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Rhizobia associated with Australian *Acacia* species (*Acacia mearnsii*, *Acacia dealbata* and *Acacia decurrens*) in South Africa as determined by Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis

by

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Submitted in partial fulfilment of the requirements for the degree of

MAGISTER SCIENTIA AGRICULTURA
(MICROBIOLOGY)

in the

Department of Microbiology and Plant Pathology,
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University of Pretoria,
Pretoria, South Africa

February 2002

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

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Date: 07/06/2002

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BEDANKINGS

Ek wil graag my dank betuig aan die volgende persone:

Prof P. L. Steyn vir die vertroue wat hy in my gestel het en al sy waardevolle insette, leiding en raad gedurende die projek, asook met die afhandeling van die verhandeling.

Fanus Venter vir sy hulp, raad en leiding met die afhandeling van die projek en veral met die skryf van die verhandeling.

Marinda Oosthuizen vir haar bereidwilligheid om haar kennis te deel en te help, veral wat die elektroferese tegniek betref, asook vir haar belangstelling en ondersteuning.

Julian Jaftha vir sy belangstelling en ondersteuning, en sy hulp en insette wat betref verskeie aspekte van die projek.

Bridgitta Steyn vir haar hulp op verskeie maniere gedurende die projek, asook haar belangstelling en ondersteuning.

My laboratoriumgenote - Marleen Kock, Jaco le Roux en Michelle Lindeque - en al my vriende vir hulle belangstelling en ondersteuning.

Gert van der Westhuizen vir sy vertroue in my, sy vriendskap, geduld, aanmoediging en ondersteuning.

My ouers vir hulle oneindige geduld, liefde en belangstelling gedurende my studies. Ook Christelle en Deon vir hulle liefde en ondersteuning.

Aan my Hemelvader, sonder wie se genade en liefde ek tot niks in staat is nie.

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SUMMARY

The projected exponential growth of the human population necessitates a concomitant increase in food supplies, and by implication an increase in fixed nitrogen for crops and pastures. This can to a large extent be supplied by biological nitrogen fixation (BNF). However, to achieve this goal improved effectivity of the legume-rhizobium symbiosis is required, implicating improvement in the macro- as well as the microsymbiont. Therefore the search for more effective microsymbionts is a *sine qua non* to provide better matching and tolerance to stress conditions.

The aim of this study was to investigate the range of rhizobia associated with the exotic Australian *Acacia* species (*A. mearnsii*, *A. dealbata* and *A. decurrens*) in South Africa and to determine whether these species could be useful to provide rhizobial strains for application in the South African inoculant industry in order to improve local existing biological nitrogen-fixing systems. Although these *Acacia* species are geographically

widespread throughout South Africa, their root nodule bacteria have never been investigated in depth. Their widespread occurrence and presumed promiscuity suggested that they might form nitrogen-fixing symbioses with a wide range of indigenous rhizobial strains with different ecological adaptations.

In this study nodulated plants of the three *Acacia* spp. were collected from diverse geographic areas with diverse climatic conditions and different soil pH's. Isolates were obtained from root nodules, purified and the putative rhizobial isolates characterized with sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), supplemented at the genomic level with 16S rDNA sequence data of selected isolates.

The majority of the isolates investigated were members of the genus *Bradyrhizobium*, whilst some isolates showed close relationships to the genera *Agrobacterium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. As a result of their predominant association with the slow-growing strains of the genus *Bradyrhizobium*, the legume spp. *A. mearnsii*, *A. dealbata* and *A. decurrens* as trap plants would not play a significant role as a source of diverse rhizobia for application in the South African inoculant industry.

Rhizobiums geassosieer met *Acacia* spesies (*Acacia mearnsii*, *Acacia dealbata* en *Acacia decurrens*) in Suid-Afrika soos bepaal deur Natriumdodesielsulfaat Poliakrielamied Jel Elektroforese

deur

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OPSOMMING

Die geprojekteerde eksponensiële groei van die menslike bevolking noodsaak 'n gepaardgaande toename in voedselvoorrade, en by implikasie 'n toename in gebonde stikstof vir gesaaides en weiding. Biologiese stikstofbinding kan tot 'n groot mate in hierdie aanvraag voorsien. Die effektiwiteit van die peulplant-rhizobium simbiose, en dus by implikasie beide die makro- en mikrosimbiont, sal egter verbeter moet word om dit te bereik. Daarom is die soeke na meer effektiewe mikrosimbionte 'n *sine qua non* om beter paring en toleransie van spanningstoestande te voorsien.

Die doel van hierdie studie was om die omvang van die diverse rhizobiums geassosieer met die uitheemse Australiese *Acacia* spesies (*A. mearnsii*, *A. dealbata* and *A. decurrens*) in Suid-Afrika te ondersoek en om te bepaal of hierdie spesies nuttig kan wees in die voorsiening van rhizobiumstamme vir benutting in die Suid-Afrikaanse inokulantindustrie met die doel om die plaaslike biologiese stikstofbindingsisteme te

verbeter. Hoewel hierdie *Acacia* spesies geografies wydverspreid in Suid-Afrika voorkom, is hulle wortel-knoppiesbakterieë nog nooit in 'n diepgaande studie ondersoek nie. Hulle wydverspreide voorkoms en veronderstelde promiskuïteit suggereer egter dat hulle moontlik stikstofbindende simbioses vorm met 'n wye reeks inheemse rhizobiumstamme met verskillende ekologiese aanpassings.

In hierdie studie is genoduleerde plante van hierdie spp. van uiteenlopende geografiese gebiede met verskillende klimaatstoetstande en grondpHs verkry. Bakterieë is uit die wortelknoppies geïsoleer, gesuiwer en vermoedelike rhizobium-isolate gekarakteriseer deur natriumdodesielsulfaat poliakrielamied jel elektroforese (SDS-PAGE), aangevul deur genomiese data (16S rDNS volgordebepaling) van geselekteerde isolate.

Die meerderheid van die isolate wat ondersoek is, was lede van die genus *Bradyrhizobium*, terwyl sekeres wel naby-verwantskappe aan lede van die genusse *Agrobacterium*, *Mesorhizobium*, *Rhizobium* en *Sinorhizobium* getoon het. Die peulplantspesies *A. mearnsii*, *A. dealbata* and *A. decurrens* sal daarom, as gevolg van hulle oorwegende assosiasie met die stadiggroeiende stamme van die genus *Bradyrhizobium*, geen noemenswaardige rol as lokplante vir die voorsiening van verskillende rhizobiums vir benutting in die Suid-Afrikaanse inokulantindustrie speel nie.

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LIST OF ABBREVIATIONS

AP-PCR	arbitrarily primed-PCR
ARC	Agricultural Research Council
ARDRA	amplified ribosomal DNA restriction analysis
AFLP	amplified fragment length polymorphism
BNF	biological nitrogen fixation
bp	base pairs
bv	biovar
DAF	DNA amplification fingerprinting
°C	degrees Celsius
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
ERIC-PCR	enterobacterial repetitive intergenic consensus-PCR
h	hour
IAR typing	intrinsic antibiotic resistance typing
IGS	intergenic spacer region
kbp	kilobase pairs
LFRFA	low frequency restriction fragment analysis
LMG	Laboratorium voor Microbiologie Gent Culture Collection
LP-RAPD	long primer RAPD
µl	microlitre
MLEE	multilocus enzyme electrophoresis
m/v	mass per volume
NCBI	National Center for Biotechnology Information
NFU	Nitrogen Fixation Unit
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
RAPD	randomly amplified polymorphic DNA analysis

rep-PCR	repetitive-sequence-based PCR (including BOX-, ERIC- and REP-PCR)
REP-PCR	repetitive extragenic palindromic PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sp.	species
spp.	species (plural)
STB	sample treatment buffer
TY	tryptone yeast extract medium
UPGMA	unweighted pair group method of arithmetic averages
USDA	United States Department of Agriculture - ARS National Rhizobium Culture Collection
var	variety
v/v	volume per volume
YEB	yeast extract beef medium
YMA	yeast extract mannitol agar
YMB	yeast extract mannitol broth

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CHAPTER 1

CHAPTER 1
INTRODUCTION

The projected exponential growth of the human population necessitates a concomitant increase in food supplies, and by implication an increase in fixed nitrogen for crops and pastures. This can to a large extent be supplied by biological nitrogen fixation (BNF). (Rockefeller Foundation, 1997)

BNF is restricted to procaryotes of which some achieve nitrogen fixation on their own, whereas others must establish a symbiotic relationship with a eucaryotic partner to support fixation. Most of the world's land-based biological nitrogen fixation can be accounted for by the symbiotic nitrogen fixation relationship between leguminous plants and rhizobia. Thus, the main practical impact of BNF has been the development of rhizobial inoculants for crop and pasture legumes. (Rockefeller Foundation, 1997)

Agriculturally important legumes are often imported from their native habitats and cultivated as introduced crops. The rhizobial strains specific for the introduced crop may be transmitted along with the legume host and become established in the new soil environment, or these strains may be replaced by indigenous rhizobia able to establish a successful symbiosis with the introduced crop (Bromfield *et al.*, 1986; Sullivan *et al.*, 1996; Wang *et al.*, 1999b).

In South Africa legume inoculation has been practised since the first half of the 20th century by transferring soil from the vicinity of a well-established legume to soil in which it was cultivated for the first time. Imported inoculants, however, in general proved unsatisfactory (Strijdom, 1998). Therefore, in order to provide inoculant strains that are better adapted to local soil and environmental conditions, it is necessary to characterize the indigenous rhizobia, as well as to establish their host ranges and factors that influence their distribution and dynamics (Bromfield *et al.*, 1986; Odee *et al.*, 1997). The host promiscuity of the indigenous strains will play an important role in their ability to

establish a symbiosis with an introduced crop (Wang *et al.*, 1999b). The indigenous strains should especially be characterized with regard to their effectivity (in terms of nitrogen fixation) when developing inoculants for local application (Odee *et al.*, 1997), particularly when agriculture extends into regions which are marginal because of low and/or erratic rainfall or poor soil fertility. In such instances the inoculant strains should have a wide effective host range and should be able to survive a wider range of environmental conditions to provide better matching of the rhizobia with host plants and to stress conditions.

In South Africa the switch away from commercial to small-scale farming, as well as the curtailing of subsidies to commercial farmers will enhance a culture of 'low input' agriculture favourable for the exploitation of biologically fixed nitrogen (Strijdom, 1998). Research supporting the increased utilisation and optimisation of BNF in agriculture must therefore be promoted. A comprehensive research programme was initiated at the Agricultural Research Council (ARC)-Plant Protection Research Institute, where a unit for biological nitrogen fixation (NFU) was established in 1965. Forming part of this research programme, a long-term project aimed at determining the taxonomic status of rhizobia indigenous to South Africa was established in 1995 (Dagutat, 1995). Strains obtained from this project are maintained in the culture collection of the Nitrogen Fixation Unit (NFU) of the Agricultural Research Council-Plant Protection Research Institute (Strijdom, 1998). These strains are screened for specific properties in attempts to identify inoculant strains for local application.

Acacia species are often a prominent component of ecosystems in which they occur, especially in pioneer communities that become established after deforestation (Dreyfus and Dommergues, 1981; Lafay and Burdon, 1998; Odee *et al.*, 1997). This may reflect the competitive advantage that these legumes gain in soils of low fertility from symbiotic nitrogen-fixing associations with rhizobia (Lafay and Burdon, 1998). The wide geographic distribution of the acacias also suggests that they may form nitrogen-fixing symbioses with a wide range of rhizobial strains with different ecological adaptations (Marsudi *et al.*, 1999).

Acacias may be either promiscuous, i.e. nodulated non-specifically by more than one species of rhizobia, or restricted in their association with rhizobia (Dreyfus and Dommergues, 1981). In South Africa observations have suggested that the exotic Australian *Acacia* spp., *A. mearnsii*, *A. dealbata* and *A. decurrens*, might be promiscuous in their association with rhizobia (B. W. Strijdom, personal communication). This, together with their wide geographic distribution throughout South Africa, make these legume species especially suitable candidates for trap plants for rhizobia for application in the South African inoculant industry.

In this study, which forms part of the long-term project, the suitability of the exotic Australian *Acacia* spp. (*A. mearnsii*, *A. dealbata* and *A. decurrens*) as trap plants was evaluated. The rhizobia associated with these *Acacia* spp. were characterized using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), supplemented with genotypic data (16S rDNA sequencing) of selected isolates.

CHAPTER 2

CHAPTER 2

LITERATURE REVIEW

2.1 THE RHIZOBIA

2.1.1 A brief overview of the development of the rhizobial taxonomy

The classification of the root nodule bacteria started with the isolation of these bacteria by Beijerinck in 1888. He established these organisms as the causative agents of dinitrogen assimilation and proposed the name *Bacillus radicicola*. The organism was renamed *Rhizobium leguminosarum* in 1889 by Frank. Early researchers considered all bacteria that formed nitrogen-fixing root nodules on legumes as belonging to the genus *Rhizobium*. However, in 1929 Baldwin and Fred proposed a taxonomic characterization of rhizobia based on bacteria-plant cross inoculation groups. The cross inoculation groups consisted of collections of legume species that would develop effective nodules when inoculated with the rhizobia obtained from the nodules of any member of that legume group. This concept, however, gradually became less acceptable as a result of cross inoculation(s) with rhizobia from outside the assigned group and failure to cross inoculate within a group. The designation of new species of *Rhizobium* on the basis of cross inoculation was discontinued following the publication of a paper by Wilson (1944). Although the cross inoculation system cannot be regarded as a taxonomic classification system, the current taxonomy still retains some of this concept. (Elkan, 1992; Van Berkum and Eardly, 1998; Young and Haukka, 1996)

Since the host-based classification system was abandoned, the focus of classification has shifted to other approaches. Löhnis and Hansen (1921) first suggested the division of the rhizobia according to growth rate on laboratory media. Since then the data that were accumulated with regard to serology, extracellular polysaccharide composition, nutrition, metabolism, deoxyribonucleic acid (DNA) base ratios, DNA hybridization, ribonucleic acid (RNA) analysis and phage susceptibility were incorporated into large numerical

taxonomic studies. The increase in taxonomic information led to the interim reorganization of the rhizobia published in *Bergey's Manual of Systematic Bacteriology* (Jordan 1984).

However, the rapid development of new molecular techniques has led to the description of several new genera and species since the publication of the interim taxonomy. The molecular data also indicated that the rhizobia are polyphyletic: no branch of the evolutionary tree carries all the rhizobia and no other bacteria. The data confirmed that the genera *Bradyrhizobium* and *Azorhizobium* belonged to distinct separate phylogenetic lineages. The genera comprising the fast-growing rhizobia and the genus *Agrobacterium* are, however, phylogenetically heterogeneous, and several subgroupings in which these genera are intermixed are evident. (Young, 1996). Sequence comparison of 16S ribosomal RNA (rRNA) genes, which is generally accepted as the standard method to assess phylogenetic relationships among bacteria, clearly shows the diversity of the rhizobia (Sawada *et al.*, 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993; Young *et al.*, 2001; Young, 1996; Young and Haukka, 1996). Although all the known rhizobia are members of the alpha subdivision of the *Proteobacteria*, the branch that includes them all also carries other non-root nodule bacteria of different genera. It is likely that, as more rhizobia are isolated and described, new genera will be created to accommodate new isolates. One must also take into account that the 16S rRNA gene is not an infallible guide to evolutionary relationships, particularly among closely related taxa at the family level and below (Sneath, 1989; Haukka *et al.*, 1996). To compare closely related species, hybridization of total genomic DNA will provide a more credible estimate of overall genetic similarity, although even this method is not without problems (Young, 1996).

