falcata*	Papilionoideae
PL10 groot knop[*]	*Argyrolobium tomentosum*	Papilionoideae
PL18b[#]	*Medicago sativa*	Papilionoideae
PL19b[#]	*Indigofera verrucosa*	Papilionoideae
PL20a[#]	*Lessertia annularis*	Papilionoideae
PL20b onseker[#]	*Lessertia annularis*	Papilionoideae
PL27a[#]	*Chamaecrista mimosoides*	Caesalpinoideae
PL3[*]	*Tephrosia grandiflora*	Papilionoideae
TK1[#]	*Senna petersiana*	Caesalpinoideae
W1c[#]	*Vigna unguiculata*	Papilionoideae

*   Received from the Plant Protection Research Institute, Agricultural Research Council, Roodeplaat.
\#   Putative rhizobia isolated by Kruger (1998)

3.2 MAINTENANCE OF CULTURES

The isolates were maintained on yeast mannitol agar (YMA) [1% (m/v) mannitol (UniVar), 0,5% (m/v) K_2HPO_4 (Merck), 0,02% (m/v) $MgSO_4.7H_2O$ (Merck), 0,01% (m/v) NaCl (NT Chemicals), 0,04% (m/v) yeast extract (Biolab) and 1,5% (m/v) bacteriological agar (Biolab)] slants and the long-term storage of the isolates was done in glycerol. The isolates were grown in yeast mannitol broth (YMB) for 5 to 7 d at 25 - 28°C with vigorous shaking. The broth cultures were mixed 1:1 with sterile 50% (v/v) glycerol (Merck) in sterile cryotubes and stored in duplicate at -20°C and -70°C.

3.3 EXTRACTION OF GENOMIC DNA

Each of the strains was used to inoculate 5 ml YMB in a screw-cap tube. The broth cultures were incubated on a rotary shaker for 5 to 7 d at 25 - 28°C. After the growth period the broth appeared turbid; 2 ml of the turbid broth culture was used to inoculate 15 ml sterile tryptone yeast (TY) broth [0,5% (m/v) tryptone (Difco), 0,3% (m/v) yeast extract (Biolab), 0,13% (m/v) $CaCl_2.6H_2O$ (UniLab)] in a screw-cap tube. TY broth reduces slime formation by the rhizobia. The broth cultures were incubated for 2 d at 25 - 28°C with vigorous shaking.

A modified method described by Ehlers (1995) was used to extract genomic DNA. The method is useful for organisms such as rhizobia, which produce large amounts of slime. Before harvesting the cells, the broth culture was cooled to 4°C for 30 min on ice to reduce the activity of the cells' enzymes. The cells were then harvested by centrifugation at 8 000 rpm for 15 min. After centrifugation, the supernatant was discarded. The pellet was resuspended in 1 ml of STE buffer (10 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8,0) (4°C) with gentle swirling motions, while keeping it on ice to prevent the cells from bursting. After resuspension of the pellet, 150 µl of 20% (m/v) SDS (UniVar) and 50 µl of proteinase K (20 mg/ml) (Roche Molecular Biochemicals) were added to the cell suspension. The mixture was incubated overnight at 50°C. If, after the incubation period, the mixture was still not clear because undigested proteins were left, the incubation was repeated. Two gentle extractions with equal volumes of mixed phenol:chloroform:isoamylalcohol [25:24:1 (v/v) phenol to chloroform to isoamylalcohol] were carried out. The extraction mixture was mixed with gentle movements of the wrist. The extraction mixture was centrifuged for 10 min at 8 000

rpm to separate the phases. The aqueous DNA-containing top layer was removed with a wide bore pipet without disturbing the white protein-rich interface. A final extraction with an equal volume of chloroform:isoamylalcohol [24:1 (v/v) chloroform to isoamylalcohol] removed all phenol from the DNA suspension. The mixture was centrifuged for 10 min at 8 000 rpm and the aqueous DNA layer removed with a wide bore pipet. The aqueous DNA top layer was adjusted to 0,3 M sodium acetate with a 3 M sodium acetate stock solution (pH 5,2) (SAARchem). Two volumes of absolute ethanol (Merck) (-20°C) were added to the mixture, mixed gently and DNA precipitated overnight at -20°C, or at -70°C for two hours. The suspension was centrifuged for 10 min at 8 000 rpm to pellet the DNA. The supernatant was discarded and the DNA pellet was washed with 300 µl 70% (v/v) ethanol (Merck) (-20°C) to remove the salt from the DNA. After centrifugation for 6 min at 8 000 rpm, the supernatant was discarded. The washing step was repeated. After the second washing step the pellet was vacuum dried. The pellet was then dissolved in 100 µl sterile double-distilled water and stored at -20°C until used.

Aliquots (1 µl) of genomic extractions were examined by horizontal agarose gel electrophoresis (Sambrook, Fritsch and Maniatis, 1989) using 0,9% (m/v) agarose gels (Promega) in 1x TAE buffer (40 mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA pH 8,5), stained with ethidium bromide (10 mg/ml) to determine the success of the extraction. The genomic DNA was visualised by UV fluorescence. Any contaminating RNA was removed from the extract by incubation with RNaseI (Epicentre Technologies) for 90 min at 37°C.

3.4 AMPLIFICATION OF THE 16S rDNA GENE

The universal primers fD1 and rP2 (Weisburg *et al.*, 1991) (Table 3) were used to amplify the 16S rDNA gene. The primers were synthesized by Roche Molecular Biochemicals. The PCR reaction was carried out in a volume of 50 µl with approximately 50 ng of genomic DNA, 50 pmole each of the universal primers fD1 and rP2, 1,5 mM $MgCl_2$, 10x Buffer [20 mM Tris-HCl (pH 8), 100 mM KCl, 0,1 mM EDTA, 1 mM DTT, 0,5% Tween®20, 0,5% Nonidet P-40® and 50% glycerol], 2,5 mM each of dATP, dCTP, dGTP, dTTP and 0,5 U *Takara Taq* polymerase (TaKaRa Biomedicals, Japan). The 50 µl reaction volume was overlaid with two drops of sterile mineral oil (Sigma). Amplification of the reactions was carried out in a Hybaid Omnigene Thermocycler with the temperature profile described by Laguerre *et al.*

(1994). This consisted of an initial "hot start" at 95°C for 3 min to denature the DNA, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. A final extension step at 72°C for 3 min was performed after the 35 cycles were completed.

Aliquots (5 µl) of the amplification reactions were examined by horizontal agarose gel electrophoresis (Sambrook *et al.*, 1989) using 0,9% (m/v) agarose gels (Promega) in 1x TAE buffer (40 mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA pH 8,5) and stained with ethidium bromide (10 mg/ml). The amplified gene was visualised by UV fluorescence and the size of the product was estimated using DNA molecular weight standards (Molecular Weight Marker VI, Roche Molecular Biochemicals).

TABLE 3   **Universal primers used for amplification of the 16S rDNA gene.**

Universal Primer	Primer sequence (5' to 3')	Reference
Forward primer: fD1 (8 -27)[*]	AGAGTTTGATCCTGGCTCAG	Weisburg *et al.*, 1991
Reverse primer: rP2 (1491 - 1506)[*]	ACGGCTACCTTGTTACGACTT	Weisburg *et al.*, 1991

[*]   The numbers indicate the positions relative to the *E. coli* 16S rRNA gene sequences (GenBank accession number J01859)

3.5 PURIFICATION OF THE AMPLIFIED 16S rDNA GENE

The amplified products were purified using the High Pure PCR Product Purification kit from Roche Molecular Biochemicals. The PCR product (40 µl) was transferred into a sterile Eppendorf tube and 400 µl of the binding buffer was added. The mixture was mixed and transferred to the assembled High Pure filter tube and collection tube and centrifuged for 30 sec at 13 000 rpm. After centrifugation, the filtrate (flow through) was discarded and the same filter tube and collection tube were reassembled. To the filter tube, 500 µl of the wash buffer was added. The tubes were centrifuged for 30 sec at 13 000 rpm, the filtrate discarded and 200 µl of the wash buffer added to the assembled tubes. Again the tubes were centrifuged at 13 000 rpm for 30 seconds, the filtrate discarded and the filter tube placed into a new sterile Eppendorf tube. The PCR product was eluted with 30 µl of sterile double-distilled water. The efficiency of the purification and the concentration of the DNA was determined by horizontal agarose gel electrophoresis (Sambrook *et al.*, 1989) using 0,9% (m/v) agarose gels (Promega) in 1x TAE buffer (40 mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA pH 8,5) and stained with ethidium bromide (10 mg/ml).