It is perceived that the taxonomy of the rhizobia will still undergo many changes until more of the legumes species have been studied with regard to their associated rhizobia. Presently only the economically important genera or those that are grown over large areas, have been examined for their ability to form nodules with rhizobia. As the information with regard to the systematics of the root-and stem-nodulating bacteria

develops, it will become even more important to balance phylogenetic differences with selected phenotypic characteristics (Woese, 1987). Therefore a set of universal standards for the description of new genera and species of root-and stem-nodulating bacteria was proposed (Graham *et al.*, 1991).

2.1.2 The rhizobium-legume association

The rhizobia are classified together by virtue of their ability to nodulate members of the *Fabaceae*. The family *Fabaceae* constitutes one of the largest and most widely distributed families of flowering plants, occupying habitats ranging from rain forests to arid zones throughout the world. Many members of the family are of ecological and economic significance, which is partly due to their ability to form nitrogen-fixing symbioses with the rhizobia. The family *Fabaceae* is divided into three subfamilies, namely *Caesalpinoideae*, *Mimosoideae* and *Papilionoideae* (Van Berkum and Eardly, 1998). These subfamilies comprise about 750 genera, containing 16 000 to 19 000 species (Elkan, 1992).

Only a very small number of species (23% of those examined) of the subfamily *Caesalpinoideae* are known to be nodulated. The subfamily *Mimosoideae* seems to have evolved earlier from the *Caesalpinoideae* than the subfamily *Papilionoideae*. However, less is known about the symbiotic bacteria from the *Mimosoideae* than from the *Papilionoideae*. The species of the subfamily *Mimosoideae* are nodulated at a higher frequency (90% of those examined) than the members of the *Caesalpinoideae*. *Leucaena*, *Acacia* and *Prosopis* are important genera within the *Mimosoideae*. Species in certain genera may be nodulated by either *Rhizobium* or *Bradyrhizobium*. Most of the genera of the subfamily *Papilionoideae* are nodulated (97% of those examined) and well studied. (Somasegaran and Hoben, 1994a; Van Berkum and Eardly, 1998)

For a summary of the rhizobia and their principal host legumes, see Table 2.1.

Table 2.1 **Members of the family *Rhizobiaceae* and their principal host legumes**

Member	Principal host legume(s)	Reference
The genus *Allorhizobium*		
A. undicola	*Neptunia natans*	De Lajudie *et al.*, 1998a
The genus *Azorhizobium*		
A. caulinodans	*Sesbania rostrata*	Dreyfus *et al.*, 1988
The genus *Bradyrhizobium*		
B. elkanii	*Glycine max*	Kuykendall *et al.*, 1992
B. japonicum	*Glycine* spp., *Macroptilium artropurpureum*	Jordan, 1982
B. liaoningense	*Glycine max, G. soja*	Xu *et al.*, 1995
The genus *Mesorhizobium*		
M. amorphae	*Amorpha fruticosa*	Wang *et al.*, 1999b
M. chacoense	*Prosopis alba, P. chilensis, P. flexuosa*	Velázquez *et al.*, 2001
M. ciceri	*Cicer arietinum*	Nour *et al.*, 1994
M. huakuii	*Astragalus sinicus*	Chen *et al.*, 1991
M. loti	*Lotus* spp., *Lupinus densiflorus, Anthyllis vulneraria*	Jarvis *et al.*, 1982
M. mediterraneum	*Cicer arietinum*	Nour *et al.*, 1995
M. plurifarium	*Acacia* spp., *Leucaena leucocephala, Neptunia oleracea*	De Lajudie *et al.*, 1998b
M. tianshanense	*Glycine max, Glycyrrhiza* spp. and other	Chen *et al.*, 1995
The genus *Rhizobium*		
R. etli bv *mimosae*	*Mimosa affinis, Phaseolus vulgaris, Leucaena leucocephala*	Wang *et al.*, 1999a
R. etli bv *phaseoli*	*Phaseolus vulgaris*	Segovia *et al.*, 1993
R. galegae	*Galega officinalis, G. orientalis*	Lindström, 1989
R. gallicum	*Phaseolus vulgaris*	Amarger *et al.*, 1997
R. giardinii	*Phaseolus vulgaris*	Amarger *et al.*, 1997
R. hainanense	*Desmodium sinuatum*	Chen *et al.*, 1997

Table 2.1 (continued)

Member	Principal host legume(s)	Reference
The genus *Rhizobium* (continued)		
R. huautlense	*Sesbania herbacea, Leucaena Leucocephala*	Wang *et al.*, 1998
R. leguminosarum bv *viciae*	*Pisum, Vicia, Lathyrus, Lens*	Jordan, 1984
R. leguminosarum bv *trifolii*	*Trifolium* spp.	Jordan, 1984
R. leguminosarum bv *phaseoli*	*Phaseolus vulgaris*	Jordan, 1984
R. mongolense	*Medicago ruthenica, Phaseolus vulgaris*	Van Berkum *et al.*, 1998
R. tropici	*Phaseolus vulgaris, Leucaena* spp.	Martinez-Romero *et al.*, 1991
R. yanglingense	*Coronilla varia, Gueldenstaedtia multiflora, Amphicarpaea trisperma*	Tan *et al.*, 2001
The genus *Sinorhizobium*		
S. arboris	*Acacia senegal, Prosopis chilensis*	Nick *et al.*, 1999b
S. fredii	*Glycine max, G. soja, Vigna unguiculata, Cajunus cajan*	Scholla and Elkan, 1984
S. kostiense	*Acacia senegal, Prosopis chilensis*	Nick *et al.*, 1999b
S. medicae	*Medicago polymorpha* and other annual *Medicago* spp.	Rome *et al.*, 1996b
S. meliloti	*Medicago, Melilotus, Trigonella*	Jordan, 1984
S. saheli	*Acacia seyal, Sesbania* spp., *Leucaena leucocephala, Neptunia oleracea*	De Lajudie *et al.*, 1994
S. terangae	*Acacia* spp., *Sesbania* spp., *Leucaena leucocephala, Neptunia oleracea*	De Lajudie *et al.*, 1994

2.1.2.1 The genus Allorhizobium

The species *A. undicola* includes a group of fast-growing strains that are capable of efficient nitrogen-fixing symbiosis with the aquatic plant *Neptunia natans* via adventitious root nodules rather than true stem nodules (De Lajudie *et al.*, 1998a).

2.1.2.2 The genus Azorhizobium

Stem nodulation occurs in four legume genera, namely *Aeschynomene*, *Neptunia*, *Discolobium* and *Sesbania*. The *Aeschynomene* stem nodule isolates of the genus *Bradyrhizobium* are the only symbionts to produce bacteriochlorophyll and perform photosynthesis.

The fast-growing strains that very specifically nodulate the stems and roots of *Sesbania rostrata* are grouped in the species *A. caulinodans* (Dreyfus *et al.*, 1988). Although this species is the only named species within the genus, a second species has been recognised by DNA-DNA hybridization assays (Rinaudo *et al.*, 1991). This species is unusual in its ability to grow with dinitrogen as sole source of nitrogen; thus they can actively fix atmospheric nitrogen both symbiotically and *ex planta*.

2.1.2.3 The genus Bradyrhizobium

The genus *Bradyrhizobium* represents an extremely heterogeneous group of symbionts. The symbionts of soybean belong to one of three groups, namely the fast-growing strains of the species *Sinorhizobium fredii* (Scholla and Elkan, 1984; Chen *et al.*, 1988), the slowly growing strains of the species *B. japonicum* (Skerman *et al.*, 1980; Jordan, 1982) and the extra-slowly growing strains of the species *B. liaoningense* (Xu *et al.*, 1995). After the description of the species *B. japonicum* it was, however, evident that this species consisted of several groups which were too diverse to fit into a single species (Hollis *et al.*, 1981; Kuykendall *et al.*, 1988; Minamisawa *et al.*, 1992; Stanley *et al.*, 1985). Hollis *et al.* (1981) recognised three DNA homology groups of which groups I and Ia corresponded to the current species *B. japonicum*. Strains of DNA homology group II, which formed a fairly clear subgroup within the soybean bradyrhizobia, were described as *B. elkanii* (Kuykendall *et al.*, 1992, 1993). The remaining *B. japonicum* strains are, however, still very diverse and further speciation might be needed.

The many *Bradyrhizobium* strains that do not nodulate soybean, but clearly belong within this genus, are simply known as *Bradyrhizobium* sp., followed by the name of the legume host. The photosynthetic symbionts of *Aeschynomene* spp. are included within this group

of bradyrhizobia. Apart from the fact that this group of strains is mostly known to be distinct from the three named species, their taxonomic status and interrelationships are unresolved. However, several studies have revealed a large diversity among *Bradyrhizobium* isolates (Doignon-Bourcier *et al.*, 2000; Dupuy *et al.*, 1994; Moreira *et al.*, 1993; Lafay and Burdon, 1998, 2001; Willems *et al.*, 2000, 2001b, 2001c), with the potential of many more species than the current three.

2.1.2.4 The genus Mesorhizobium

Due to the polyphasic approach to the classification of the rhizobia, it has become evident that there are three genetic groups within the genus *Rhizobium*. The separation of the third branch was well supported by 16S rRNA sequence data (Sawada *et al.*, 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993), as well as phenotypic data (Jarvis *et al.*, 1996). Therefore, the formerly described species *R. loti* (Jarvis *et al.*, 1982), *R. huakuii* (Chen *et al.*, 1991), *R. ciceri* (Nour *et al.*, 1994), *R. mediterraneum* (Nour *et al.*, 1995) and *R. tianshanense* (Chen *et al.*, 1995) were transferred to a new genus, *Mesorhizobium* (Jarvis *et al.*, 1997). Strains from this genus generally form nitrogen-fixing nodules on the roots of a restricted range of leguminous plants and cross inoculation between the strains of one species of this genus and the plant hosts associated with another species is not known.

M. loti forms effective nodules on *Lotus*, *Lupinus* and *Anthyllis* spp., though not necessarily on all species (Jarvis *et al.*, 1982). The same host legumes can also be nodulated by *Bradyrhizobium*. *M. huakuii* strains nodulate their original host, *Astragalus sinicus*, as well as a few other hosts (Chen *et al.*, 1991). *Cicer arietinum* (chickpea) is nodulated by *M. ciceri* (Nour *et al.*, 1994) and *M. mediterraneum* (Nour *et al.*, 1995). The species *M. tianshanense* includes a collection of strains from various legumes (including soybean) in Xinjiang Province, China (Chen *et al.*, 1995).

De Lajudie *et al.* (1994) identified three distinct clusters, namely clusters S, T and U, based on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell proteins for strains isolated from different *Acacia* and *Sesbania*

spp. from Senegal (West Africa). Cluster U belonged to the genus *Mesorhizobium* and a new species, *M. plurifarium*, was proposed for these strains isolated from *Acacia*, *Leucaena*, *Prosopis* and *Chamaecrista* spp. in West Africa (Senegal), East Africa (Sudan) and South America (Brazil) (De Lajudie *et al.*, 1998b). These strains are able to nodulate various *Acacia* spp., *Leucaena leucocephala* and *Neptunia oleracea*.

The species *M. amorphae* includes the moderately slow- to slow-growing, acid-producing symbionts of *Amorpha fruticosa* (Wang *et al.*, 1999b), whilst strains included in the species *M. chacoense* form effective nitrogen-fixing symbioses with several *Prosopis* spp. (Velázquez *et al.*, 2001).

2.1.2.5 The genus Rhizobium

The genus *Rhizobium* is phylogenetically heterogeneous and contains eleven accepted and proposed species. Young *et al.* (2001) proposed the transfer of the species *Agrobacterium* biovar (bv) 1, *A. rhizogenes*, *A. rubi*, *A. vitis* and *Allorhizobium undicola* to this genus.

The species *R. leguminosarum* has been subdivided into three biovars to distinguish their plant affinities: biovar *viciae* nodulates *Pisum*, *Vicia*, *Lathyrus* and *Lens* spp.; biovar *trifolii* nodulates *Trifolium* spp. (clover) and biovar *phaseoli* nodulates *Phaseolus vulgaris* (common bean) (Jordan, 1984). The host ranges of the three biovars are quite distinct and seem to be mutually exclusive.

The recognition of several species was warranted within the genetically heterogeneous symbionts of bean (Beynon and Josey, 1980; Crow *et al.*, 1981; Pinero *et al.*, 1988; Roberts *et al.*, 1980). Two major groups were recognised within the species *Rhizobium leguminosarum* bv *phaseoli*, namely type I and type II. The type II strains that effectively nodulated both *P. vulgaris* and *Leucaena* spp. and were distinct from *R. leguminosarum* bv *phaseoli*, were classified as *R. tropici* (Martínez-Romero *et al.*, 1991). It was also proposed that the type I strains which differed from typical *R. leguminosarum* in its 16S rDNA sequence and nonsymbiotic isolates of American origin be reclassified as *R. etli*

(Segovia *et al.*, 1993). At least one biovar, *R. etli* bv *phaseoli*, which nodulate and fix nitrogen on *P. vulgaris* exclusively, was distinguished. Later studies indicated that *R. etli* strains are not restricted to the Americas and that these strains can nodulate hosts other than common bean, including tree legumes (Amarger *et al.*, 1997; Dagutat, 1995; Diouf *et al.*, 2000). A second biovar, *R. etli* bv *mimosae*, was described for the isolates from *Mimosa affinis* which could form nitrogen-fixing nodules on *L. leucocephala* in addition to *P. vulgaris* (Wang *et al.*, 1999a).

In France two other species nodulating *P. vulgaris*, namely *R. gallicum* and *R. giardinii*, were also recognized (Amarger *et al.*, 1997). Both species were subdivided into two biovars based upon symbiotic characteristics. *R. gallicum* bv *gallicum* nodulates *Phaseolus* spp., *L. leucocephala*, *Macroptilium artropurpureum* and *Onobrychis viciifolia* and fixes nitrogen with *P. vulgaris*, whilst *R. gallicum* bv *phaseoli* nodulates *Phaseolus* spp. and *M. artropurpureum*. In the species *R. giardinii*, *R. giardinii* bv *giardinii* strains nodulate *Phaseolus* spp., *L. leucocephala*, and *M. artropurpureum* and are not able to fix nitrogen with *P. vulgaris*, whilst *R. giardinii* bv *phaseoli* strains nodulate *Phaseolus* spp. and *M. artropurpureum* and are weakly efficient in nitrogen fixation with *P. vulgaris*.

The rhizobia which form a very specific nitrogen-fixing symbiosis with either *Galega orientalis* or *G. officinalis* were classified as *R. galegae* (Lindström, 1989). Strains from one *Galega* sp. form only ineffective nodules on the other host species and no nodules on other legumes. The species *R. hainanense* includes fast-growing strains isolated from various tropical legumes in Hainan Province, China (Chen *et al.*, 1997). The type strain could only nodulate its original host, *Desmodium sinuatum*, and the universal host *Vigna unguiculata*.

Commonly, symbionts associated with *Medicago* belong to the genus *Sinorhizobium*. However, in 1998 Van Berkum *et al.* demonstrated that isolates of *M. ruthenica* belonged to the genus *Rhizobium* based upon phenotypic and genotypic data. These authors concluded that at least three genomic species of rhizobia, represented by *R. tropici*, a

single isolate USDA 1920 and isolate USDA 1844, form nitrogen-fixing symbioses with *M. ruthenica*. The name *R. mongolense* was proposed for the genomic species represented by USDA 1844.

The species *R. huautlense* includes symbionts of *Sesbania herbacea* which nodulate and form nitrogen-fixing symbioses with *S. herbacea* and *L. leucocephala* (Wang *et al.*, 1998). The fast-growing, acid-producing isolates from *Amphicarpaea trisperma*, *Coronilla varia* and *Gueldenstaedtia multiflora* were classified as *R. yanglingense* (Tan *et al.*, 2001).

2.1.2.6 The genus Sinorhizobium

As mentioned previously, it has become evident that there are three genetic groups within the genus *Rhizobium*. In 1988 Chen *et al.* proposed that soybeans are nodulated by a distinct group of fast-growing, acid-producing rhizobia which were classified as *R. fredii* (Scholla and Elkan, 1984), but which they renamed *Sinorhizobium fredii*. However, *Sinorhizobium* as defined by Chen *et al.* (1988) was never widely accepted.

In 1994 De Lajudie *et al.* demonstrated that the proposal by Chen *et al.* (1988) for the description of the new genus *Sinorhizobium* had merit. These authors proposed to emend the genus *Sinorhizobium* to include *R. meliloti* [consisting of, at that time, the genetically diverse symbionts of *Medicago* (perennial and annual medics), *Melilotus* (sweet clover) and *Trigonella* (fenugreek)] as *Sinorhizobium meliloti*, in addition to *S. fredii*. *S. fredii* can form effective nodules on *Glycine max* (soybean), *Vigna unguiculata* (cowpea) and *Cajanus cajan* (pigeon pea). The species *S. xinjiangense* (correction of epithet: Euzéby, 1998) as proposed by Chen *et al.* (1988) has not been commonly used.

The cluster S and T strains isolated from *Acacia* and *Sesbania* spp. from Senegal (West Africa) were also described as new species (De Lajudie *et al.*, 1994). The cluster S strains, isolated from *Sesbania* spp., were included in the species *S. saheli* and are able to nodulate *Sesbania* spp., *A. seyal*, *L. leucocephala* and *N. oleracea*. The species *S. terangae* (correction of epithet: Trüper and De' Clari, 1997) included the cluster T strains

isolated from *Sesbania* and *Acacia* spp.. These strains have a host range similar to that of *S. saheli* and were subdivided into two biovars, namely biovars *sesbaniae* and *acaciae*, on the basis of their different host ranges (Lortet *et al.*, 1996). Strains isolated from *A. senegal* and *Prosopis chilensis* were described as the species *S. arboris* and *S. kostiense* (Nick *et al.*, 1999b).

The annual *Medicago* species exhibited various degrees of specificity in their symbioses that suggested that there is a subspecific sinorhizobial structure. Several studies using different techniques indicated that two genetically distinct groups could be distinguished (Brunel *et al.*, 1996; Eardly *et al.*, 1990; Rome *et al.*, 1996a). The first group corresponded to the species *S. meliloti*, whereas the members of the second group belonged to a separate genomic species. This group was described as a new species, *S. medicae* (Rome *et al.*, 1996b). In addition, a third group of isolates represented by strain *Rhizobium* sp. Or 191 can symbiotically fix nitrogen with *M. sativa* (Eardly et al., 1992).

2.2 THE ASSOCIATION BETWEEN *ACACIA* SPECIES AND RHIZOBIA, WITH SPECIAL EMPHASIS ON THE AFRICAN AND AUSTRALIAN CONTEXTS

In general, leguminous trees are abundant in savannah and arid regions of Africa, Australia, Southeast Asia, South America and North America where they provide fodder, firewood and gum and prevent soil erosion. Most of the leguminous trees form effective nitrogen-fixing symbioses with root nodule bacteria. Traditionally it was thought that the leguminous trees were only infected by slow-growing rhizobia of the *Bradyrhizobium* cowpea-type. However, later studies concerning the rhizobial symbionts of leguminous trees in Mexico and South America (Martinez-Romero *et al.*, 1991), Brazil (De Lajudie *et al.*, 1998b; Moreira *et al.*, 1993), Kenya (Haukka *et al.*, 1996; Nick *et al.*, 1999a, 1999b; Odee *et al.*, 1997; Zhang *et al.*, 1991), Sudan (De Lajudie *et al.*, 1998b; Haukka *et al.*, 1996; Nick *et al.*, 1999a, 1999b; Zhang *et al.*, 1991), Senegal (De Lajudie *et al.*, 1994, 1998a, 1998b; Dreyfus and Dommergues, 1981; Dupuy *et al.*, 1994) and Australia (Barnet and Catt, 1991; Barnet *et al.*, 1985; Lafay and Burdon, 1998; Lafay and Burdon,

2001; Lawrie, 1983; Marsudi *et al.*, 1999) indicated that there is a great diversity among tree rhizobia and that these trees are infected as much by fast-growing rhizobia as by slow-growing rhizobia. This has lead to the description of several new rhizobial species (De Lajudie *et al.*, 1994, 1998a, 1998b; Martinez-Romero *et al.*, 1991; Nick *et al.*, 1999b). The tropical legumes are, however, predominantly nodulated by slow-growing strains (Moreira *et al.*, 1993). The tree-nodulating rhizobia are either host specific or have a very wide host range (Dreyfus and Dommergues, 1981; Zhang *et al.*, 1991).

Acacia species in Africa have been shown to be nodulated by either slow- or fast-growing strains or by both (Dreyfus and Dommergues, 1981). Therefore, it was proposed that these species could be classified in three groups: (i) Group 1 *Acacia* spp. which are nodulated by slow-growing strains only, for example *A. mearnsii* (Dreyfus and Dommergues, 1981), *A. albida* (Dreyfus and Dommergues, 1981; Dupuy and Dreyfus, 1992; Dupuy *et al.*, 1994) and *A. mangium* (Zhang *et al.*, 1991); (ii) Group 2 *Acacia* spp. which are nodulated by fast-growing strains only, for example *A. senegal* (De Lajudie *et al.*, 1994, 1998b; Dreyfus and Dommergues, 1981; Haukka *et al.*, 1996; Nick *et al.*, 1999b; Odee *et al.*, 1997; Zhang *et al.*, 1991), *A. nilotica* (Dreyfus and Dommergues, 1981; Odee *et al.*, 1997), *A. raddiana* (Dreyfus and Dommergues, 1981) and *A. farnesiana* (Dreyfus and Dommergues, 1981); and (iii) Group 3 *Acacia* spp. which are nodulated by both fast- and slow-growing strains, for example *A. seyal* (Dreyfus and Dommergues, 1981).

However, in a study by Odee *et al.* (1997) in which they used *Acacia* spp. as trap plants for indigenous rhizobia from soils collected from different sites in Kenya, they found contradictory results for *A. albida* and *A. seyal.* Although the majority of the isolates (71%) were trapped with the *Acacia* spp. and the trap hosts were also native to the sites from where the soils were collected, one does not know how reliable these results are, especially since they differ from results by previous workers. Odee *et al.* (1997) found that *A. albida* is also nodulated by fast-growing strains and that *A. seyal* is nodulated by fast-growing strains alone and not by both fast- and slow-growing strains (Dreyfus and Dommergues, 1981).

Members of the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* nodulate *Acacia* spp. in African countries such as Kenya (Haukka *et al*., 1996; Nick *et al*., 1999a, 1999b; Odee *et al*., 1997; Zhang *et al*., 1991), Sudan (De Lajudie *et al*., 1998b; Haukka *et al*., 1996; Nick *et al*., 1999a, 1999b; Zhang *et al*., 1991) and Senegal (De Lajudie *et al*., 1994, 1998b; Dreyfus and Dommergues, 1981; Dupuy *et al*., 1994). However, not only rhizobia have been isolated from root nodules of *Acacia* spp. in Africa, but also *Agrobacterium* biovar 1 strains (De Lajudie *et al*., 1999).

In South Africa the tree rhizobia also exhibited a great diversity, as well as a predominance of members of the genus *Bradyrhizobium* (Dagutat, 1995; Kruger, 1998). Isolates from *A. sieberana* var *woodii* and *A. caffra* showed a high degree of relatedness to the genus *Bradyrhizobium*, whilst isolates from *A. dealbata* (exotic to SA), *A. xanthoploea* and *A. robusta* belonged to the genera *Rhizobium* and *Sinorhizobium*. No isolations were made from *A. decurrens* in these studies and the number of isolates from *A. mearnsii* in the study by Dagutat (1995) was not sufficient to allow separate consideration.

In Australia the family *Mimosoideae* is overwhelmingly represented by the genus *Acacia* with about 850 species occurring naturally on the continent. The acacias are widespread and represent a dominant component of many ecosystems, including forests, arid zone woodlands and rain forests. Both slow-and fast-growing isolates were obtained from *A. longifolia* var *sophorae* (Barnet *et al*., 1985; Lawrie, 1983), *A. suavoelens* (Barnet *et al*., 1985), *A. saligna* (Barnet *et al*., 1985), *A. victoreae* (Barnet and Catt, 1991), *A. obliquinervia* (Lafay and Burdon, 1998), *A. dealbata* (Lafay and Burdon, 2001), *A. mearnsii* (Lafay and Burdon, 2001) and *A. melanoxylon* (Lafay and Burdon, 2001) from many environments in south-eastern Australia, as well as from *A. saligna* in south-western Australia (Marsudi *et al*., 1999). So far, only slow-growing isolates have been isolated from *Acacia* spp. in northern Australia (Bowen, 1956).

Although earlier studies (Barnet and Catt, 1991) suggested marked geographic localisation of the various rhizobial types according to their growth rate, later studies

could not find such a partitioning (Lafay and Burdon, 1998, 2001; Marsudi *et al.*, 1999). Rather, it was suggested that the soil type might strongly influence the type of rhizobia most common in the soil, with acid or near-neutral soil favouring *Bradyrhizobium* species. In South Africa no association could be found between the geographic origin of host legumes and the groupings of their rhizobial isolates (Kruger, 1998).

Rhizobium, *Mesorhizobium* and *Bradyrhizobium* appear to be the only three genera represented among Australian *Acacia* rhizobial isolates, with *Bradyrhizobium* dominant throughout the continent (Barnet and Catt, 1991; Barnet *et al.*, 1985; Bowen, 1956; Lafay and Burdon, 1998, 2001; Lawrie, 1983; Marsudi *et al.*, 1999). In south-western Australia the rhizobial strains isolated from *A. saligna* were related to *B. japonicum*, *Bradyrhizobium* spp. (*Lupinus*), *R. leguminosarum* bv *phaseoli* or *R. tropici* (Marsudi *et al.*, 1999). *Bradyrhizobium*, *R. leguminosarum* and *Mesorhizobium* genomospecies were isolated from *A. obliquinervia* (Lafay and Burdon, 1998) and *Bradyrhizobium* and *R. tropici* genomospecies from 13 other *Acacia* spp., including *A. dealbata* and *A. mearnsii* (Lafay and Burdon, 2001), in south-eastern Australia. The findings of Lafay and Burdon (2001) differ from those of previous workers with regard to African *A. mearnsii* and South African *A. dealbata*, respectively: according to Dreyfus and Dommergues (1981) *A. mearnsii* is nodulated by slow-growing strains only, whilst *A. dealbata* is nodulated by fast-growing strains only according to Dagutat (1995) and Kruger (1998). The number of isolates from *A. decurrens* in the study by Lafay and Burdon (2001) was not sufficient to allow separate consideration and was restricted to a single region in south-eastern Australia.

2.3 METHODS USED FOR THE CHARACTERIZATION AND TYPING OF BACTERIA AT THE SPECIES AND SUBSPECIES LEVELS

2.3.1 DNA fingerprinting techniques

Several DNA fingerprinting techniques, which involve the display of a set of DNA fragments from a specific DNA sample, are currently available. The choice of which fingerprinting technique to use, is dependent on the application and the organism under investigation. Ideally, a fingerprinting technique should require no prior knowledge of the nucleotide sequence or investments in terms of primer synthesis and probe characterization.

2.3.1.1 Restriction fragment length polymorphism (RFLP) analysis

If two DNA molecules are essentially the same, but nonetheless have small differences in their nucleotide sequence, then the fact that they are not absolutely identical may be determined by comparison of their restriction maps. Restriction fragment length polymorphism (RFLP) analysis basically involves such a comparison in which polymorphisms are generated by events such as point mutations or rearrangements in the genome which alter the distribution of specific endonuclease recognition sites within defined regions of the genome (Botstein *et al.*, 1980).

Classical RFLP involves the digestion of extracted genomic DNA with restriction enzymes and the separation of the resulting fragments by agarose gel electrophoresis (Botstein *et al.*, 1980; Vandamme *et al.*, 1996). The number and location of the restriction sites are unique for each genome and so the pattern of groups of fragments of different size is also unique for each. These fragment classes form the specific restriction pattern or fingerprint of an individual organism. Different endonucleases will yield different patterns whose taxonomic values will vary with the group concerned. Although the technique is simple, rapid and requires very little DNA, it often generates complex patterns of DNA fragments making differentiation between closely related strains

difficult. Also, if a plasmid-specific DNA probe is not used, it will be impossible to detect and characterize plasmid-linked sequences (Demezas *et al.*, 1991).

The number of bands for analysis could be reduced by identifying particular fragments using a probe in a hybridization analysis (Botstein *et al.*, 1980; Van Berkum and Eardly, 1998). If the probe consists of portions of either the 16S rRNA gene, 23S rRNA gene or both, with or without the spacer region or a conserved part of the rRNA gene, the technique is referred to as ribotyping (Vandamme *et al.*, 1996). However, the use of rDNA-based data for phylogenetic analyses is sometimes questionable.