3.6 SEQUENCING OF THE 16S rDNA GENE

The 16S rDNA gene was sequenced with the primers 16SRNAII-S and 16SRNAVI-S (Kuhnert, Capaul, Nicolet and Frey, 1996) (Table 4). The primers are internal. Primer 16SRNAII-S is a forward primer binding from position 682 to 702 and 16SRNAVI-S is a reverse primer starting from the same position. The sequencing reaction was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (with AmpliTaq® DNA Polymerase, FS) (Perkin Elmer Applied Biosystems). The PCR sequencing reaction was performed in volumes of 5 µl in 0,2 ml MicroAmp PCR tubes (Perkin Elmer Applied Biosystems). The reaction mix consisted of 3,2 pmole primer (16SRNAII-S or 16SRNAVI-S), 2 µl terminator ready reaction mix [A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dITP, dATP, dCTP, dTTP, Tris-HCl (pH 9,0), $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA polymerase] and 50 ng template DNA. The PCR reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler and consisted of 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension for 4 min at 60°C. The sequencing products were precipitated with 20 µl of 60% (v/v) ethanol (Merck) for 15 min at room temperature. The tubes were centrifuged for 15 min at 13 000 rpm to pellet the sequencing product, supernatants discarded and the sequencing products washed with 25 µl of 70% (v/v) ethanol (Merck) (-20°C). The tubes were centrifuged for 10 min at 13 000 rpm and the supernatants discarded. To remove all traces of ethanol the sequencing products were vacuum dried and stored at -20°C. For analysis, the purified products were resuspended in 3,5 µl Blue dextran/EDTA loading buffer (Perkin Elmer Applied Biosystems). The loading buffer was prepared by combining deionized formamide and 25 mM EDTA (pH 8,0) containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran. The resuspended products were denatured for 2 min at 90°C and loaded onto the ABI Prism model 377 DNA sequencer gel.

TABLE 4   **Universal primers used for the partial sequencing of the 16S rDNA gene.**

Universal primers	Primer sequence (5' to 3')	Reference
16SRNAII-S (682 - 702)[*]	GTGTAGCGGTGAAATGCGTAG	Kuhnert *et al.*, 1996
16SRNAVI-S (702 - 682)[*]	CTACGCATTTCACCGCTACAC	Kuhnert *et al.*, 1996

[*]   The numbers indicate the positions relative to the *E. coli* 16S rRNA gene sequence (GenBank accession number J01859)

3.7 PHYLOGENETIC ANALYSIS OF SEQUENCE DATA

The sequencing gels were analysed and sequences edited with the ABI Prism Sequencing Analysis 3.1 and the ABI Prism Sequencing Navigator 1.0.1 computer programmes (Perkin Elmer Applied Biosystems). All the sequences (direction 5' to 3') and the reference sequences (Table 5) were aligned with *Bradyrhizobium japonicum* (GenBank accession number: X87272) and edited. The sequences obtained from primer 16SRNAII-S were edited to a total length of 450 bp. The sequences obtained from primer 16SRNAVI-S were edited to a total length of 440 bp. The ClustalX programme was used to analyse the sequences, the reverse and forward sequences were analysed separately. A distance matrix was constructed by the pairwise alignment of the sequences. Scores were calculated as the number of identical residues in the best alignment of two sequences, minus a fixed gap penalty of ten. All scores were converted to distance by dividing percent identity by 100 and subtracting from 1.0 to give the amount of difference between the sequences being compared (Thompson, Higgins and Gibson, 1994). The neighbour-joining method (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the distance matrix. Branch lengths were proportional to the estimated divergence along each branch. Confidence levels of the phylogenies were estimated with the bootstrap method (Felsenstein, 1985). The bootstrap method also tests the topology of the tree. The data was randomly resampled, creating a new data table, which was then analysed. A record was kept of all the groups of species that form monophyletic subsets in the resulting estimated phylogeny. The resampling and estimation procedure was repeated several times and only groups appearing in 95% or more of the trees were considered statistically significant. Bootstrap-supported trees were constructed using a random seed generator of 111 and 100 bootstrap trials. The phylogenetic trees were displayed using NJPlot which is an interactive visualiser of phylogenetic data.

TABLE 5   List of reference strains included in the analysis of the 16S rDNA sequence data obtained from the GenBank[1].

Current classification	Culture collection no.	Host plant	GenBank accession no.[1]
The genus *Allorhizobium*			
Allorhizobium undicola	LMG 11875[T]	*Neptunia natans*	Y17047
The genus *Azorhizobium*			
Azorhizobium caulinodans	ORS 571[T]	*Sesbania rostrata*	X94200
The genus *Bradyrhizobium*			
Bradyrhizobium elkanii	USDA 76	*Glycine max*	U35000
Bradyrhizobium japonicum	DSM 30131[T]	NS	X87272
The genus *Mesorhizobium*			
Mesorhizobium amorphae	ACCC 19665	*Amorpha fruticosa*	AF041442
Mesorhizobium ciceri	UPM-Ca7[T]	*Cicer arietinum*	U07934
Mesorhizobium huakuii	IFO 15243	NS	D13431
Mesorhizobium loti	LMG 6123	*Lotus divaricatus*	Y14159
Mesorhizobium plurifarium	LMG 11892	NS	Y14158
Mesorhizobium tianshanense	USDA 3592	*Glycyrrhiza pallidiflora*	AF041447
The genus *Rhizobium*			
Rhizobium etli	USDA 9032	*Phaseolus vulgaris*	U28916
Rhizobium galegae	LMG 6214[T]	*Galegae orientalis*	X67226
Rhizobium gallicum	Strain R602sp[T]	*Phaseolus vulgaris*	U86343
Rhizobium giardinii	Strain H152[T]	*Phaseolus vulgaris*	U86344
Rhizobium huautlense	USDA 4900[T]	*Sesbania herbacea*	AF025852
Rhizobium leguminosarum	IAM 12609	NS	D14513/D01269
Rhizobium mongolense	USDA 1832	*Medicago ruthenica*	U89816
Rhizobium tropici	IFO 15247	NS	D11344
The genus *Sinorhizobium*			
Sinorhizobium fredii	LMG 6217[T]	*Glycine max*	X67231
Sinorhizobium meliloti	IAM 12611	NS	D14509/D01265
Sinorhizobium saheli	LMG 7837[T]	*Sesbania cannabina*	X68390
Sinorhizobium teranga	LMG 6463	*Sesbania rostrata*	X68387
Sinorhizobium xinjiangensis	IAM 14142	NS	D12796

[1]	The GenBank database website address: *www.ncbi.nlm.nih.gov/GenBank/*
[T]	Type strain
ACCC	Agricultural Culture Collection of China
DSM	Deutsche Sammlung von Mikroorganismen, Germany
IAM	Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan
IFO	Institute for Fermentation, Osaka, Yodogawa-ku, Osaka 532, Japan
LMG	Laboratorium voor Mikrobiologie Gent Culture Collection, State University Gent, Belgium
ORS	ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal
UPM	Universidad Politécnica Madrid, Spain
USDA	United States Department of Agriculture, Agriculture Research Service, Beltsville, USA
NS	not stated

CHAPTER 4

……the sure and definite determination of species of bacteria requires so much time, so much acumen of eye and judgement, so much perseverance and patience that there is hardly anything else so difficult. - Mueller

CHAPTER 4
RESULTS

4.1 EXTRACTION OF GENOMIC DNA

The genomic DNA obtained after extraction was of a high quality (results not shown). The purification method removed bacterial polysaccharides, which can have a negative effect on the amplification reaction. Each DNA preparation appeared intact as only one large fragment was observed on electrophoresis. The concentration of RNA was generally very low.

4.2 AMPLIFICATION OF 16S rDNA GENE

The primers amplified a fragment of approximately 1 500 bp from each DNA preparation (results not shown). The concentration of the unpurified PCR product was approximately 250 ng/μl as determined by the method of Sambrook *et al.* (1989). Amplification products of a lower concentration generally did not give acceptable results after purification.

4.3 PURIFICATION OF AMPLIFIED PRODUCT

After purification, the concentration of the amplified fragment was less than 250 ng/μl and in the region of 180 to 200 ng/μl (results not shown). Some of the PCR product was regularly lost during purification. Problems were also experienced with the purification kit. Some of the filter tubes did not bind the DNA in the initial purification step and often all DNA was lost in the subsequent steps. Some of the tubes also did not purify the product. The dNTP's and other components of the PCR reaction remained in the "purified product".