The fingerprint patterns could also be simplified by using a variant of the classical RFLP technique that utilizes polymerase chain reaction (PCR) technology, namely PCR-RFLP. With PCR-RFLP specific regions of the genome are amplified and the amplification products digested with restriction enzymes to obtain fingerprint patterns (Van Berkum and Eardly, 1998). If the target DNA to be amplified by PCR-RFLP is rDNA (16S or 23S rDNA or parts of both genes with or without the spacer region), the technique is also referred to as amplified rDNA restriction analysis (ARDRA) (Vandamme *et al.*, 1996; Vaneechoutte *et al.*, 1993). The rDNA is amplified by using universal primers located in the conserved regions of the rRNA genes and is subsequently digested with a combination of restriction enzymes. ARDRA is a rapid, technically less demanding identification method than direct sequencing or hybridization with specific probes and is able to distinguish between closely related species (Vandamme *et al.*, 1996; Vaneechoutte *et al.*, 1993). As a result of the universality of the primers used, a range of bacteria can be included in a single analysis (Vaneechoutte *et al.*, 1993). However, as mentioned before, the use of rDNA-based data for phylogenetic analyses is sometimes questionable and is not generally sufficient to define a species when DNA-DNA hybridization or sequence data are not available to support the translation of ARDRA profiles into species designations. However, once species designations are established, it should be possible to rapidly and preliminary classify strains using ARDRA profiles without recourse to serology or DNA-DNA hybridization (Ralph *et al.*, 1993).

By identifying specific bands by hybridization with a probe or by amplifying specific regions of the genome by PCR, sequence divergence is estimated across very small regions of the genome which may not be representative of the sequence divergence across the entire genome (Van Berkum and Eardly, 1998). Therefore, classical RFLP should rather be used for the useful differentiation of closely related strains at and below the species level, as well as to assess genetic diversity among strains. Estimates of sequence divergence from RFLP fingerprints may, however, be influenced by the presence of polymorphic insertion sequences (Van Berkum and Eardly, 1998).

2.3.1.2 Low frequency restriction fragment analysis (LFRFA)

Low frequency restriction fragment analysis (LFRFA), also referred to as pulsed-field gel electrophoresis (PFGE), also involves the reduction of the number of DNA fragments generated by classical RFLP by selecting restriction enzymes that only rarely cut DNA. Endonucleases containing the tetranucleotide CTAG or the trinucleotides CCG or CGG in their recognition sites will rarely cut bacterial genomes with G+C contents above or less than 45%, respectively (McClelland *et al.*, 1987).

By carefully choosing the rare-cutting restriction enzymes, fragments are generated which are ideally greater than 100 kilobase pairs (kbp). These DNA fragments are too large to separate by conventional agarose gel electrophoresis and are therefore separated by PFGE. PFGE involves the separation of large DNA molecules by periodical changes in the direction of the current according to a predetermined pattern. The electrophoretic pattern yielded by this electrophoretic process reflects an array of conserved traits that fingerprints the organism at the species or strain level.

Although LFRFA is often considered to be the most discriminative DNA-based typing method (McClelland *et al.*, 1987; Vandamme *et al.*, 1996) and has been applied to taxonomic studies, it requires expensive restriction enzymes, cell preparations in agarose-embedded plugs and lengthy electrophoretic separations (Janssen *et al.*, 1996).

2.3.1.3 Randomly amplified DNA fingerprinting (AP-PCR, RAPD, DAF)

Arbitrarily primed (AP)-PCR fingerprinting, randomly amplified polymorphic DNA (RAPD) analysis and DNA amplification fingerprinting (DAF) generates genomic fingerprints of species of which little is known about the target sequence to be amplified by employing short arbitrary sequences as primers in PCR assays. The length of the primers used varies with each of the techniques: (i) Primers of 20 bases are used in AP-PCR fingerprinting (Welsh and McClelland, 1990); (ii) Primers of 9-10 bases in RAPD analysis (Williams *et al.*, 1990); and (iii) Primers of as short as 5 bases, but typically 8-10 bases in DAF (Caetano-Anollés *et al.*, 1991).

Both AP-PCR fingerprinting and RAPD involve the amplification of random DNA segments by PCR using single primers of arbitrary nucleotide sequence in order to reveal differences as DNA polymorphisms or fingerprints (Welsh and McClelland, 1990; Williams *et al.*, 1990). The amplification step is characterised by low stringency and the resulting fingerprint will be determined by the nucleotide sequence of the primer and the nature of the template DNA. Both techniques are generally applicable to a wide variety of organisms as a result of the universality of the primers and provide fingerprints that are species or subspecies specific.

DAF differs from RAPD in terms of its reaction conditions and its separation and detection systems. DAF is able to detect a large number of amplification products and profiles can be tailored to vary in complexity (Caetano-Anollés *et al.*, 1991; Prabhu *et al.*, 1997). An additional advantage of DAF compared to RAPD is that the silver stained DAF gels are archivable (Prabhu *et al.*, 1997).

These techniques should, however, be optimised and used with care and consistency to ensure reproducibility within laboratories. Differences in equipment, enzyme and PCR conditions may lead to artifactual variation that can interfere with the amplification fragments generated (Jutras *et al.*, 1995; Selenska-Pobell *et al.*, 1995). Therefore, AP-PCR fingerprinting data alone is generally insufficient to define a species unless it is supported by DNA-DNA hybridization or sequence data (Ralph *et al.*, 1993). RAPD

i 16050095
b 1542585X

analysis and DAF also suffer from a lack of portability between laboratories (Gillings and Holley, 1997b; Power, 1996). The reproducibility of RAPD has been improved by the development of long primer (LP)-RAPD that involves the use of longer primers (18 to 24 bases). LP-RAPD is sensitive to intraspecific and interspecific genetic variation (Gillings and Holley, 1997b).

2.3.1.4 Repetitive-sequence-based PCR (rep-PCR)

Enterobacteria contain families of short interspersed repetitive elements, these being the repetitive extragenic palindromic (REP) element, the enterobacterial repetitive intergenic consensus (ERIC) sequence and the BOX element. The REP and ERIC elements contain highly conserved central inverted repeats, do not show significant homology to each other, and are normally found in intergenic transcribed, but not translated, regions. REP- and ERIC-like sequences, with a significant degree of homology to those found in enterobacteria, were also detected in a large variety of eubacterial species (De Bruijn, 1992; Versalovic *et al.*, 1991). Their relative position in the genome of a particular bacterial isolate appear to be conserved in closely related strains and are distinct in diverse species (genera). Thus, by using consensus primers to each of these elements in PCR assays, the regions between neighbouring repetitive elements which are within ~ 5 kb from each other are amplified to generate DNA fingerprints that are species and strain specific (Versalovic *et al.*, 1991). Repetitive-sequence-based (rep)-PCRs performed on non-enterobacterial targets are not necessarily directed at genuine repetitive elements, but rather represents a highly reproducible variant of the RAPD technique (Gillings and Holley, 1997a).

REP-PCR fingerprints allow the recognition of strains between laboratories (Judd *et al.*, 1993; Laguerre *et al.*, 1997) and REP-PCR and ERIC-PCR analyses yield similar levels of discrimination (Laguerre *et al.*, 1997). The REP- and ERIC-PCR procedures are extremely rapid, do not require the generation of bacterial cultures or the extraction of genomic DNA and have the advantage that a single set of primers could be used for the analysis of both closely related and widely divergent strains (De Bruijn, 1992). The complexity of the REP- and ERIC-PCR patterns can also be manipulated to enable the

differentiation of closely related strains of the same species, distinct species or different genera (De Bruijn, 1992). There is a fairly good correlation between data derived from REP-and ERIC-PCR analyses and phylogenetic data generated by multilocus enzyme electrophoresis (MLEE) (De Bruijn, 1992), as well as between data derived from BOX-, ERIC- and REP-PCR analyses and DNA-DNA hybridization data (Nick *et al.*, 1999a; Rademaker *et al.*, 2000) for a given bacterial species. Combined BOX-, ERIC- and REP-PCR fingerprinting data provide the most significant and consistent results as the genome is more extensively covered, than any of these fingerprinting techniques alone (Rademaker *et al.*, 2000). While none of these techniques alone can provide unambiguous classification of bacterial strains, the combination of genomic and phenotypic data may determine phylogenetic relationships precisely. However, rep-PCR could be used as a rapid screening technique to determine taxonomic diversity and phylogenetic structure, especially of large collections of bacterial isolates (Rademaker *et al.*, 2000).

2.3.1.5 Amplified fragment length polymorphism (AFLP) analysis

Amplified fragment length polymorphism (AFLP) analysis is basically a RFLP analysis followed by a PCR amplification step to selectively amplify particular restriction fragments from the total digest of genomic DNA (Vandamme *et al.*, 1996; Vos *et al.*, 1995). The restriction fragments are selected by performing the restriction using two restriction enzymes (ideally a rare cutter and a frequent cutter) which would yield DNA fragments with two different types of sticky ends to which adapters are ligated to provide templates for the amplification step. The selective amplification is achieved by using two different primers consisting of the same sequence as the adapters as well as one or more selective nucleotides next to the restriction site of the enzyme. Under the stringent primer annealing conditions used, only the fragments in which the selective nucleotides completely match the primer extensions, are amplified. The amplification process results in an array of fragments, of which some are group-specific and others are strain-specific, which could be detected by denaturing polyacrylamide gel electrophoresis. The number of resulting amplified DNA fragments is determined by the choice of restriction enzymes, the number and base composition of the selective nucleotides in the primers, as well as

the complexity of the genomic DNA (Vandamme *et al.*, 1996; Vos *et al.*, 1995). For small bacterial and fungal genomes a single PCR amplification with one or two selective nucleotides, respectively, on both primers is sufficient (Janssen *et al.*, 1996; Rademaker *et al.*, 2000; Savelkoul *et al.*, 1999).

The AFLP technique is a robust, reliable and very powerful high-resolution DNA fingerprinting technique for DNA of any origin or complexity and could be used simultaneously for identification and typing purposes of highly related bacterial strains without prior knowledge of the nucleotide sequence (Savelkoul *et al.*, 1999; Vandamme *et al.*, 1996; Vos *et al.*, 1995). It is especially useful as a rapid screening technique for large collections of bacterial isolates as it yields results which are comparable to DNA-DNA hybridization (Janssen *et al.*, 1996; Rademaker *et al.*, 2000). Although AFLP fingerprinting has the same taxonomic range for bacteria as other genomic fingerprinting techniques, it has several advantages compared to ribotyping, ARDRA analysis, LFRFA, randomly amplified PCR fingerprinting, rep-PCR fingerprinting, classical RFLP and RFLP-based techniques (Janssen *et al.*, 1996; Savelkoul *et al.*, 1999).

2.3.2 Protein electrophoresis

Apart from the complete DNA base sequence, which is considered an absolute reference standard for the estimation of relationships between bacteria, cellular proteins also form an information source for the identification and differentiation of bacteria. The amino acid sequence of a protein is an indirect copy of a part of the bacterial genome. The primary structure of the proteins is reflected by parameters such as molecular weight, net electrical charge and spatial conformation. One or a combination of these parameters is used to separate proteins by gel electrophoresis. When the cellular proteins of a bacterial strain are electrophoresed, it produces a complex banding pattern called a protein electrophoregram. Each bacterial strain, when grown under identical conditions, produces a constant electrophoregram which is reproducible and represents a fingerprint of that specific strain which could be used for comparative purposes. (Kersters, 1985; Priest and Austin, 1993; Vauterin *et al.*, 1993)

The taxonomic information yielded by different electrophoretic techniques will depend on the principles underlying the protein sample preparation and electrophoretic separation. When the sample is prepared under non-denaturing conditions, ribosomal, membrane and nucleic acid-bound proteins are usually not included in the electrophoretic analysis. Protein patterns of such extracts reflect size (molecular weight), as well as charge and primary conformation differences of the proteins. Under these conditions the proteins retain their enzymatic activity and can be detected on the basis of this activity. Patterns obtained from samples prepared under denaturing conditions, most commonly by treatment of cells with sodium dodecyl sulphate (SDS), include the majority of the cellular proteins (including ribosome- and membrane-bound proteins) and reflect size differences only. As the molecular weight of homologous proteins are more conserved than their net charge or isoelectric point, SDS electrophoretic patterns should theoretically detect broader taxonomic relationships. (Kersters, 1985; Vauterin *et al.*, 1993)

However, the discriminatory level of protein electrophoresis from a taxonomic viewpoint does not only depend on the type of proteins extracted or the electrophoretic system used, but also on the organisms under study (Kersters, 1985; Vauterin *et al.*, 1993). The level of discrimination generally ranges from the single mutation level (two-dimensional PAGE and isoenzyme electrophoresis) to the species level (whole cell protein patterns).

The different types of electrophoresis that are available to explore the relationships between bacterial strains at the protein level, are the following: (i) Electrophoresis of cellular proteins; (ii) Electrophoresis of ribosomal proteins; (iii) Electrophoresis of cell envelope proteins; and (iv) Enzyme electrophoresis.

2.3.2.1 Electrophoresis of cellular proteins

Several types of polyacrylamide gel electrophoretic separation systems have found application in bacterial systematics, i.e. discontinuous PAGE, isoelectric focusing and two-dimensional PAGE.

The most widely used discontinuous PAGE system in bacterial systematics is the SDS-PAGE system. Although discontinuous PAGE of non-denatured proteins leads to simpler and easier interpretable protein patterns which have the same discriminatory value than the SDS method to differentiate and characterize closely related bacteria, it is limited with regard to its reproducibility. When applying one-dimensional gel electrophoresis, it is important to realize that each protein band would usually consist of a number of structurally different proteins with identical electrophoretic mobility and that only a fraction of the proteins are detected. (Vauterin *et al.*, 1993)

Isoelectric focusing separates proteins on the basis of their isoelectric points, i.e. the pH at which the net charge of a protein is zero. This method has a very high resolving capacity and is very useful for the characterization of bacteria. It is mainly used for enzyme studies, as it allows the separation of isoenzymes differing in only a single charge unit. However, its application in bacterial systematics is limited, probably due to difficulties in standardizing pH gradients. (Vauterin *et al.*, 1993)

Two-dimensional PAGE involves the separation of proteins in two successive dimensions: In the first dimension the proteins are separated according to their isoelectric points and in the second dimension according to molecular weight. This system has a high resolving power and has been used to characterize several bacterial strains. However, the preparation of reproducible two-dimensional gels is difficult and the interpretation and comparison of the protein profiles are complicated. (Vauterin *et al.*, 1993)

2.3.2.2 Electrophoresis of ribosomal proteins

The highly conserved nature of ribosomes is reflected in both the sequence of the rRNAs and the primary structure of the ribosomal proteins, making ribosomes an excellent tool to study phylogeny. Therefore, the electrophoretic patterns of ribosomal proteins could be applied in phylogenetic studies and has the advantage over soluble cytoplasmic proteins that they do not vary with environmental conditions. However, this method is not really suitable for routine diagnosis. (Vauterin *et al.*, 1993)

2.3.2.3 Electrophoresis of cell envelope proteins

The cell envelope of bacteria consists of a cell wall and a cell membrane ("inner membrane") for which the protein composition is well described for several Gram-negative bacteria. Electrophoresis of cell envelope proteins is especially useful for the characterization and identification of medically important bacteria. Total cell envelope proteins could be extracted by subjecting disrupted washed cells to ultracentrifugation and treatment with SDS or another detergent-containing buffer. Characterization of the bacteria is carried out by either one-dimensional electrophoresis or by high-resolution two-dimensional electrophoresis. (Vauterin *et al.*, 1993)

Ethylenediaminetetraacetate (EDTA) is used to release non-covalently bound cell surface compounds, including proteins, of Gram-positive bacteria. Also, comparative studies of outer membrane protein patterns have proven to be useful in epidemiological studies of pathogenic and clinically important Gram-negative bacteria and provide an alternative to serotyping techniques. Proteins can be selectively extracted from the outer membrane by the EDTA-lysozyme method, by ultracentrifugation of cell envelopes in a discontinuous sucrose gradient, by lithium chloride extraction or by the treatment of cell envelopes with sarkosyl. (Vauterin *et al.*, 1993)

2.3.2.4 Multilocus enzyme electrophoresis (MLEE)

Rather than analysing the total proteins in a cell, the electrophoretic properties of several enzymes can be compared. Such studies focus on enzymes that are common to a group and involve the electrophoretic separation of native (non-denatured) proteins, followed by specific staining that results in a colour reaction that reveals the enzymatic activity. Thus, strains can be classified into groups on the basis of the presence or absence of particular enzymes, as well as by comparing their electrophoretic mobilities. (Priest and Austin, 1993; Vandamme *et al.*, 1996; Vauterin *et al.*, 1993)

Isoenzymes/isozymes (all distinguishable forms of an enzyme) which could be detected by differences in their electrophoretic mobility are called "allozymes" or "electromorphs". Thus, the underlying rationale for enzyme electrophoresis is that a

given enzyme species showing different electrophoretic mobilities is a parameter for genetic diversity: minor changes in gene structure result in variation in enzyme structure. Subsequently, electromorphs or allozymes of an enzyme can be directly equated with alleles at the corresponding structural gene locus. Thus, in effect, gene sequences are compared at the level of expression, revealing even smaller genetic divergences between strains than DNA-DNA hybridization. Enzyme electrophoresis is, therefore, applied to taxonomy at the intraspecific level and is an excellent tool to study population genetics. (Priest and Austin, 1993; Selander *et al.*, 1986; Vandamme *et al.*, 1996; Vauterin *et al.*, 1993). However, it is important to keep in mind that the number of electrophoretic types recognized in a sample of isolates obviously would depend on the number of loci analyzed. If only a few loci are analyzed, it may seriously underestimate the full extent of genotypic variation in a population (Pinero *et al.*, 1988).

The method has the following limitations: (i) Enzyme electrophoresis is an indirect measure for genomic diversity as enzyme variants resulting from post-translational modifications are given an equal value for genetic divergence as isoenzymes generated by different alleles; (ii) Only a limited number of representative enzymes can be assessed (Selander *et al.*, 1986; Vauterin *et al.*, 1993).

2.3.3 Phage typing

Phage typing is useful for the identification and differentiation of bacterial strains and species and has found wide application in epidemiological studies to identify bacterial species, differentiate between pathogenic and saprophytic strains, to determine specific subgroups or types and to determine the virulence of bacteria.

Phage typing is based on the susceptibility of a certain bacterial strain to a particular bacteriophage, which is a specific interaction. Thus, in order to type a bacterial strain, a group of phages with different host specificities is used. The bacteria can then be placed into groups based on their different patterns of susceptibility to the group of phages used (Somasegaran and Hoben, 1994b). Bacteriophages usually lyse strains from the same

species, occasionally from other species from within the same genus and rarely from strains of different genera.

However, discrimination of isolates using phage typing is limited by the number of phage types used. Discrimination also depends on the presence or absence of susceptibility to a particular phage and can only be usefully applied to the typing of strains within a species. Therefore this technique could not be used to group different species. Phage typing, as well as serological typing, is also vulnerable to single mutational events that alter the cell surface. The selection for these alterations in cell surface properties may occur inadvertently in nature, for example phage resistance (Roberts *et al.*, 1980).

2.3.4 Intrinsic antibiotic resistance (IAR) typing

Intrinsic antibiotic resistance typing is useful for the identification and characterization of bacterial strains and basically involves the same principles as phage typing. Therefore, IAR typing suffers from the same limitations as phage typing and can only be usefully applied to strains within the same species. The technique also suffers from a few other drawbacks: (i) The resistance characteristic itself may perturb the test to be performed by, for example, altering the growth rate or cell permeability; (ii) Many bacteria already have a high incidence of resistance; (iii) Antibiotic resistance, when plasmid borne, can be transferred between strains (Demezas *et al.*, 1991; Roberts *et al.*, 1980).

2.3.5 Serology

Comparative serological studies are based on the presence of variability in the antigenic constituents of cells and the fact that specific antigenic components of one organism react more strongly with an antiserum raised against the antigens of a similar organism than with one raised against the antigens of a dissimilar organism. Therefore serology can be used for strain identification. Antigens can either be proteins or carbohydrates, thermostable or thermolabile and may include structural components such as capsules,

cell envelopes, flagella or fimbriae and intracellular molecules or secretion products such as enzymes and toxins (Somasegaran and Hoben, 1994b; Vandamme *et al.*, 1996).

Since there are many intrinsic and extrinsic sources of error in serological tests, strict standardization and quantification are essential. A wide range of techniques based upon agglutination, precipitation, neutralization, immunofluorescence and radio- and enzyme-immunoassay may be used.

Somatic or O antigens are heat stable and the most specific group of antigens. Some somatic antigens are tightly bound to the cell wall and are detected when whole bacterial cells react with the antibody, as in agglutination or immunofluorescence. The soluble somatic antigens are detected by precipitation in gels. The "internal antigens", which are widely cross-reactive within and between species, are taxonomically significant and are detected by gel immunodiffusion. Agglutination or immunofluorescence is used to detect the heat labile flagellar or H antigens. The capsular or K antigens are extracellular surface antigens. (Somasegaran and Hoben, 1994b).

The main advantage of serological tests is speed. With certain methods, e.g. ELISA, there is also the potential for the development of kits, which could be used for identification in the field.

With respect to taxonomic studies, antigenic analysis depends to a great extent on the resolving power of the method and the nature of the antiserum. Bacteria may have several antigens in common, but are distinct species on the basis of DNA similarity and consequently it may be necessary to distinguish antigenic similarity from measures based on other criteria. It seems likely that serological analyses will have even less appeal in the future given the ease of carrying out taxonomic comparisons based on analysis of DNA and RNA. Therefore, serological data may be useful to confirm classifications based upon other characters in groups with less complex antigenic complements, provided that a reference antiserum is available for each main taxon. However, some bacterial groups has antigenically complex cell surfaces which permit the recognition of so many serovars

that objective analysis is practically impossible and make serological analysis of these groups of limited use in classification.

2.3.6 Application of these techniques to rhizobial identification

Although rRNA sequencing is an excellent tool to determine the phylogenetic relationships of bacteria at the specific level and higher (Vandamme *et al.*, 1996), the conservative nature of the 16S rRNA gene limits the discriminatory power of 16S rRNA sequencing for distinguishing closely related strains. The discrimination of closely related strains by techniques such as classical RFLP (Demezas *et al.*, 1991) and 16S ARDRA (Amarger *et al.*, 1994; Laguerre *et al.*, 1994) require several probes and/or restriction endonucleases and even then discrimination is sometimes not achieved as a result of the conserved nature of the 16S rDNA sequences (Laguerre *et al.*, 1994). In addition, estimates of sequence divergence from RFLP fingerprints may be influenced by the presence of polymorphic insertion sequences (Van Berkum and Eardly, 1998). In the rhizobial group estimates of genetic divergence between strains on the basis of ARDRA analysis of 16S rDNA are limited as a result of length variation between the 16S rRNA genes: Some *R. tropici* strains possess an insertion in these genes (Willems and Collins, 1993; Laguerre *et al.*, 1994).

In order to increase the discriminative power at the intraspecies level, less highly conserved sequences should be analyzed along with 16S rRNA genes. This can be achieved by, among other, ARDRA analysis of the 16S rRNA genes together with the intergenic spacer region (IGS) or parts thereof. 16S-IGS ARDRA analysis is sufficiently variable to differentiate between and fingerprint closely related *Rhizobium* strains and can be used to screen large numbers of strains and classify them into probable genomic species (Nour *et al.*, 1994, 1995).

Greater variability can also be obtained by ARDRA analysis of the highly variable 5'-half of 23S rDNA and especially of the IGS between the 16S rRNA and 23S rRNA genes in order to examine chromosomally encoded genetic variations at the intraspecies level

(Laguerre *et al.*, 1996; Selenska-Pobell *et al.*, 1996). However, as a result of length variability of the IGS rDNA between and within species and the fact that three rRNA operon copies occur in *Rhizobium* species, length polymorphisms of restriction fragments between IGS types do not necessarily correspond to differences in restriction sites and distances between genotypes could be overestimated (Laguerre *et al.*, 1996). Therefore, AP- and REP-PCR which are more informative and discriminative than ARDRA analysis of the IGS and 23S rDNA, could be used (Laguerre *et al.*, 1996; Selenska-Pobell *et al.*, 1996).

Both AFLP fingerprinting and IGS ARDRA are recommended as initial screening techniques for bradyrhizobial strains, of which IGS ARDRA is simpler and less laborious (Doignon-Bourcier *et al.*, 2000; Willems *et al.*, 2000, 2001b). Both techniques almost differentiate at the strain level and are more sensitive than 16S ARDRA, which is limited in its discriminative powers because of the highly conserved nature of the 16S rDNA of bradyrhizobia (Doignon-Bourcier *et al.*, 2000; Janssen *et al.*, 1996; Willems *et al.*, 2000). AFLP fingerprinting is also more discriminative than SDS-PAGE analysis of whole cell proteins, which corresponds poorly with genotypic data (Willems *et al.*, 2000). However, DNA-DNA hybridization remains essential to fully evaluate the discriminative power of these techniques in the case of *Bradyrhizobium*. Direct sequencing of the IGS region could also be used for the initial grouping of the highly related bradyrhizobial strains within a particular DNA homology group or at least limit the number of DNA-DNA hybridizations that will be needed to identify a strain (Willems *et al.*, 2001b, 2001c).

Phage typing has been used for the identification of strains isolated from plants (Bromfield *et al.*, 1986; Lesley, 1982; Novikova *et al.*, 1993) and is able to distinguish between strains of the same serogroup (Kowalski *et al.*, 1974; Somasegaran and Hoben, 1994b). In some instances typing systems were developed to identify a specific species, for example the phage typing system for *R. meliloti* (Lesley, 1982).

Strain-specific antisera have been used extensively in field trials for strain identification, especially in precipitation reactions of somatic antigens and ELISA. However, the

production of strain-specific antibodies is a laborious process, particularly if many strains are being used. Within the rhizobial group, the surface somatic antigens are more strain-specific than the flagellar or internal antigens (Graham, 1963, 1969; Pankhurst, 1979; Vincent, 1982). Although agglutination reactions were originally used extensively for strain identification, positive identification was made difficult by the frequency with which cross-reactions occurred rendering the technique unable to distinguish between antigenically identical and closely related nonidentical strains (Dudman, 1964, 1971; Vincent, 1982). Gel immune-diffusion techniques are capable of giving more detailed information of antigenic structure and have assisted in more direct strain recognition, demonstrating antigens not detectable by agglutination (Dudman, 1964; Vincent, 1982). However, agglutination remained a basic technique to detect surface-located antigens (Vincent, 1982). Immune-diffusion methods can be applied directly to identify strains in larger nodules (Dudman, 1971) and a modified agglutination method to small nodules (Parker and Grove, 1970). The use of fluorescently labelled antibody has been applied to the direct detection of strains occupying a nodule (Schmidt *et al.*, 1968; Trinick, 1969) or present in soil (Schmidt *et al.*, 1968). Immunoblot assays and ELISA can be used to analyze a large number of samples simultaneously, rendering these techniques especially useful to establish strain occupancy in root nodules (Somasegaran and Hoben, 1994b; Vincent, 1982).

Methods that derive information from the whole genome are preferable for identification purposes. De Bruijn (1992) indicated that REP- and ERIC-like sequences are highly conserved in rhizobia and agrobacteria and could be used to identify and classify even closely related rhizobial strains. There is a close agreement between data derived from rep-PCR and DNA-DNA hybridization (Nick *et al.*, 1999a), as well as between data derived from REP- and ERIC-PCR and MLEE (De Bruijn, 1992). Several studies established REP-PCR fingerprinting is an excellent tool to determine genomic relatedness and to identify strains within rhizobia (De Lajudie *et al.*, 1998b; Judd *et al.*, 1993; Laguerre *et al.*, 1996, 1997; Nick *et al.*, 1999a; Selenska-Pobell *et al.*, 1995, 1996). REP- and ERIC-PCR are, however, not widely applicable to all rhizobia. The relatively low number of REP and ERIC consensus sequences found in some of the bradyrhizobia limits

the effective use of these techniques in the classification of these strains. However, combined REP-plus-ERIC-PCR fingerprinting could be used to differentiate between highly diverse strains of *Bradyrhizobium* spp. (Judd *et al.*, 1993). The species *S. meliloti* does not have ERIC or ERIC-like sequences as an integral part of its genome (Niemann *et al.*, 1999). AP-PCR fingerprinting has also been applied to the taxonomical identification and discrimination of rhizobial strains (Selenska-Pobell *et al.*, 1995, 1996).

The use of LFRFA has proven to be a powerful technique to map bacterial genomes (McClelland *et al.*, 1987). However, for rhizobia it has also found application as identification methods that could be used to identify rhizobia in both nodules and inoculants. A LFRFA identification method developed for different fast-growing rhizobia, especially *R. leguminosarum* bv *viciae*, can differentiate field isolates at the species, biovar and strain levels (Corich *et al.*, 1991), while another method distinguishes between *R. galegae* strains (Huber and Selenska-Pobell, 1994).

2.4 THE APPLICATION OF SODIUM DODECYL-SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) TO BACTERIAL SYSTEMATICS

The utility of protein gel electrophoresis in bacterial systematics is well established (Kersters, 1985; Vauterin *et al.*, 1993). SDS-PAGE of whole cell proteins gives highly discriminatory results, especially at the intrasubspecific level. The objective comparison of the whole cell electrophoretic protein patterns allows the same level of discrimination of relatedness between strains than DNA-DNA hybridization, since bacteria are being compared on the translational products of most of the cell's chromosome (Kersters, 1985; Kersters and de Ley, 1975; Priest and Austin, 1993; Vauterin *et al.*, 1993).

Generally, bacterial strains with 90 to 100% DNA sequence homology usually produce almost identical protein patterns, whilst strains with at least 70% of DNA sequence homology tend to have protein patterns with minor heterogeneity but an overall

similarity. Therefore, comparative protein electrophoresis is of little value when comparing distantly related bacteria (Kersters, 1985). Protein electrophoresis is, however, more rapid than DNA reassociation and has the advantage that clusters are formed from complete similarity matrices.

All culture conditions should be standardised when cultivating bacteria for protein extraction as these conditions may influence the protein composition of the bacterial cell and thus ultimately influence the fingerprint produced. Therefore, the bacteria should be cultured on a medium that supports optimum growth of all or most strains and medium composition, incubation temperature and growth time should be kept as constant as possible (Jackman, 1985; Kersters and de Ley, 1975; Vauterin *et al.*, 1993).