4.4 SEQUENCING OF 16S rDNA GENE

Sequenced regions of between 450 and 600 bp were obtained with both primers. The primary structures (5' to 3') of the sequenced products are shown in Fig. 2 (primer 16SRNAVI-S) and Fig. 3 (primer 16SRNAII-S).
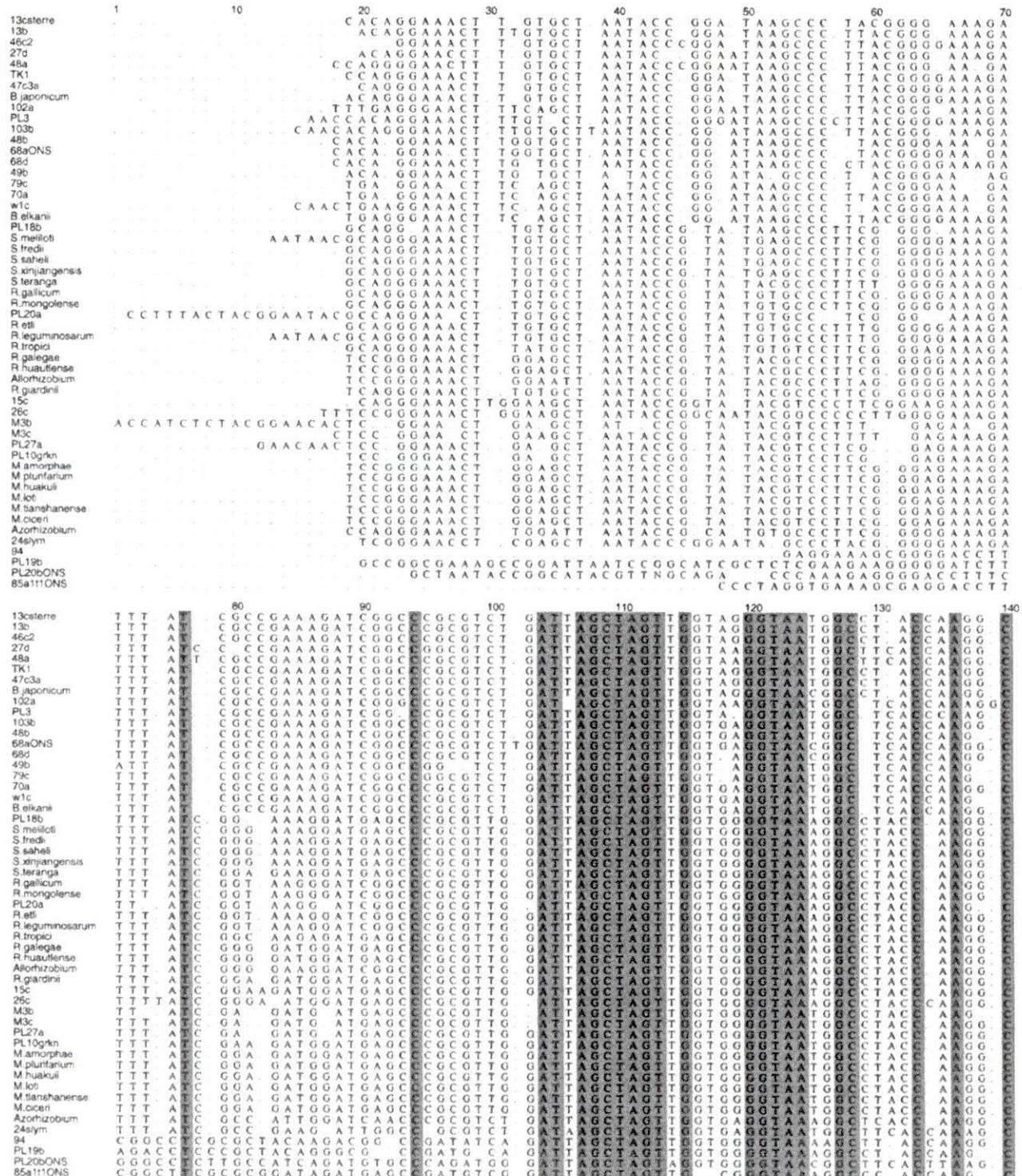
FIGURE 2   ALIGNMENT OF THE SEQUENCES OF THE 440 bp REGIONS OF THE 16S rDNA OF THE ISOLATES AND REFERENCE STRAINS INCLUDED IN THE STUDY. ALIGNMENTS WERE GENERATED BY ClustalX AND DISPLAYED WITH THE Shadybox COMPUTER PROGRAMME. THE REGIONS IN COLOUR ARE 100% HOMOLOGOUS.