Numerical techniques are best to analyse protein electrophoretic patterns, as visual comparison is only feasible when a few strains are to be compared and it cannot estimate quantitative resemblance. The first step in the numerical analysis of the gels involves the densitometric measurement of the photometric absorbency of the stained gels, after which normalization takes place. Normalization involves the standardization of the gel lengths and compensation for discrepancies within and between gels. These are inherent to the system and could be caused by factors such as variations in acrylamide and buffer composition, polymerization and running conditions. Interpolation of the traces is carried out by alignment of patterns of a reference sample (consisting of a reference bacterial extract or a mix of molecular weight markers that produce well-delineated and easily recognizable peaks) run on each gel. The similarity between protein electrophoregrams is calculated best using the Pearson product-moment correlation coefficient (*r*). Alternatively, selected peaks can be searched for and recorded and similarity calculated on these selected peaks using the Dice coefficient, the correlation coefficient or a simple matching coefficient. The resulting similarity matrix is usually clustered using the unweighted pair group method using arithmetic averages (UPGMA)-algorithm and the groupings presented as a dendrogram (Kersters and de Ley, 1975; Vauterin *et al.*, 1993).

By far the most critical and limiting factor of the effective utilization of protein electrophoresis in bacterial classification and identification is reproducibility as it depends not only on the experimental conditions, but also on factors such as the purity and source of the chemicals used. Therefore it is important to rigorously control not only the culture conditions, but all experimental conditions to attain reproducible results, especially when automated numerical analysis is involved (Jackman, 1985; Kersters and de Ley, 1975; Vauterin *et al.*, 1993). Factors affecting reproducibility also depend on the resolution of the electrophoretic system. In general, fast-run gels yield sharper patterns, and hence provide finer discrimination than slow-run gels, but are more difficult to standardize. For minor variation in sample concentration, the concentration does not affect the similarity of the protein patterns. The reproducibility of the electrophoresis system could be controlled by comparing protein electrophoretic patterns of independently grown cultures of the same strain and reference strains in each gel or by comparing replicates of a single sample in independently run gels (Jackman, 1985; Vauterin *et al.*, 1993). Generally, correlations above $r = 0.90$ between repeated control samples are accepted as reproducible (Vauterin *et al.*, 1993). However, experience has shown that inter-laboratory comparisons could be quite difficult and are not always possible.

One of the advantages of total cellular protein electrophoresis is that identification of strains is based on the overall genetic background of the cell rather than a few selected properties. Thus, the relatedness between very similar and different strains could be assessed and strains that are identical by traditional methods could be differentiated (Kishore *et al.*, 1996).

The computer-aided numerical analysis of protein patterns also provides another advantage in that large numbers of bacterial strains could be compared by a relatively rapid, inexpensive but reliable high-resolution method. Thus, once the electrophoretic groups have been established, the number of strains that have to be investigated by genomic methods can be substantially reduced to representatives of each group. The digitally processed electrophoretic patterns of representative strains can be stored in

databanks and used as references to allocate an unknown microorganism to a group. Other advantages of the electrophoretic technique include its application to a wide variety of bacteria from different ecological niches, as well as its use to quickly determine whether two colony types in a culture are due to variation or contamination and the provision of information on the epidemiological spreading of animal and plant pathogens (Jackman, 1985; Kersters, 1985; Kersters and de Ley, 1975; Priest and Austin, 1993; Vauterin *et al.*, 1993).

The electrophoretic technique is, however, more time-consuming than other fingerprinting techniques and cannot be used to establish the identity of a single colony within a few hours after isolation. The technique also requires a large amount of cells, as well as a large number of experimental steps. Each of the experimental steps introduce some experimental error; therefore, standardization is absolutely necessary (Kersters, 1985; Vauterin *et al.*, 1993).

2.5 THE PLACE OF 16S rRNA GENE SEQUENCE ANALYSIS IN BACTERIAL SYSTEMATICS

rRNA molecules show a high degree of functional consistency, occur in all organisms, are large and consist of variable and more conserved regions, making them excellent molecular tools to infer phylogenetic lines of descent. The random changes that occur in the nucleic acid sequence of these molecules, become fixed in time and act as an evolutionary clock (Woese, 1987).

The primary structure of the 16S rRNA molecule is highly conserved and the variability in the sequence is concentrated in certain hypervariable regions which are taxon specific (Stackebrandt and Goebel, 1994). Universal primers, which bind to conserved regions, are used to sequence the molecule (Lane *et al.*, 1985; Weisburg *et al.*, 1991). Comparison of the sequences of the variable regions could be used to determine the relationships between more closely related organisms, whereas those of the more conserved regions

could be used to determine the relationships between distantly related organisms. However, when comparing highly related organisms (97% or more sequence similarity), the resolution power of 16S rRNA sequences is limited (Fox *et al.*, 1992; Stackebrandt and Goebel, 1994). At this level DNA hybridization remains the optimal method to determine the degree of relatedness between the organisms. Sequence analysis of 16S rRNA genes is only superior to DNA hybridization from the level of domains to moderately related species (Stackebrandt and Goebel, 1994).

Phylogeny should be based on the comparison of complete rather than short, incomplete sequences. Information will be lost when comparing only the hypervariable regions, since a substantial amount of difference is not concentrated in these regions. Multiple changes, which are highly likely to accumulate in these regions, may result in "false" identities that will obscure the genealogy of the molecule. In addition, the statistical sampling error is greater for smaller sequences; thus, the stability of phylogenetic trees is negatively influenced by very short sequences. If analyses have to be restricted to only a few hundred nucleotides, the regions need to be selected with care in order to obtain the same degree of similarity as that obtained from analyses of full sequences (Sneath, 1989; Stackebrandt and Goebel, 1994).

Although rDNA is ubiquitous, phylogenies based on sequence analyses of these molecules are not necessarily accurate, particularly among closely related taxa at the family level and below (Sneath, 1989). Analyses give differing results depending on the algorithm chosen and particularly on the sequences included (Young, 2001; Young *et al.*, 2001). In addition, phylogenies based on 16S rDNA are supported by only a small number of other gene sequences (Young, 2001). It is, therefore, increasingly recognized that analyses based on this single sequence should be supported by other sequence data.

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CHAPTER 3

CHAPTER 3

CHARACTERIZATION OF PUTATIVE RHIZOBIAL ISOLATES FROM EXOTIC AUSTRALIAN *ACACIA* SPECIES WITH SODIUM DODECYL-SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

3.1 INTRODUCTION

Locally existing biological nitrogen-fixing systems may be improved by utilizing biodiversity in strains of indigenous rhizobia which might provide better matching and tolerance to stress conditions. Insufficient information regarding the rhizobia indigenous to South Africa lead to the establishment of a long-term project aimed at determining the taxonomic status of the indigenous rhizobia and providing a culture collection of potential inoculant strains (Dagutat, 1995).

The culture collection of potential inoculant strains could be expanded by using trap plants, i.e. legume hosts used to bait indigenous rhizobia. In order to be applied effectively to provide rhizobia for the inoculant industry in South African, these trap plants should be promiscuous, nodulated by a wide range of rhizobia, and geographically widespread. The exotic Australian *Acacia* species, *A. mearnsii*, *A. dealbata* and *A. decurrens*, may satisfy these requirements: they are widespread throughout South Africa and are presumed to be promiscuous. This study was, therefore, undertaken to determine if these exotic *Acacia* spp. are indeed promiscuous or if they would predominantly nodulate with indigenous *Bradyrhizobium* spp. as was indicated for tree legumes in South Africa (Dagutat, 1995; Kruger, 1998).

The aim of this study was to investigate the diversity of the rhizobia associated with the exotic Australian *Acacia* species (*A. mearnsii*, *A. dealbata* and *A. decurrens*) in South Africa by characterizing putative rhizobial isolates from these species with SDS-PAGE

analysis of their whole cell proteins. Reference strains of the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* were included in the SDS-PAGE analysis. The SDS-PAGE data is supplemented with genotypic data (16S rDNA sequencing) of selected isolates.

3.2 MATERIALS AND METHODS

3.2.1. Isolation of rhizobia from nodulated *Acacia* spp. growing in uninoculated soils

Forty nine specimens from the three studied *Acacia* species (*A. mearnsii*, *A. dealbata* and *A. decurrens*) were collected from diverse geographic areas in South Africa in order to isolate rhizobial strains associated with these trees over a wide range of climatic conditions and soils (especially different soil pH's) (Fig. 3.1 and Table 3.1).

At least two nodules were excised from each specimen (where possible) and surface sterilized with NaOCl:H_2O (1:1) for 2 to 5 minutes according to size and washed twice with sterilized distilled water (dH_2O). Nodules were squashed individually in 300 µl sterile dH_2O. Loopsful of these suspensions were streaked onto yeast extract mannitol agar (YMA) plates [containing 1% (m/v) mannitol (BDH GPR), 0.05% (m/v) K_2HPO_4 (Merck), 0.02% (m/v) $MgSO_4.7H_2O$ (Merck), 0.01% (m/v) NaCl (BDH GPR), 0.04% (m/v) yeast extract (OXOID) and 1.5% (m/v) bacteriological agar (Biolab)], supplemented with 1% (v/v) of a 0.25% (m/v) Congo red (Merck) stock solution. The excess suspension was poured onto YMA plates as well. The plates were incubated at 28°C and examined after 5, 8 and 20 days for growth. Culture purity was verified by repeated streaking (at least three times or until pure cultures were obtained) of single colony isolates on the same medium, as well as by microscopic examination of Gram-stained cells and phase contrast microscopy of wet preparations.

Figure 3.1 Locations from which *Acacia* specimens were collected. The red dot (●) indicates the location from which specimens of all three the *Acacia* species were collected. Blue (●), yellow (●) and green (●) dots indicate locations from which specimens of the species *Acacia mearnsii*, *Acacia dealbata* and *Acacia decurrens* were collected, respectively.

Table 3.1 ***Acacia* host species and their geographic origin and soil pH**

***Acacia* species**	**Specimen no.**	**Geographic origin**	**Soil pH**
Acacia decurrens	1	Boschkop, Pretoria, Gauteng	5.11
	17	Evaton, Gauteng	5.25
	34	Centurion, Pretoria, Gauteng	6.24
	35	Centurion, Pretoria, Gauteng	5.87
	36	Tonteldoos, Mpumalanga	4.66
Acacia dealbata	2	Pretoria, Gauteng	5.73
	3	Klipkop, Pretoria, Gauteng	5.27
	4	Pienaars River bridge, Pretoria, Gauteng	5.44
	5	Pretoria, Gauteng	5.23
	6	Pretoria, Gauteng	5.11
	9	Vandyksdrif, Mpumalanga	5.51
	10	Vandyksdrif, Mpumalanga	**4.42***
	11	Vandyksdrif, Mpumalanga	4.43
	12	Vandyksdrif, Mpumalanga	5.44
	13	Vandyksdrif, Mpumalanga	5.55
	14	Vandyksdrif, Mpumalanga	6.03
	15	Vandyksdrif, Mpumalanga	7.57
	16	Vandyksdrif, Mpumalanga	7.19
	20	Kliprivier, Gauteng	7.18
	21	Swartkopjes, Gauteng	7.10
	28	Underberg, Kwazulu-Natal	5.16
	31	Muldersdrift, Gauteng	7.07
	33	Krugersdorp district, Gauteng	6.62
	49	Magoebaskloof Pass, Northern Province	6.47
Acacia mearnsii	7	Laingsnek, Kwazulu-Natal	5.71
	8	Vryheid, Kwazulu-Natal	6.30
	18	Evaton, Gauteng	5.28
	19	Evaton, Gauteng	5.10
	22	Rietvlei Dam, Pretoria, Gauteng	**7.64***

Table 3.1 **(continued)**

Acacia species	Specimen no.	Geographic origin	Soil pH
Acacia mearnsii	23	Rietvlei Dam, Pretoria, Gauteng	6.39
	24	Rietvlei Dam, Pretoria, Gauteng	5.62
	25	Riviersonderend, Western Cape	6.07
	26	Bloukrans Pass, Eastern Cape	6.79
	27	Swellendam, Western Cape	6.64
	29	Muldersdrift, Gauteng	6.02
	30	Muldersdrift, Gauteng	5.50
	32	Krugersdorp district, Gauteng	7.03
	37	Wonderwater vicinity, Mpumalanga	4.84
	39	Wonderwater, Mpumalanga	5.77
	40	Paulpietersburg district, Kwazulu-Natal	5.15
	41	Paulpietersburg district, Kwazulu-Natal	5.27
	42	Piet Retief district, Mpumalanga	4.98
	43	Piet Retief district, Mpumalanga	4.75
	44	Wakkerstroom district, Mpumalanga	4.64
	45	Wakkerstroom district, Mpumalanga	5.02
	46	Commondale, Mpumalanga	5.64
	47	Tzaneen, Northern Province	6.14-6.61
	48	Tzaneen, Northern Province	5.77-6.49
	50	Magoebaskloof Pass, Northern Province	4.90

* The lowest and highest soil pH values are indicated in bold

3.2.2. Maintenance and preservation of cultures

Purified cultures were maintained on YMA slants at 4°C. For long-term storage, yeast extract mannitol broth (YMB) cultures were incubated at 28°C for 5 to 7 days with rigorous shaking and mixed 1:1 with 50% (v/v) glycerol (SKY CHEM) in sterile cryotubes and stored at -20°C and -70°C.

3.2.3. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins

3.2.3.1 Strains used

Fifty six reference strains (Table 3.2), obtained from the Laboratorium voor Microbiologie Gent Culture Collection (State University Gent, Belgium), the United States Department of Agriculture-ARS National Rhizobium Culture Collection (USA) and the Institut National de la Recherche Agronomique (INRA)-Laboratoire de Microbiologie des Sols (France), as well as 257 putative rhizobial strains isolated in this study were used in the SDS-PAGE analysis.

3.2.3.2 Preparation of whole cell protein extracts

YMB cultures were incubated at 28°C for 6 days with rigorous shaking and used as inoculum for fresh yeast extract beef (YEB) medium [containing 0.5% (m/v) peptone (Biolab), 0.1% (m/v) yeast extract (OXOID), 0.5% (m/v) beef extract (Biolab), 0.5% (m/v) sucrose (UnivAR, SAARCHEM) and 2% (m/v) bacteriological agar (Biolab)]. The inoculated YEB medium was incubated at 28°C for 6 days. Cells were harvested by washing the YEB medium with 0.2 M sodium phosphate buffer (pH 6.88), transferred to medium sized centrifuge tubes and washed twice in the sodium phosphate buffer by centrifugation and resuspension. The cells were finally resuspended in 1 ml 0.2 M sodium phosphate buffer (pH 6.88), transferred to Eppendorf tubes and centrifuged. The supernatants were discarded and the cell pellets suspended in 30-400 µl sample treatment buffer (STB) without sodium dodecyl sulphate (SDS) depending on pellet size. The cell pellets were disrupted with a Cole-Parmer ultrasonic homogenizer (Series 4710) at 50% maximum output (40 Watt). 10% SDS was added to the amount of 10% the STB added, mixed and the suspensions frozen at -20°C for 1 h. The suspensions were heated at 94°C for 30 minutes, cooled to room temperature, centrifuged and the supernatants transferred to clean Eppendorf tubes and stored at -20°C.