47

FIGURE 2   CONTINUED

48
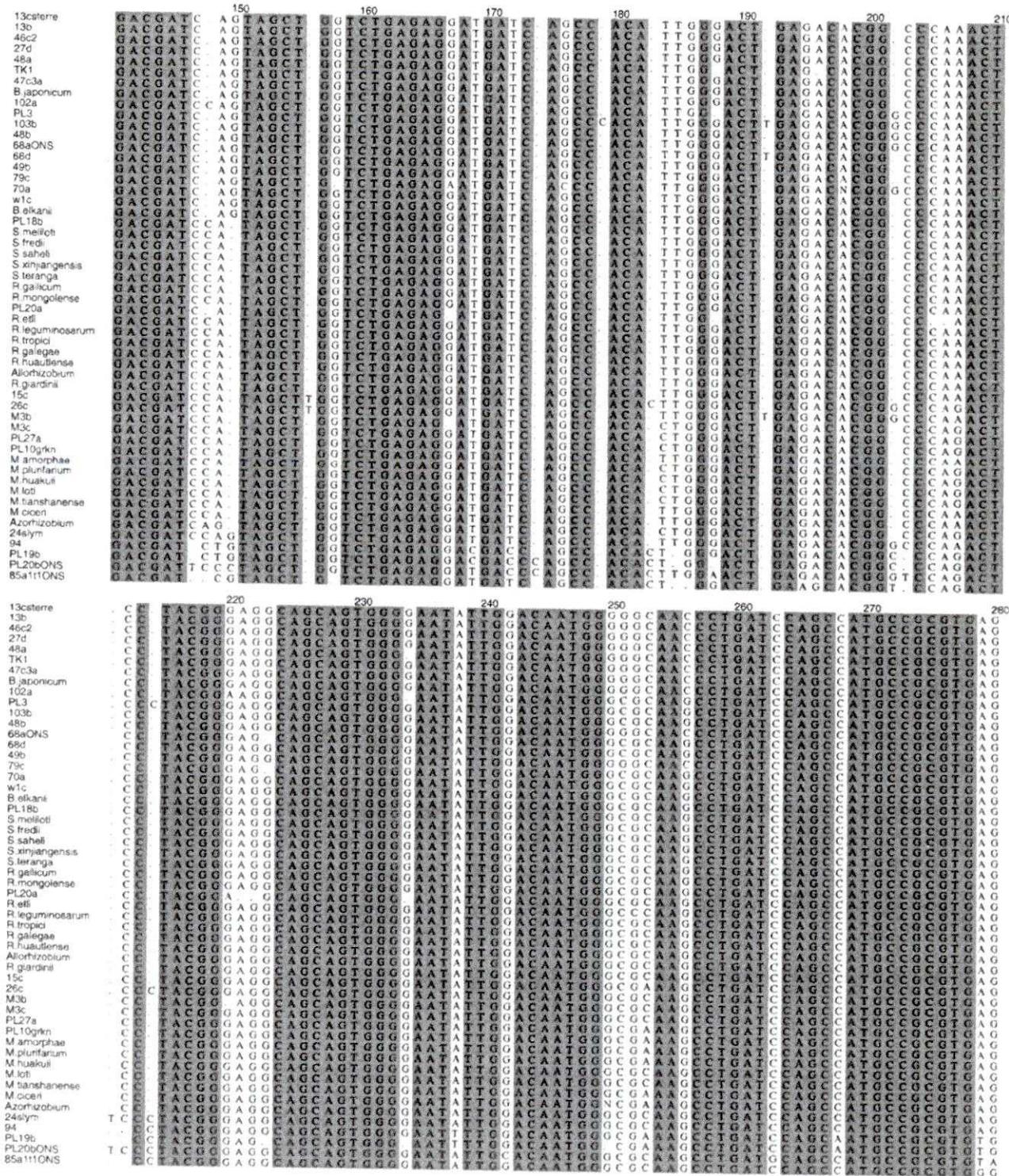
FIGURE 2   CONTINUED

FIGURE 2   CONTINUED

50
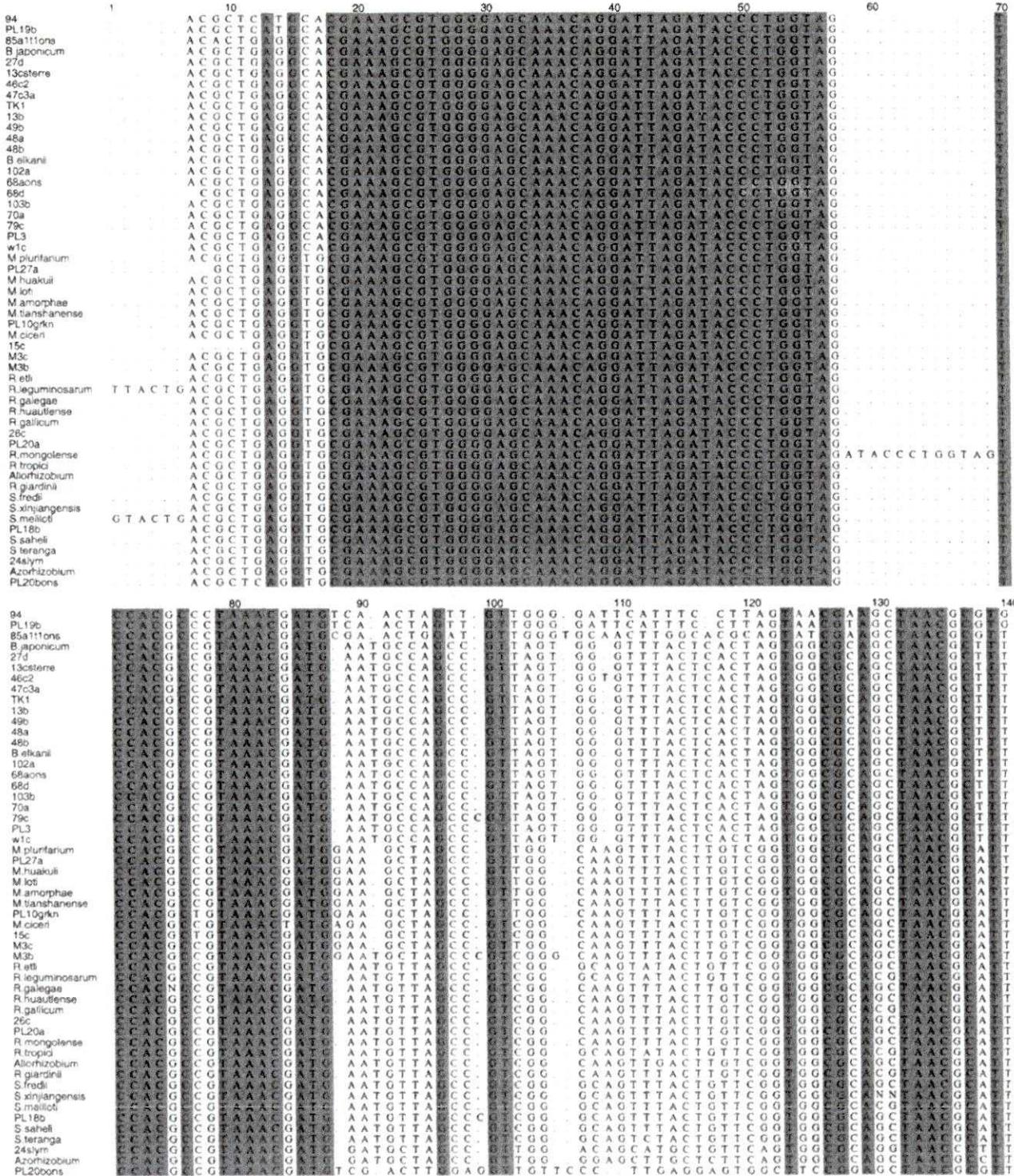
FIGURE 3   ALIGNMENT OF THE SEQUENCES OF THE 450 bp REGIONS OF THE 16S rDNA OF THE ISOLATES AND REFERENCE STRAINS INCLUDED IN THE STUDY. ALIGNMENTS WERE GENERATED BY ClustalX AND DISPLAYED WITH THE Shadybox COMPUTER PROGRAMME. THE REGIONS IN COLOUR ARE 100% HOMOLOGOUS.
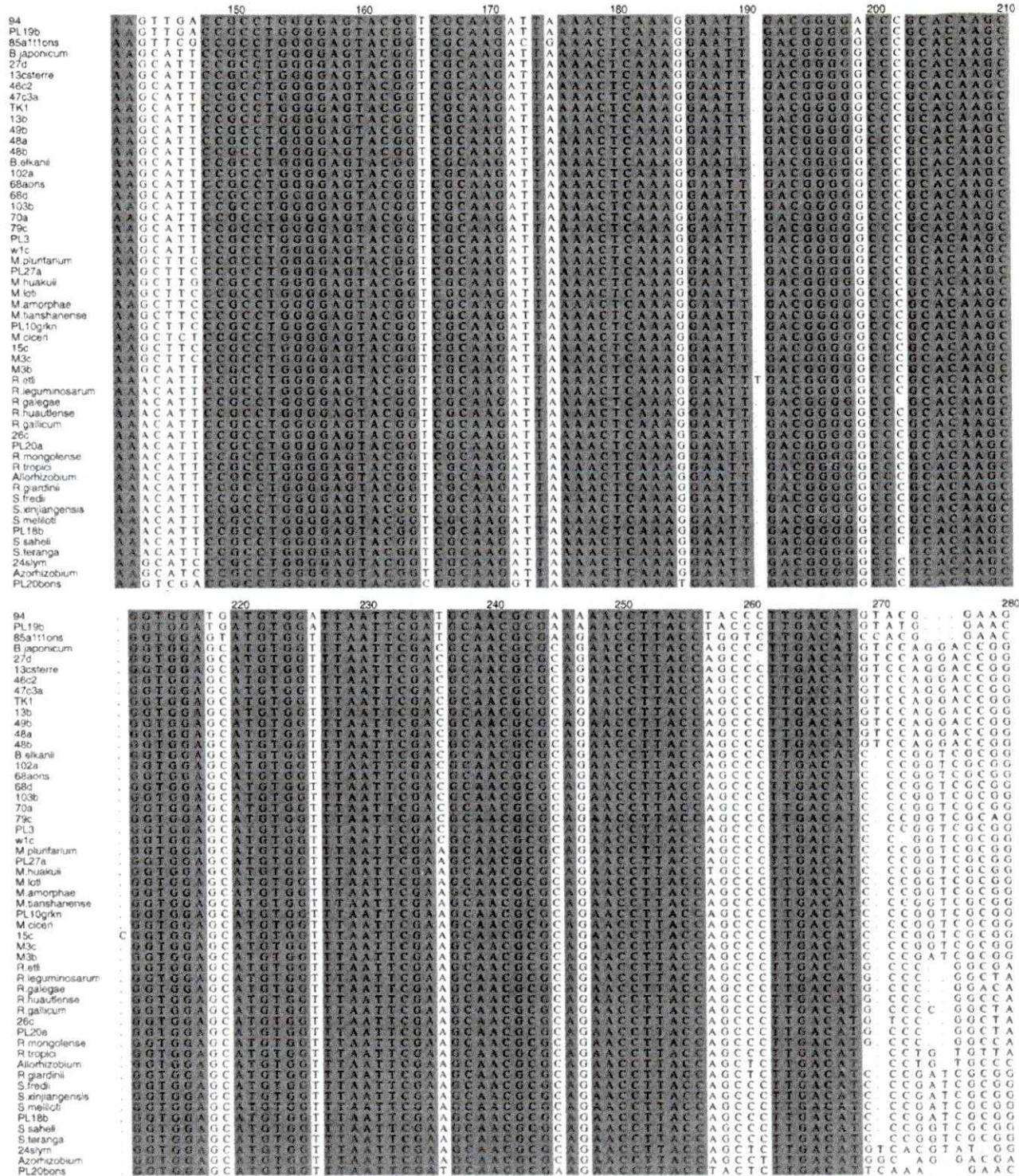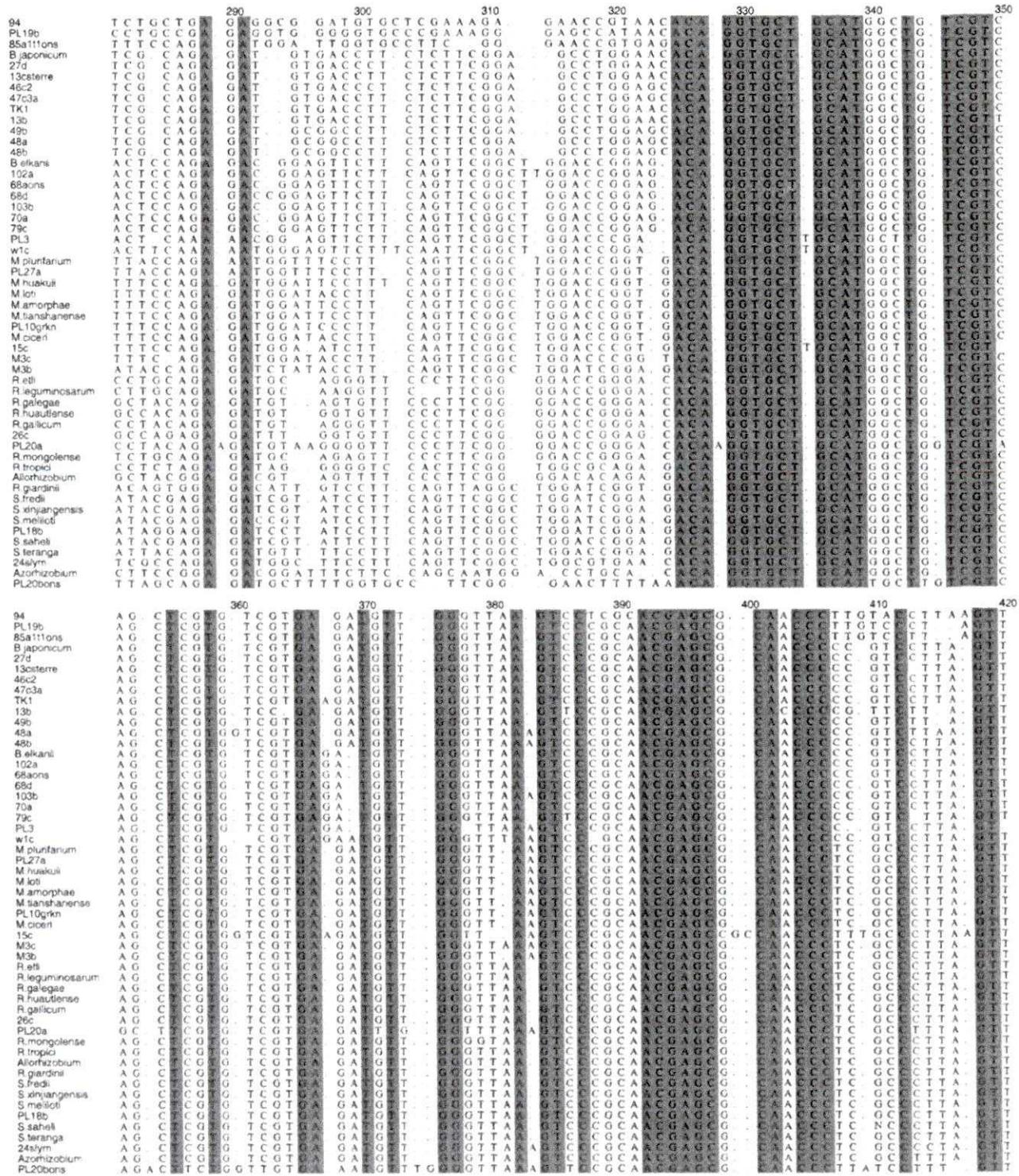
FIGURE 3   CONTINUED

FIGURE 3   CONTINUED

53
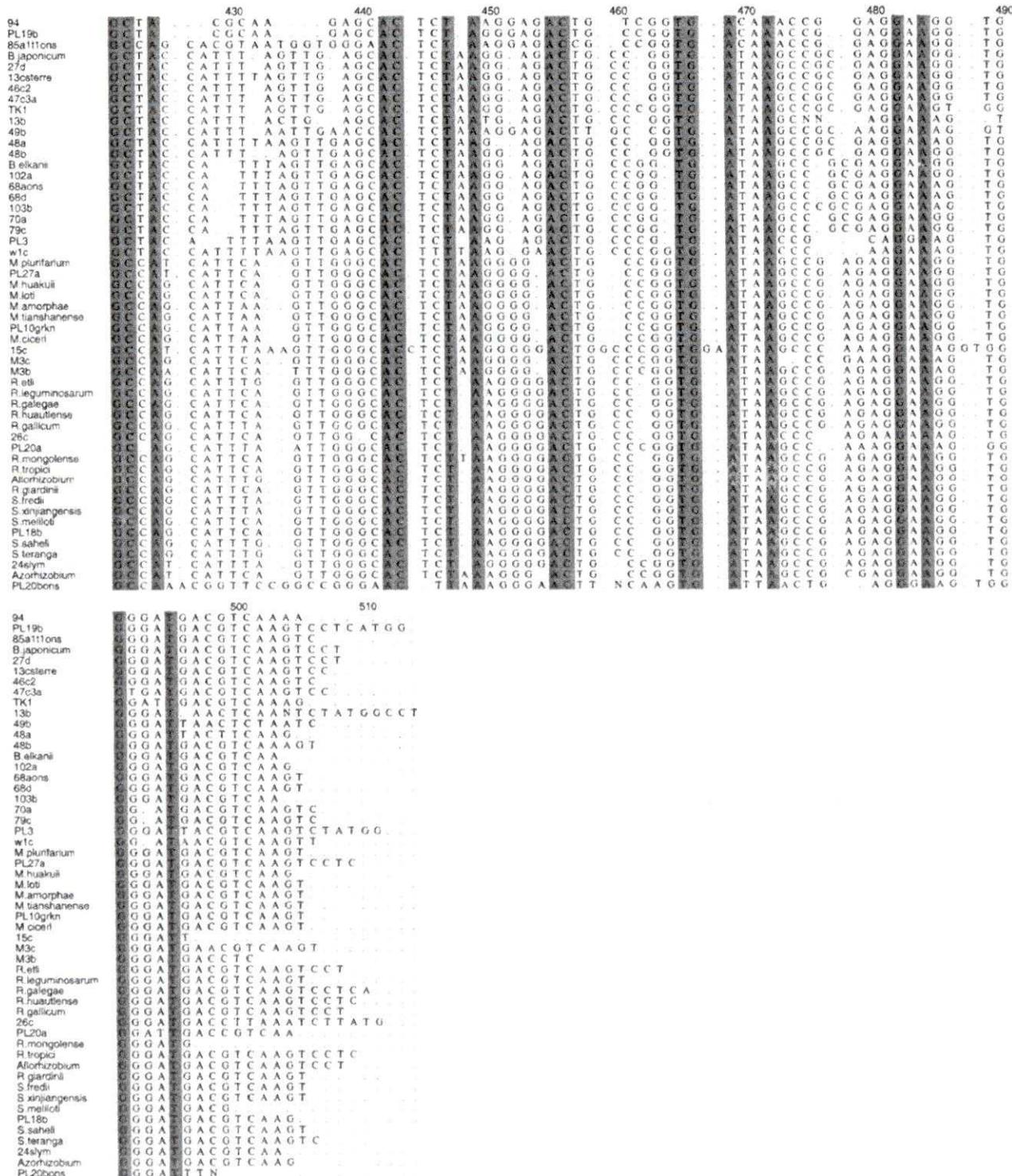
FIGURE 3   CONTINUED

Variation in the success of the sequencing reactions can be attributed to several factors: the quality of the sequencing gel, the knowledge and expertise of the operator of the ABI 377 automatic sequencer, the quality of the purified product and correct concentrations of the different reagents of the sequencing reaction.

4.5 PHYLOGENETIC ANALYSIS OF DATA

The sequencing results obtained using primer 16SRNAVI-S were edited to a total length of 440 bp. The region corresponded to positions 131-570 (numbering corresponds to that of *E. coli*). The region included two of the variable regions (V2 and V3). Primary sequences of the primer 16SRNAII-S were edited to a total length of 450 bp, corresponding to positions 691-1140 (numbering corresponds to that of *E. coli*). Three variable regions (V5-V7) were included in the region sequenced by primer 16SRNAII-S. Only part of the variable region V7 was included in the primary sequence. The afore-mentioned primer sequenced more easily than primer 16SRNAVI-S.

The trees reconstructed after the multiple alignment of the sequences with the ClustalX programme are shown in Fig. 4 (tree reconstructed from sequences obtained with primer 16SRNAVI-S) and Fig. 5 (tree reconstructed from sequences obtained with primer 16SRNAII-S). The topologies of the trees differed, but the isolates clustered in the same clusters. The relationships of the isolates within each cluster differed between the two figures. The tree in Fig. 4 was selected to represent the main tree and the numbering of the clusters of Fig. 5 corresponds to that of Fig. 4.

4.6 TREE RECONSTRUCTED FROM PARTIAL SEQUENCE RESULTS OF PRIMER 16SRNAVI-S

Six distinct groups could be distinguished in the tree reconstructed from the data obtained using primer 16SRNAVI-S (Fig. 4). Each group is described separately, referring to the relevant positions of the groups and the isolates within each group.
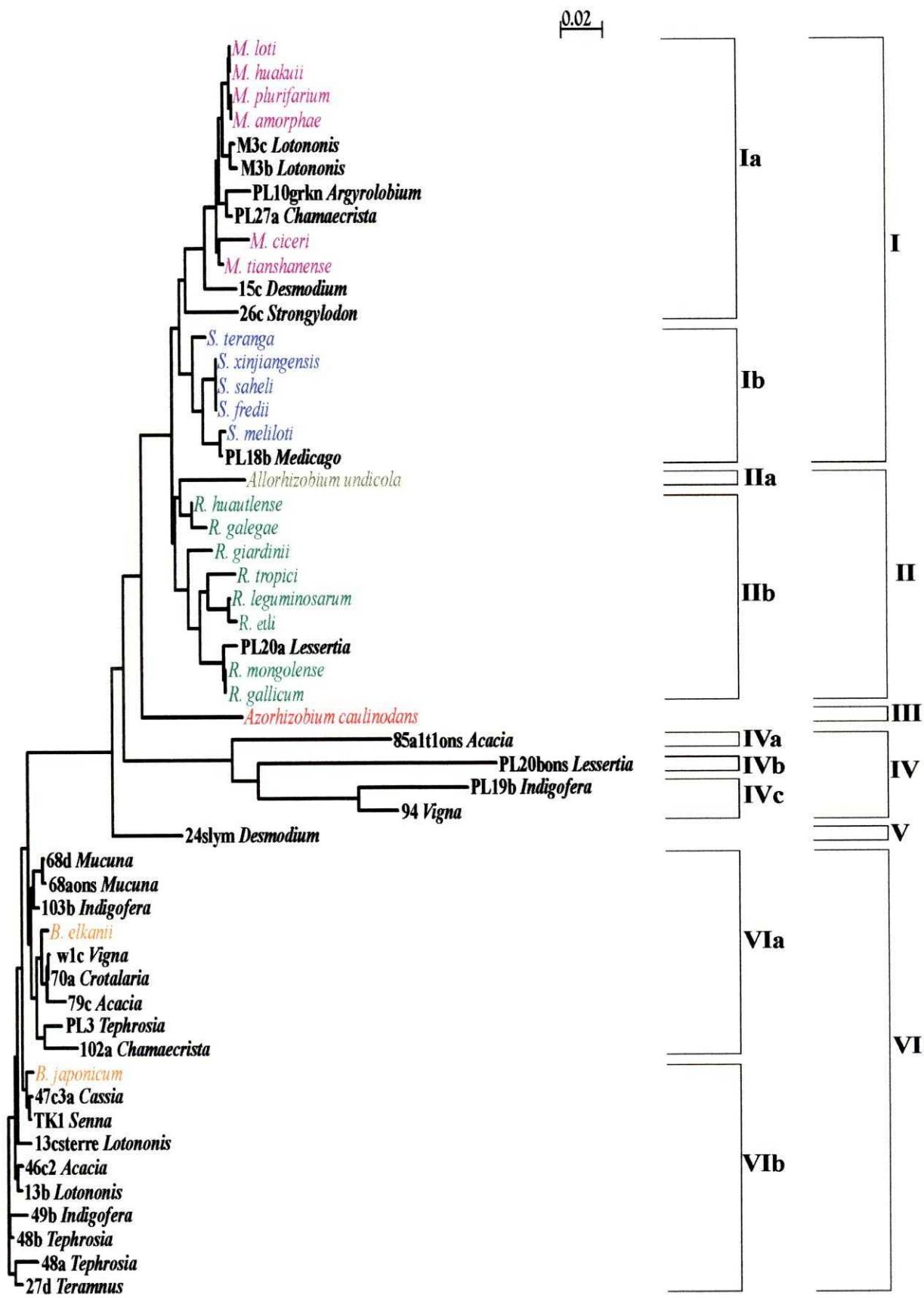
**FIGURE 4** An unrooted tree reconstructed from sequence data obtained from primer 16SRNAVI-S. Branch lengths are proportional to the estimated genetic distance between the strains. The scale represents 2% nucleotide difference. Vertical lengths are not significant and are set for clarity. Abbreviations: *M.*, *Mesorhizobium*; *S.*, *Sinorhizobium*; *R.*, *Rhizobium* and *B.*, *Bradyrhizobium*.