Table 3.2 **List of reference strains used in the SDS-PAGE analysis**

Species	Strain number#	Host plant	Source*
Agrobacterium radiobacter (*Agrobacterium* biovar 1)	LMG 140^T	NS	Belgium
Agrobacterium rhizogenes (*Agrobacterium* biovar 2)	LMG 150^T	NS	Belgium
Agrobacterium rubi	LMG 17935^T	*Rubus ursinus* var *loganobaccus*	Belgium
Agrobacterium tumefaciens (*Agrobacterium* biovar 1)	LMG 187^T	*Lycopersicon lycopersicum*	Belgium
Allorhizobium undicola	USDA 4902	NS	USA
	USDA 4903^T	NS	USA
Azorhizobium caulinodans	LMG 6465^T	*Sesbania rostrata*	Belgium
	USDA 4892^T	*Sesbania rostrata*	USA
Bradyrhizobium sp.	LMG 8319	*Macrotyloma africanicus*	Belgium
Bradyrhizobium elkanii	LMG 6134^T	*Glycine max*	Belgium
	USDA 76^T	*Glycine max*	USA
Bradyrhizobium japonicum	LMG 6138^T	*Glycine max*	Belgium
	USDA 6^T	*Glycine max*	USA
Bradyrhizobium liaoningense	LMG 18230^T	*Glycine max*	Belgium
	USDA 3622^T	*Glycine max*	USA
Mesorhizobium amorphae	USDA 10001^T	NS	USA
Mesorhizobium ciceri	LMG 14989^T	*Cicer arietinum*	Belgium
	USDA 3383^T	NS	USA
Mesorhizobium huakuii	LMG 14107^T	*Astragalus sinicus*	Belgium
	USDA 4779^T	*Astragalus sinicus*	USA
Mesorhizobium loti	LMG 4264	*Lupinus densiflorus*	Belgium
	LMG 4268t1	*Lotus americanus*	Belgium
	LMG 6123	*Lotus divaricatus*	Belgium
	LMG 6125^T	*Lotus corniculatus*	Belgium

Table 3.2 **(continued)**

Species	**Strain number**[#]	**Host plant**	**Source***
Mesorhizobium loti	USDA 3471^T	NS	USA
Mesorhizobium mediterraneum	USDA 3392^T	*Cicer arietinum*	USA
Mesorhizobium plurifarium	USDA 3707^T	NS	USA
Mesorhizobium tianshanense	LMG 18976^T	*Glycyrrhiza pallidiflora*	Belgium
	USDA 3592^T	*Glycyrrhiza pallidiflora*	USA
Rhizobium sp.	USDA 2947	NS	USA
Rhizobium etli bv *phaseoli*	LMG 17827^T	*Phaseolus vulgaris*	Belgium
Rhizobium galegae	LMG 6214^T	*Galega orientalis*	Belgium
R. gallicum bv *gallicum*	R602sp^T	*Phaseolus vulgaris*	France
R. gallicum bv *phaseoli*	PhI21	*Phaseolus vulgaris*	France
R. giardinii bv *giardinii*	H152^T	*Phaseolus vulgaris*	France
Rhizobium hainanense	USDA 3588^T	NS	USA
Rhizobium huautlense	USDA 4900^T	*Sesbania herbacea*	USA
Rhizobium leguminosarum	LMG 4260	*Vigna unguiculata*	Belgium
	LMG 6294t2	*Lathyrus* sp.	Belgium
R. leguminosarum bv *trifolii*	LMG 8820^T	*Trifolium pratens*	Belgium
R. leguminosarum bv *viciae*	LMG 14904^T	NS	Belgium
	USDA 2370^T	*Pisum sativum*	USA
Rhizobium mongolense	LMG 19141^T	*Medicago ruthenica*	Belgium
	USDA 1844^T	*Medicago ruthenica*	USA
Rhizobium tropici group B	LMG 9503^T	*Phaseolus vulgaris*	Belgium
Sinorhizobium arboris	LMG 14919^T	*Prosopis chilensis*	Belgium
	USDA 4878^T	NS	USA
Sinorhizobium fredii	LMG 6217^T	*Glycine max*	Belgium
	LMG 6219	*Glycine max*	Belgium
Sinorhizobium kostiense	LMG 19227^T	*Acacia senegal*	Belgium
Sinorhizobium medicae	LMG 18864	*Medicago truncatula*	Belgium
	USDA 1037^T	*Medicago truncatula*	USA
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*	Belgium

Table 3.2 **(continued)**

Species	**Strain number#**	**Host plant**	**Source***
Sinorhizobium meliloti	USDA 1002^T	*Medicago sativa*	USA
Sinorhizobium saheli	USDA 4893^T	NS	USA
Sinorhizobium terangae	USDA 4894^T	NS	USA

\# LMG, Laboratorium voor Microbiologie Gent Culture Collection; USDA, United States Department of Agriculture-ARS National Rhizobium Culture Collection

* Refer to text for detailed description of source

^{T} Type strain

NS Not stated

3.2.3.3 Polyacrylamide gel electrophoresis

The method of Laemmli (1970), as modified by Kiredjian *et al.* (1986), was used. The 1.5 mm thick gels consisted of a 5% stacking gel (0.5 M Tris-HCl pH 6.68, conductivity 31.0 mS/cm) and a 12% separation gel (1.5 M Tris-HCl pH 8.87, conductivity 16.02 mS/cm). Electrophoresis was conducted in a BioRad Protean II gel apparatus at a constant current of 5 Watt through the stacking gel and 10 Watt through the separation gel. Gels were stained in Coomassie Brilliant Blue solution [15% (v/v) of a 2% (m/v) Coomassie Brilliant Blue R-250 (ICN Biomedicals) stock solution, 50% (v/v) methanol (Chemical Suppliers) and 10% (v/v) acetic acid (Chemical Suppliers)] for 1 h 10 minutes at room temperature and then destained overnight in a solution containing 25% (v/v) methanol (Chemical Suppliers) and 10% (v/v) acetic acid (Chemical Suppliers).

3.2.3.4 Analysis of the gels

The protein electrophoretic patterns were scanned using a Hoefer GS300 Transmittance/Reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco). Analysis of the data was done using the GelCompar 4.0 computer program (Applied Maths, Kortrijk, Belgium). Normalized densitometric traces were grouped and

the similarities calculated between all organisms using the Pearson product moment correlation coefficient (*r*), converted to a percentage. The cluster analysis was performed using the unweighted pair group method of arithmetic averages (UPGMA).

The protein profiles of *Psychrobacter immobilis* LMG 1125 were used as references in each gel (6 tracks out of 20 per gel) to ensure reproducibility. Reproducibility was determined by comparing these six reference profiles with the *P. immobilis* protein profile selected in the GelCompar 4.0 computer program as standard. A correlation of 94% between the standard and the six reference protein profiles on successive gels was accepted as reproducible.

3.2.4 16S rDNA sequencing of selected putative rhizobial strains

3.2.4.1 Strains used

Twenty selected putative rhizobial isolates were subjected to 16S rDNA sequencing (Table 3.3).

3.2.4.2 Extraction of genomic DNA

Tryptone yeast extract medium (TY) slants [0.5% (m/v) tryptone (Biolab), 0.3% (m/v) yeast extract (OXOID), 0.065% (m/v) $CaCl_2$ (UniLAB, SAARCHEM) and 1.5% (m/v) bacteriological agar (Biolab)] were inoculated and incubated at 28°C for 3 days. Cells were harvested by gentle shaking after adding sterile distilled water to the slants and collected in a plastic tube. Optical density of the cells was measured at 620 nm using water as a blank and the number of cells to be treated further calculated by the following formula: V (ml) = $^{0.2}/_{OD620}$. The appropriate volume of cells was centrifuged at 13 000g for 5 minutes, the supernatant discarded and the cell pellet blotted dry. The cell pellet was resuspended in 100 µl ultra-high quality (UHQ) water (i.e. sterilised double distilled water), 100 µl Tris-HCl (10 mM, pH 8.2) and 10 µl proteinase K (15.6 mg/ml) and incubated overnight at 55°C. Proteinase K was inactivated by boiling for 10 minutes and the cell lysates were stored at -20°C.

Table 3.3 List of putative rhizobial isolates subjected to 16S rDNA sequencing

Acacia **host species**	**Isolates***
A. mearnsii	18AB; 18BE (ES); 23BAV; 23DH (ES); 27BBV; 29BBV; 32AAV; 40AAV; 45BCV; 46ABV
A. dealbata	4BA; 10AB; 10BAV; 15P (ES); 16BD (ES); 20BCV; 20BGVno1; 20BI
A. decurrens	17DB; 35BI

* The isolates' names are compiled as follows: The numerals refer to the *Acacia* host specimen no. from which the strain was isolated (see Table 3.1 for host specimen number), followed by a letter indicating the nodule from which the strain was isolated (namely nodule A, B, C, D, etc.). Following the letter is further descriptive information. Exceptions are host specimens numbers 43, 47 and 48 where the numerals are followed by a second specimen number to indicate more than one specimen obtained from the same location, followed by further descriptive information. Example: Isolates 18AB and 18BE (ES) were obtained from the same *Acacia* host specimen, namely specimen no. 18 (*A. mearnsii* specimen obtained from Evaton, Gauteng), but from nodules A and B, respectively.

3.2.4.3 PCR amplification of the 16S rDNA

The primers fD1SHRT and rP2SHRT [derived from the universal primers fD1 and rP2 (Weisburg *et al.*, 1991) without the restriction enzyme sites] were used to amplify the rhizobial 16S rRNA gene (Table 3.4). Amplification was carried out in 50 µl reaction volumes containing 5 µl pure genomic DNA, 5 µl 10× PCR buffer, 4 µl 25 mM $MgCl_2$, 0.5 µl 25 mM dNTP, 12.5 pmol of both fD1SHRT and rP2SHRT and 0.5 U Super-Therm DNA polymerase (Southern Cross). Amplification was conducted in a Perkin Elmer GeneAmp PCR System 2400 apparatus with the following temperature profile: an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 3 minutes (Laguerre *et al.*, 1994). The PCR products were

examined by horizontal gel electrophoresis using 1% (m/v) agarose gels containing ethidium bromide (10 mg/ml) in 1× TAE buffer (40mM Tris-HCl, 20mM NaOAc and 1mM EDTA, pH 8.5).

Table 3.4 Primers used for PCR amplification of rhizobial 16S rDNA

Primer	Sequence (5' to 3')
fD1SHRT	AGAGTTTGATCCTGGCTCAG
rP2SHRT	ACGGCTACCTTGTTACGACTT

3.2.4.4 Purification of the 16S rDNA PCR products

The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN).

3.2.4.5 Sequencing of the 3' end (approximately 700 bp) of the 16S rRNA gene

The 3' end of the 16S rRNA gene was amplified using primer II (Kuhnert *et al.*, 1996) and primer rP2SHRT (Table 3.5). The sequencing reaction was performed using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq® DNA Polymerase, FS) (PE Applied Biosystems). The amplification was carried out in 5 µl reaction volumes containing purified 16S rDNA PCR products, 2 µl BigDye™ Terminator RR mix, 10 pmol of primer II or rP2SHRT and UHQ water. Amplification was carried out in a Perkin Elmer GeneAmp PCR System 2400 apparatus with the following temperature profile: 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes. The PCR products were precipitated using the manufacturer's protocol. The DNA was stored at -20°C. Sequencing samples were run overnight on an ABI 377 Automated Sequencer.

Table 3.5 Primers used to sequence the 3' end of the rhizobial 16S rRNA gene

Primer	Sequence (5' to 3')
Primer II	GTGTAGCGGTGAAATGCGTAG
rP2SHRT	ACGGCTACCTTGTTACGACTT

3.2.4.6 Analysis of sequence data

Sequences were analysed using Sequence Navigator version 1.0.1 (Perkin Elmer). Phylogenetic relationships were determined using the Neighbour-joining algorithm in ClustalX (Thompson *et al*., 1997). Sequence data of the bacterial genera (Table 3.6) were retrieved from the GenBank database available on the website of the National Center for Biotechnology Information (NCBI). The NJPLOT computer programme was used to generate tree representations of the phylogenetic relationships.

Table 3.6 Accession numbers of sequence data retrieved from GenBank

Bacterial genus and species	Isolate no.#	Accession no.
The genus *Agrobacterium*		
Agrobacterium larrymoorei	AF3.10^T (ATCC 51759^T)	Z30542
Agrobacterium radiobacter	ATCC 19358	AJ389904
Agrobacterium rhizogenes	LMG 152	X67224
Agrobacterium rubi	IFO 13261^T	D14503
Agrobacterium tumefaciens (*Agrobacterium* bv 1)	NCPPB 2437	D14500
Agrobacterium vitis	NCPPB 3554^T	D14502
The genus *Allorhizobium*		
Allorhizobium undicola	LMG 11875^T	Y17047

Table 3.6 **(continued)**

Bacterial genus and species	**Isolate no.#**	**Accession no.**
The genus *Azorhizobium*		
Azorhizobium caulinodans	LMG 6465^T	X67221
The genus *Bradyrhizobium*		
Bradyrhizobium elkanii	USDA 76^T	U35000
Bradyrhizobium japonicum	LMG 6138^T	X66024
Bradyrhizobium liaoningense	LMG 18230^T	AJ250813
The genus *Mesorhizobium*		
Mesorhizobium amorphae	ACCC 19665^T	AF041442
Mesorhizobium chacoense	LMG 19008^T	AJ278249
Mesorhizobium ciceri	UPM-Ca7^T	U07934
Mesorhizobium huakuii	IAM 14158	D12797
Mesorhizobium loti	LMG 6125^T	X67229
Mesorhizobium mediterraneum	UPM-Ca36^T	L38825
Mesorhizobium plurifarium	LMG 11892^T	Y14158
Mesorhizobium tianshanense	A-1BS^T (CCBAU 3306^T)	U71079
The genus *Phyllobacterium*		
Phyllobacterium myrsinacearum	IAM 13584^T	D12789
Phyllobacterium rubiacearum	IAM 13587^T	D12790
The genus *Rhizobium*		
Rhizobium etli	CFN 42^T	U28916
Rhizobium galegae	ATCC 43677^T	D11343
Rhizobium gallicum	R602sp^T (MSDJ 1109^T)	U86343
Rhizobium giardinii	H152^T (MSDJ 0144^T)	U86344
Rhizobium hainanense	I66^T (CCBAU 57015^T)	U71078
Rhizobium huautlense	S02^T	AF025852
Rhizobium leguminosarum bv *trifolii*	LMG 8820^T	X67227
Rhizobium leguminosarum bv *viciae*	USDA 2449	U89828

Table 3.6 (continued)

Bacterial genus and species	Isolate no.#	Accession no.
The genus *Rhizobium* (continued)		
Rhizobium mongolense	USDA 1844T	U89817
Rhizobium tropici IIB	LMG 9517T	X67234
Rhizobium yanglingense	CCBAU 71462	AF195031
The genus *Sinorhizobium*		
Sinorhizobium arboris	HAMBI 1552T	Z78204
Sinorhizobium fredii	LMG 6217T	X67231
Sinorhizobium kostiense	HAMBI 1489T	Z78203
Sinorhizobium medicae	A 321T	L39882
Sinorhizobium meliloti	LMG 6133T	X67222
Sinorhizobium saheli	LMG 7837T	X68390
Sinorhizobium terangae	LMG 7834T	X68388
Sinorhizobium xinjiangense	IAM 14142	D12796

\# ACCC, Agricultural Center Culture Collection, Chinese Academy of Agriculture, Beijing, China; ATCC, American Type Culture Collection, Rockville, MD, USA; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autonoma de Mexico, Cuernavaca, Mexico; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, University of Gent, Gent, Belgium; MSDJ, Culture Collection of the Laboratoire de Microbiologie des Sols, Institut National de la Recherche Agronomique, Dijon, France; NCPPB, National Collection of Plant-Pathogenic Bacteria, Harpenden Laboratory, Hertfordshire, UK; UPM, Universidad Politécnica Madrid, Madrid, Spain; USDA, United States Department of Agriculture, Beltsville, MD, USA.