Group I - *Mesorhizobium* and *Sinorhizobium* group

Group I could be divided into two subgroups, Ia and Ib, corresponding to the genera, *Mesorhizobium* (subgroup Ia) and *Sinorhizobium* (subgroup Ib). Reference strains of the different species within each genus clustered in the appropriate subgroup, as expected.

Subgroup Ia

The reference strains of *M. loti*, *M. huakuii*, *M. plurifarium* and *M. amorphae* clustered in a tight grouping. The two isolates from *Lotononis falcata* M3b and M3c clearly belonged to the genus *Mesorhizobium*, showing sequence similarity of 98,09% (M3b) and 99,09% (M3c) to the cluster of the above-mentioned reference strains. The reference strains *M. ciceri* and *M. tianshanense* formed a separate cluster in the group. The isolates PL10grkn (*Argyrolobium tomentosum*) and PL27a (*Chamaecrista mimosoides*) showed sequence similarities of 98,18% and 99,09% respectively to the *M. loti*, *M. huakuii*, *M. plurifarium* and *M. amorphae* cluster. The isolate from *Argyrolobium tomentosum* (PL10grkn) showed the closest relationship to the reference strain of *M. tianshanense* (98,09%) and the isolate from *Chamaecrista mimosoides* (PL27a) shared 98,36% sequence similarity with *M. tianshanense*. The isolate 15c (*Desmodium tortuosum*) clustered into a separate branch and shared sequence similarities of 97,82% with the previously discussed cluster and 97,64% with the *M. loti*, *M. huakuii*, *M. plurifarium* and *M. amorphae* cluster. The closest reference strain was *M. tianshanense* showing sequence similarity of 97,55% with 15c. The isolate from *Strongylodon macrobotrys* (26c) formed a separate branch in the group. The sequence similarity of isolate 26c with the other members of the group was relatively low and the position of the isolate in the *Mesorhizobium* subgroup was uncertain. *Mesorhizobium tianshanense* was the closest relative of the isolate from *Strongylodon macrobotrys* (26c) in the group, sharing 95,73% sequence similarity.

Subgroup Ib

All the reference strains of *Sinorhizobium* clustered into subgroup Ib (excluding *S. medicae*, which is not available on the GenBank database). Isolate PL18b from *Medicago sativa* displayed 99,45% sequence similarity with *S. meliloti*, 98,27% sequence similarity with the *S. xinjiangensis*, *S. saheli* and *S. fredii* cluster, and 97,82% sequence similarity with *S. teranga*.

Group II - *Allorhizobium* and *Rhizobium* group

The group displayed 99,27% sequence similarity with subgroup Ia and 98,91% with subgroup Ib. The group could be divided into two subgroups: IIa and IIb.

Subgroup IIa

The genus *Allorhizobium* was the only isolate in the subgroup and shared 96,18% sequence similarity with *Rhizobium huautlense*, the closest phylogenetic relative.

Subgroup IIb

All the reference species included in the genus clustered into the subgroup. Only one indigenous isolate from *Lessertia annularis* (PL20a) clustered into the *Rhizobium* group. The closest phylogenetic relatives (99,27% sequence similarity) were *R. mongolense* and *R. gallicum*.

Group III - *Azorhizobium* group

The reference strain *Azorhizobium caulinodans* was the only member of group III. As expected the closest phylogenetic relative was quite distant. The group shared 92,91% with group I, 93,27% sequence similarity with group II, 88,55% with group IV, 90,00% with group V and 89,27% with group VI.

Group IV

No rhizobial reference strains clustered in group IV. The group displayed 92,00% sequence similarity with group II, 91,64% similarity with group I, 88,55% similarity with group III, 90,55% with group V and 89,82% with group VI. The group could be divided into three subgroups: IVa, IVb and IVc.

Subgroup IVa

The isolate from *Acacia xanthophloea* (85a1t1ons) was the only member of this subgroup. It displayed 90,91% sequence similarity with subgroup IVb and IVc.

Subgroup IVb

Only one isolate from *Lessertia annularis* (PL20bons) clustered in this subgroup. The subgroup displayed 88,18% sequence similarity with subgroup IVc and 79,09% similarity with subgroup IVa.

Subgroup IVc

Two isolates, PL19b from *Indigofera verrucosa* and 94 from *Vigna subterranea* clustered in the subgroup. The two isolates displayed a sequence similarity of 92,55%.

Group V

The only member of the group was isolate 24slym from *Desmodium tortuosum*. The closest phylogenetic relative according to the tree was group II, the *Allorhizobium* and *Rhizobium* group, displaying a sequence similarity of 93,45%.

Group VI - *Bradyrhizobium* group

The group could be divided into two subgroups (VIa and VIb), each containing one of the *Bradyrhizobium* reference strains (no 16S rDNA sequence data for the species *B. liaoningense* exist in the GenBank database).

Subgroup VIa

The reference strain *B. elkanii* clustered into the subgroup. Other isolates falling in this group were phylogenetically closely related to the reference strain. The isolates displayed the following sequence similarities with *B. elkanii*: w1c (*Vigna unguiculata*) - 99,27%, 70a (*Crotalaria brachycarpa*) - 99,36%, 79c (*Acacia caffra*) - 98,45%, PL3 (*Tephrosia*

grandiflora) - 98,09%, 102a (*Chamaecrista biensis*) - 97,36%, 68d (*Mucuna coriacea*) - 98,64%, 68aons (*Mucuna coriacea*) - 98,55% and 103b (*Indigofera hilaris*) - 98,73%.

Subgroup VIb

The *Bradyrhizobium japonicum* reference strain clustered into this subgroup. The two isolates displaying the closest phylogenetic relationship to *B. japonicum* were the isolates from *Senna petersiana* (TK1) and *Cassia floribunda* (47c3a). The sequence similarity of both with *B. japonicum* was 99,27%. The remaining isolates in subgroup VIb displayed the following sequence similarities with *B. japonicum*: 13csterre (*Lotononis bainesii*) - 98,45%, 46c2 (*Acacia sieberana* var *woodii*) and 13b (*Lotononis bainesii*) - 98,82%, 49b (*Indigofera melanadenia*) - 97,91%, 48b (*Tephrosia purpurea*) - 98,64%, 48a (*Tephrosia purpurea*) - 97,36% and 27d (*Teramnus labialis*) - 98,09%.

4.7 TREE RECONSTUCTED FROM PARTIAL SEQUENCE RESULTS OF PRIMER 16SRNAII-S

Six groups could be distinguished in the tree reconstructed from the data obtained using primer 16SRNAII-S (Fig. 5). Their numbering corresponds to that of Fig. 4.

Subgroup Ia - *Mesorhizobium* group

Five rhizobia isolates clustered in the *Mesorhizobium* group. The following rhizobia isolates were included: PL10grkn (*Argyrolobium tomentosum*), PL27a (*Chamaecrista mimosoides*), M3c (*Lotononis falcata*), 15c (*Desmodium tortuosum*) and M3b (*Lotononis falcata*). The branching pattern of the isolates differed from that in Fig. 4. PL10grkn displayed a sequence similarity of 99,65% with the reference strains *M. tianshanense* and *M. amorphae*. The closest relative (nearly 100% sequence similarity) of PL27a was *M. plurifarium. Mesorhizobium loti* was the closest phylogenetic relative of the isolates M3c (96,29%), 15c (96,18%) and M3b (96,65%).
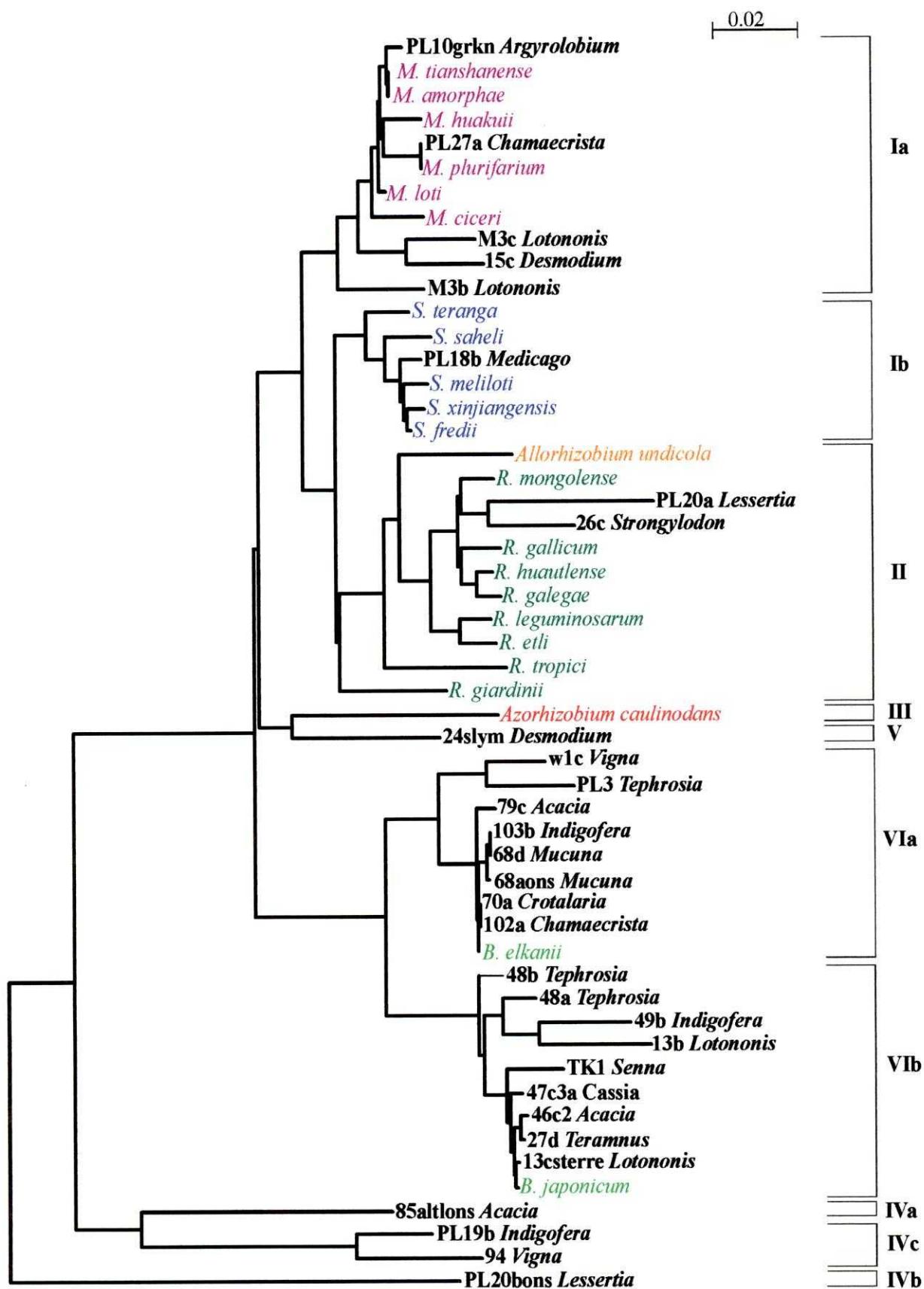
FIGURE 5 An unrooted tree reconstructed from sequence data obtained from primer 16SRNAII-S. Branch lengths are proportional to the estimated genetic distance between the strains. The scale represents 2% nucleotide difference. Vertical lengths are not significant and are set for clarity. Abbreviations: *M.*, *Mesorhizobium*; *S.*, *Sinorhizobium*; *R.*, *Rhizobium* and *B.*, *Bradyrhizobium*.

61

Subgroup Ib - *Sinorhizobium* group

All the species of *Sinorhizobium* clustered in the group together with the isolate PL18b from *Medicago sativa*. The isolate displayed different degrees of similarity with the reference strains: 98,94% (*S. meliloti*), 99,06% (*S. xinjiangensis*), 99,41% (*S. fredii*), 98,00% (*S. saheli*) and 97,77% (*S. teranga*).

Subgroup IIa - *Allorhizobium* group

The reference strain *A. undicola* was the only member of the group, no indigenous isolate clustered in the group.

Subgroup IIb - *Rhizobium* group

All the reference strains clustered in the group, except that the relative positions of the different species differed from Fig. 4, as well as the phylogenetic distance between the species. Two isolates clustered in the group, PL20a (*Lessertia annularis*) and 26c (*Strongylodon macrobotrys*). Isolate PL20a shared sequence similarity of 94,47% with *R. mongolense*, 94,35% with *R. gallicum* and *R. galegae* and 94,59% with *R. huautlense*. The isolate 26c was phylogenetically more related to the reference strains named above than PL20a. Sequence similarities of 96,35% (*R. mongolense*), 96,24% (*R. gallicum* and *R. galegae*) and 96,47% (*R. huautlense*) were displayed by the different reference strains.

Group III - *Azorhizobium* group

Azorhizobium caulinodans was the only member of group III, the closest phylogenetic relative being quite distant. The group displayed the following sequence similarities: 92,18% (subgroup Ia), 91,53% (subgroup Ib), 92,24% (group II), 81,41% (subgroup IVa), 77,18% (subgroup IVb), 82,94% (subgroup IVc), 91,29% (group V) and 90,82% (group VI). The group was the most similar to group II.

Subgroup IVa

The only isolate in the subgroup was from *Acacia xanthophloea* (85alt1ons), with no reference strain grouping close to the isolate. The isolate was distantly related to subgroup IVb (79,29% sequence similarity) and to subgroup IVc (88,12% sequence similarity).

Subgroup IVb

Isolate PL20bons (*Lessertia annularis*) was the only member of the subgroup, differing significantly from any reference strain.

Subgroup IVc

Two isolates PL19b (*Indigofera verrucosa*) and 94 (*Vigna subterranea*) belonged to this subgroup. The two isolates shared 95,06% sequence similarity.

Group V

The isolate 24slym (*Desmodium tortuosum*) was the only member of this group. All the rhizobial reference strains in other subgroups were quite distant to the isolate displaying the following sequence similarities: 93,59% (subgroup Ia), 92,94% (subgroup Ib), 93,65% (group II), 91,29% (group III), 82,82% (subgroup IVa), 78,59% (subgroup IVb), 84,35% (subgroup IVc) and 92,24% (group VI). The group shared the highest similarity with group II, the *Allorhizobium* and *Rhizobium* cluster.

Subgroup VIa

Eight indigenous rhizobia isolates belonged to the subgroup, which also contained the *B. elkanii* reference strain. The indigenous isolates were w1c (*Vigna unguiculata*), PL3 (*Tephrosia grandiflora*), 79c (*Acacia caffra*), 103b (*Indigofera hilaris*), 68d (*Mucuna coriacea*), 68aons (*Mucuna coriacea*), 70a (*Crotalaria brachycarpa*) and 102a (*Chamaecrista biensis*). Two isolates w1c and PL3 were phylogenetically more distant from *B. elkanii* than the other isolates, sequence differences varying from 3,53% (w1c) to 4,35% (PL3). The isolates from *Crotalaria brachycarpa* (70a) and *Chamaecrista biensis* (102a) displayed nearly

100% sequence similarity with the reference strain. All the other isolates were more distantly related, but still shared more than 99% sequence similarity with the reference strain. The isolates displayed the following sequence similarities with the reference strain: 99,53% (79c), 99,76% (103b and 68d) and 99,65% (68aons).

Subgroup VIb

The reference strain *B. japonicum* clustered in the subgroup, together with nine indigenous rhizobia isolates. The isolates 13csterre (*Lotononis bainesii*), 27d (*Teramnus labialis*), 46c2 (*Acacia sieberana* var *woodii*), 47c3a (*Cassia floribunda*), TK1 (*Senna petersiana*), 13b (*Lotononis bainesii*), 49b (*Indigofera melanadenia*), 48a (*Tephrosia purpurea*) and 48b (*Tephrosia purpurea*) belonged to the subgroup. Phylogenetically, most of the isolates were closely related to *B. japonicum*, displaying the following sequence similarities: 99,76% (13csterre), 99,59% (27d), 99,47% (46c2), 99,53% (47c3a), 98,47% (TK1), 98,00% (48a) and 98,59% (48b). Two of the isolates displayed less than 97% similarity with the reference strain. Isolate 13b from *Lotononis bainesii* shared 96,41% sequence similarity with *B. japonicum* and isolate 49b from *Indigofera melanadenia* shared 95,71% sequence similarity with *B. japonicum*.

CHAPTER 5

CHAPTER 5
DISCUSSION

The purpose of this investigation was to characterise and identify selected indigenous rhizobia by means of 16S rDNA sequence analysis. With five exceptions, they were unequivocally identified as members of valid rhizobia genera.

Trees reconstructed from the data obtained using the primers 16SRNAVI-S and 16SRNAII-S support the separation of the rhizobial isolates and reference strains into the six genera that are currently recognised. Although data in Fig. 4 was based on sequences from positions 131 to 570 and Fig. 5 on sequences from positions 691 to 1140 groupings within the two trees were remarkably similar. Some isolates, however, clustered differently in the two trees.

It has previously been shown that strains of a species showing 70% or more DNA-DNA relatedness usually display more than 97% sequence similarity between their 16S rDNA sequences (Stackebrandt and Goebel, 1994), while strains with 95% sequence similarity may belong to the same genus (Ludwig *et al.*, 1998). With this in mind it is clear that the isolates from *Argyrolobium tomentosum* (PL10grkn) and *Chamaecrista mimosoides* (PL27a) belonged to the genus *Mesorhizobium* since they clustered in the same genus in both trees (Fig. 4 and Fig. 5). The isolates from *Lotononis falcata* (M3b and M3c) and *Desmodium tortuosum* (15c) also belonged to the genus *Mesorhizobium*, but their degree of similarity in the two trees differed. In Fig. 4, isolate 15c displayed 97,55% sequence similarity with *M. tianshanense*, which was the closest reference strain. In Fig. 5, isolate 15c shared 96,18% sequence similarity with *M. loti*. The two isolates from *Lotononis falcata* (M3b and M3c) shared 98,09% and 99,09% sequence similarity with the *M. loti, M. huakuii, M. plurifarium* and *M. amorphae* cluster in Fig. 4. In Fig. 5, however, *M. loti* was the closest relative, displaying 96,65% and 96,29% sequence similarity with M3b and M3c, respectively.

The results of this study clearly showed that seventeen of the indigenous isolates belonged to the genus *Bradyrhizobium*. Eight of the isolates belonged to the species *B. elkanii* and nine to the species *B. japonicum*. Two isolates, 49b (*Indigofera melanadenia*) and 13b (*Lotononis bainesii*), displayed less than 97% (95,71% and 96,41% respectively) sequence similarity with

the *B. japonicum* reference strain (Fig. 5). In Fig. 4, however, the isolates 49b and 13b displayed sequence similarities of 97,91% and 98,82% respectively.

Only one isolate (PL18b *Medicago sativa*) could be assigned to the genus *Sinorhizobium*, as indicated in both trees (Fig. 4 and Fig. 5).

The results of the present study, using 16S rDNA sequence analysis, supported those of other workers using other methods including SDS-PAGE of WCP by Dagutat (1995) and Kruger (1998), and Biolog and 16S rDNA-RFLP (Kruger, 1998). The position of the isolates that grouped in the genus *Bradyrhizobium* corroborated results previously obtained by Kruger (1998). Inclusion of isolate PL18b (*Medicago sativa*) in the genus *Sinorhizobium* also corroborated the results obtained by Kruger (1998) using SDS-PAGE of WCP and 16S rDNA-RFLP dendrograms.

However, the different methods used as part of a polyphasic approach to characterise indigenous rhizobia did not always give the same results and common ground must be found in order to reach justifiable conclusions. For example, present results showed that the isolate from *Desmodium tortuosum* (15c) clustered differently in the different techniques used by Kruger (1998). According to her results the isolate either belonged to the genus *Mesorhizobium* or to the genus *Rhizobium* depending on the techniques used for identification.

The dendrograms obtained by SDS-PAGE of WCP and 16S rDNA-RFLP failed to elucidate the possible taxonomic position of isolate 24slym (*Desmodium tortuosum*) (Kruger, 1998). The isolate from *Desmodium tortuosum* (24slym) should therefore be further characterised in order to clarify the taxonomic position and identity of the isolate. The determination of the G + C content and DNA hybridisation results should indicate the taxonomic position of the isolate. Legume nodulation studies should confirm or disprove the nodule-forming ability of the isolate.

The position of the isolates from *Lessertia annularis* (PL20a) and *Strongylodon macrobotrys* (26c) were also not clear. The tree reconstructed from the first part of the gene (position 131 to 570) (Fig 4) suggested that isolate 26c belonged to the *Mesorhizobium* cluster and PL20a to the *Rhizobium* cluster. The results displayed in Fig 5 placed both the isolates in the genus

Rhizobium. However, sequence similarities with the reference strains were lower than 97%, suggesting that the isolates belonged to a new species. Isolate PL20a (*Lessertia annularis*) clustered loosely in the *Sinorhizobium* cluster with SDS-PAGE of WCP, in the *Rhizobium* cluster with 16S rDNA-RFLP and in a *Rhizobium-Sinorhizobium* cluster according to Biolog results (Kruger, 1998). The isolate from *Strongylodon macrobotrys* (26c) may belong to the genus *Mesorhizobium* or the genus *Rhizobium* according to SDS-PAGE of WCP and Biolog results (Kruger, 1998). Further polyphasic characterisation of isolates PL20a and 26c is therefore required before their taxonomic position can be determined.

The isolates from group IV [85a1t1ons (*Acacia xanthophloea*), PL20bons (*Lessertia annularis*), PL19b (*Indigofera verrucosa*) and 94 (*Vigna subterranea*)] were either not true rhizobia or unknown rhizobia as they differed significantly from the rhizobial reference strains. It is possible the isolates were either contaminants of the nodules or true nitrogen fixing bacteria or bacteria promoting the nitrogen-fixing ability of the rhizobia strains in the nodules. However, colony morphology and inability to absorb Congo Red from YMA suggested that they were rhizobia. In order to determine the possible identity of the isolates, the sequences of the isolates were compared with the sequence data in the GenBank database. The following interesting results were obtained: isolate 85a1t1ons showed similarity with *Xanthomonas campestris*, isolates 94 and PL19b were related to *Burkholderia* (previously included in the genus *Pseudomonas*) and isolate PL20bons might belong to the genus *Klebsiella*. *Xanthomonas campestris* and *Burkholderia* are plant pathogens causing a wide variety of diseases (Agrios, 1997). All the species of the genus *Xanthomonas* are plant pathogens and the bacteria are only found in close association with plants or plant material (Agrios, 1997). Both are Gram-negative obligate aerobes, whereas *Klebsiella* is a member of the *Enterobacteriaceae*, a family of facultatively anaerobic Gram-negative enteric bacteria (Ørskov, 1984). The four isolates require further investigation by legume nodulation studies before any definite conclusion is reached concerning their identity.

Rhizobial strains were isolated from supposedly non-nodulating legumes. The isolates from *Senna petersiana* (TK1) and *Cassia floribunda* (47c3a) could be assigned to the genus *Bradyrhizobium*. Results from SDS-PAGE of WCP, Biolog and 16S rRNA-RFLP studies of Kruger (1998) also indicated that the isolate from *Senna petersiana* (TK1) belongs to the genus *Bradyrhizobium*. Other techniques of a polyphasic approach need to be applied to confirm the taxonomic position of the isolate from *Cassia floribunda* (47c3a). More work

should be done on the supposedly non-nodulating legumes in order to determine whether the nodulation of non-nodulating legumes is a once off happening or something that regularly occurs in nature.

This study again demonstrated that more than one strain of rhizobium can nodulate a specific host plant, as was found by Dagutat (1995) and Kruger (1998). It is clearly an oversimplification to say that a specific host plant genus is nodulated only by rhizobia belonging to a specific genus as rhizobia from taxonomic different groups were found to nodulate the same host plant genera. The legume genus *Chamaecrista* was found to be nodulated by the isolates PL27a and 102a, which respectively clustered in the *Mesorhizobium* cluster and the *Bradyrhizobium* cluster (Fig. 4 and Fig. 5).

Nodulation studies using selected hosts (*Medicago sativa, Pisum sativum, Phaseolus vulgaris, Trifolium repens, Lotus corniculatus, Glycine max, Vigna unguiculata, Leucaena leucocephala, Macroptilium atropurpureum* and *Galega officinalis*) is a phenotypic method, that must be included in the polyphasic approach to describe a possible new species or genus (Graham *et al.*, 1991). The ability to nodulate is often determined by plasmids. In the case of *M. loti, Bradyrhizobium* and *Azorhizobium*, the symbiotic genes are situated on the chromosome (Van Rhjin and Vanderleyden, 1995). The bacteria can exchange their symbiotic genes or lose the genes (lose their plasmids). Inability to nodulate is thus not enough reason to discard an isolate as not a rhizobium if all molecular techniques indicate the contrary. Nevertheless determination of nodulation and cross-inoculation ability is essential considering possible practical application of isolates as inoculants.

Results of the partial sequencing approach in this study were not suited for the determination of the evolution of the isolates. However, the data could be used for a preliminary positioning of isolates within phylogenetic trees (Ludwig *et al.*, 1998). The evolutionary pathways of the isolates can only be correctly determined using complete 16S rDNA sequences. The method used to analyse the sequence data determines whether the tree is phylogenetic or phenetic (in which case the molecular sequences are the different characters). Phylogenetically based algorithms should be used for a tree to truly represent the evolutionary pathway of the isolates. Even then, it is not certain that the phylogenetic tree is the correct one (Priest and Austin, 1993). Even though phylogenetic conclusions could not be made from the trees, the phylogenetically distant position of *Bradyrhizobium* and *Azorhizobium* was again clear.

Partial sequencing of the 16S rDNA enabled positive conclusions to be made regarding the taxonomic position of most of the rhizobial isolates included in the present study. Ludwig *et al.* (1998) suggested that partial sequence data could be used to identify organisms or to assign isolates to well-established phylogenetic groups. My results showed that the method was able to differentiate between genera, species and more distantly related strains within a species. Stackebrandt and Goebel (1994) state that it is not possible to distinguish between strains and recently diverged species using 16S rDNA sequence analysis. This limitation was confirmed in the present study. Alternative techniques such as REP-PCR, 16S-23S IGS RFLP and RAPD that possess better resolution should be used to augment the results of the 16S rDNA sequencing analysis.

CHAPTER 6

CHAPTER 6
CONCLUSIONS

- More than one rhizobium strain can nodulate the same host plant.

- Rhizobia from different taxonomic groups can nodulate the same legume genus.

- Isolates from nodules of supposedly non-nodulating legume genera are members of the genus *Bradyrhizobium*.

- Supposedly non-nodulating legume genera should be systematically investigated to determine their nodulation status.

- The partial sequencing approach supported the separation of the stem- and root-nodulating bacteria into six genera.

- No conclusions about the evolution of the rhizobia included in this study could be drawn from the trees obtained using the partial sequencing approach.

- The two different partial approach methods are equally suited for the identification and characterisation of the isolates.

- Most of the indigenous isolates examined are slow-growers belonging to the genus *Bradyrhizobium*.

- Some of the indigenous isolates belong to the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*.

- The sequencing results corroborate results obtained by other workers as part of the polyphasic approach to identify and determine the diversity of indigenous rhizobia.

- Isolates that did not group with the reference strains should be further investigated to determine their taxonomic position.

- Legume nodulation studies must be done on all the isolates to confirm their nodulating ability.

CHAPTER 7

CHAPTER 7
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