T Type strain

3.3 RESULTS

3.3.1 Isolation of putative rhizobial strains from the exotic *Acacia* species

More than 250 strains were isolated from nodules of *A. mearnsii*, *A. dealbata* and *A. decurrens* in this study. In several instances more than one rhizobial strain were isolated from the same nodule, for example 10BAV, 10BB, 10BC, 10BD and 10BE from *A. dealbata*. One isolate from *Acacia mearnsii* was interesting in that it absorbed congo red, growing dark red on YMA, but exhibited the typical cell morphology of *Bradyrhizobium*. The isolate, 48asilindrAV, was isolated from a cylindrical nodule from the host specimen. Another isolate, 48arondAV, isolated from a round nodule of the same host specimen, exhibited the typical colony and cell morphology of *Bradyrhizobium*.

3.3.2 SDS-PAGE of whole cell proteins

SDS-PAGE is an extremely reliable method for comparison and grouping of large numbers of closely related isolates and a correlation exists between high similarity in whole cell protein profiles and total DNA sequence homology (Jackman, 1985; Kersters, 1985; Kersters and de Ley, 1975; Priest and Austin, 1993; Vauterin *et al.*, 1993). Therefore, SDS-PAGE was the method of choice to characterize the large number of putative rhizobial isolates obtained from the three *Acacia* spp. by including reference strains of the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. The dendrogram obtained from the SDS-PAGE data is supplemented by indicating the genera to which the twenty sequenced isolates were related. In accordance with previous findings (Dagutat, 1995; Dupuy *et al.*, 1994; Kruger, 1998; Moreira *et al.*, 1993) the bands in the protein profiles of the slow-growing strains were less distinct and less sharp than the bands in the profiles derived from fast-growing strains.

The isolates and reference strains grouped into 22 clusters, which formed three major sections and two loosely associated clusters and a single isolate (Fig. 3.2). Separation into

these sections is to facilitate discussion of the results and does not imply a closer relationship between isolates in the sections. Section I contained 108 isolates and *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium leguminosarum* reference strains. Section II contained reference strains of the genera *Agrobacterium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* (one of the seven species included in the analysis) and 73 isolates. Section III contained four of the seven *Sinorhizobium* spp. reference strains included, several *Rhizobium* and *Mesorhizobium* reference strains, as well as one *Agrobacterium rhizogenes* and three *Bradyrhizobium* reference strains. Sixty one isolates were included. Fourteen isolates, as well as the *Agrobacterium rubi*, *S. arboris*, *S. saheli* and one of the *S. meliloti* reference strains, were included in the two loosely associated clusters. A single isolate was loosely associated with the rest of the isolates and reference strains included in the dendrogram.

For the following detailed discussion of the sections and clusters, see Fig. 3.3.

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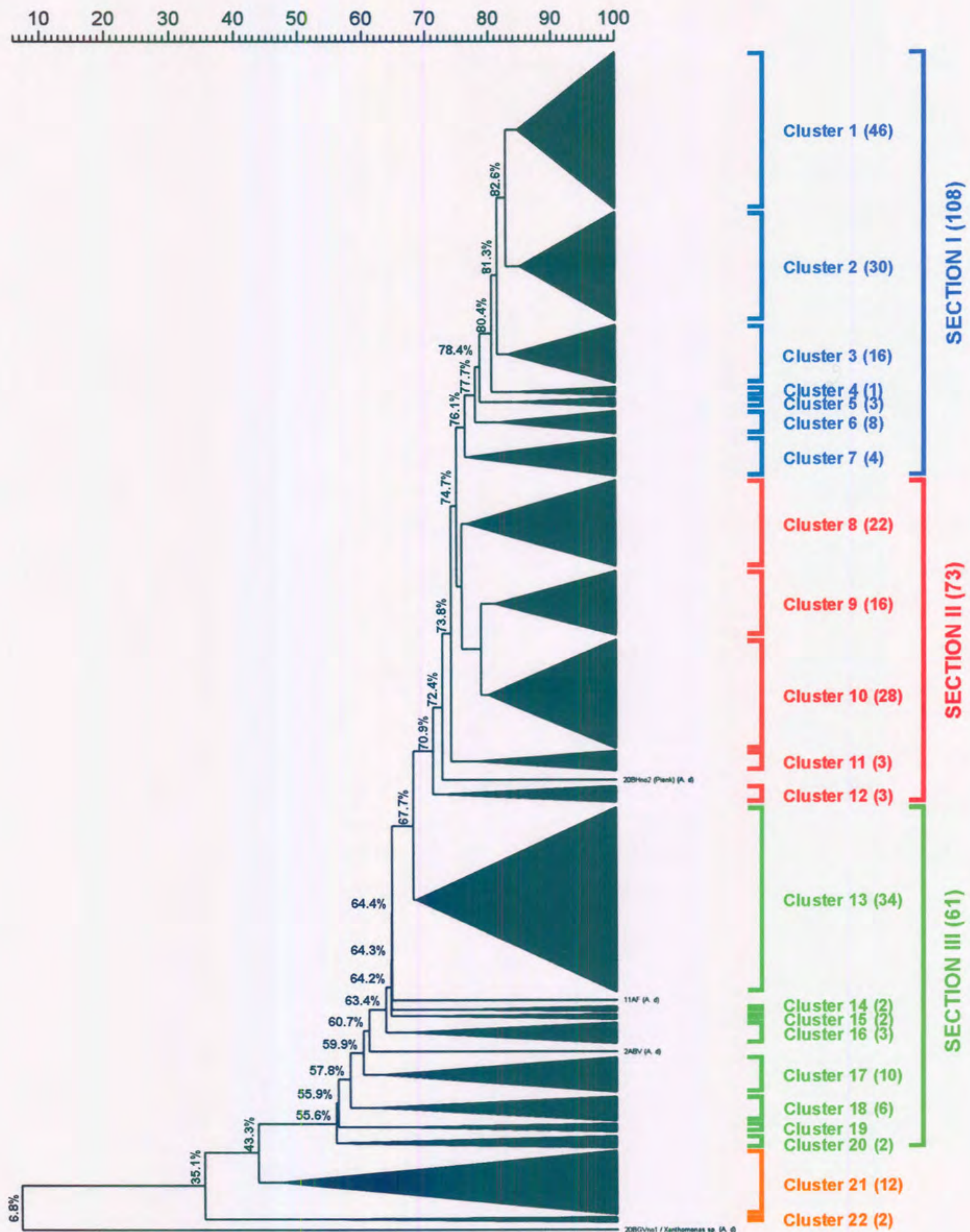

Figure 3.2 Simplified dendrogram based on UPGMA analysis of the correlation coefficients (*r*) between protein profiles of *Acacia* isolates and reference strains of the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. Correlation (r), where r (%) = *r* x 100, is represented on the x-axis. The number of *Acacia* isolates in each group is indicated in parenthesis.

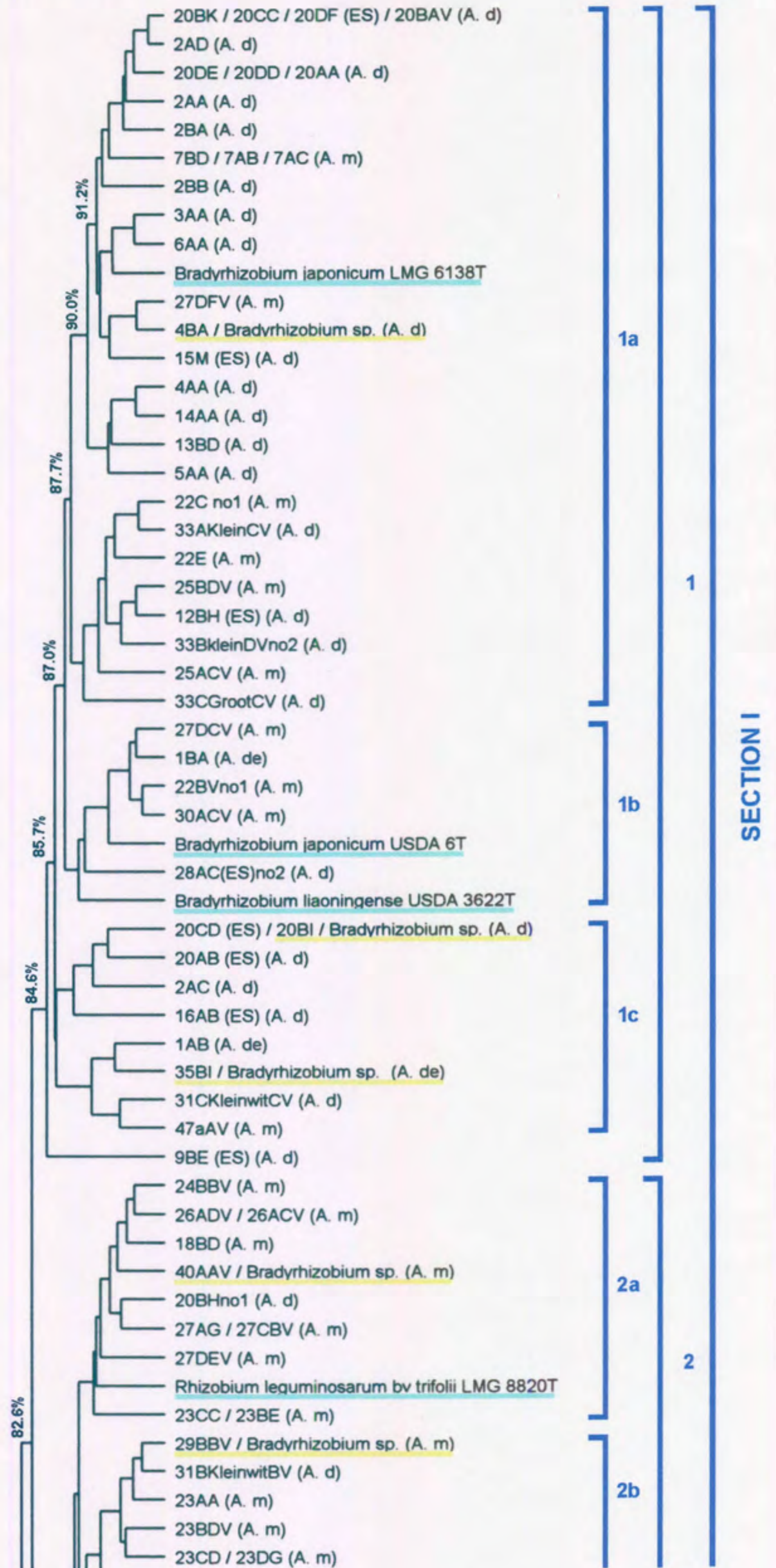

Figure 3.3

Figure 3.3 (continued)

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74.7%

12AD (A. d)
48bAV (A. m)
39AF (A. m)
37BAV (A. m)
(8b)

23AD(ES)no2 (A. m)
23AC (A. m)
23DH (ES) / Bradyrhizobium sp. (A. m)
23AB (A. m)
23DEV (A. m)
(8c)

29AAV (A. m)
A. tumefaciens (Agrobacterium biovar 1) LMG 187T
31CKleinwitE (A. d)
23CAV (A. m)
(8d)

(8)

42BAV (A. m)
34DGrootBno1 (A. de)
35ACno2 (A. de)
42ACV (A. m)
46BAV (A. m)
15I(ES)no1 (A. d)
9AE (ES) (A. d)
50AV (A. m)
37AAV (A. m)
41BAV (A. m)
31CKleinwitD (A. d)
10BE (A. d)
27BBV / Bradyrhizobium sp. (A. m)
12BI (ES) (A. d)
35ACno1 (A. de)
(9a)

Rhizobium gallicum bv gallicum R602spT
Rhizobium gallicum bv phaseoli Phl 21
32BAV (A. m)
(9b)

(9)

73.8%

11BC (A. d)
24AA (A. m)
16AA (A. d)
45AAV (A. m)
48arondAV (A. m)
43no1AKleinAV (A. m)
41AAV (A. m)
33BKleinDVno1 (A. d)
46AAV (A. m)
40ACV (A. m)
33AF (ES) (A. d)
Bradyrhizobium liaoningense LMG 18230T
39AAV (A. m)
33BE (ES) (A. d)
44AV (A. m)
39BC (A. m)
48asilindrAV (A. m)
27BDVno1 (A. m)
47bAV (A. m)
33BKleinG (ES) (A. d)
(10a)

40ABV (A. m)
40BDroogBV (A. m)
(10b)

29BEV (A. m)

17CB (A. de)
17CC (A. de)
8BC (A. m)
31CKleinwitBV (A. d)
33BKleinAV (A. d)
18AE (ES) (A. m)
(10c)

(10)

72.4%

70.9%

Rhizobium mongolense USDA 1844T
Rhizobium mongolense LMG 19141T
Rhizobium etli bv phaseoli LMG 17827T
32ACV (A. m)
34AKleinCV (A. de)
39ABV (A. m)
(11)

20BHno2 (Pienk) (A. d)

SECTION II

Figure 3.3 (continued)

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Sinorhizobium medicae USDA 1037T
Sinorhizobium medicae LMG 18864
20BJ (Oranje) (A. d)
20BAVno2 (A. d)
20BJ (Pienk) (A. d)
22G (A. m)
20AC (ES) (A. d)
14AB (ES) (A. d)
Bradyrhizobium sp. LMG 8319
30BC (A. m)
Bradyrhizobium elkanii USDA 76T
35BGV (A. de)
2ADV (A. d)
16BE (ES) (A. d)
19AI (ES) (A. m)
18BF (ES) (A. m)
34BKleinB (A. de)
28ABno3 (A. d)
18AB / Bradyrhizobium sp. (A. m)
19AC (A. m)
Bradyrhizobium elkanii LMG 6134T
33AKleinAV (A. d)
43no1BGrootBV (A. m)
10BAV / Mesorhizobium sp. (A. d)
15O (ES) (A. d)
Mesorhizobium huakuii LMG 14107T
Mesorhizobium huakuii USDA 4779T
Mesorhizobium loti LMG 6123
Mesorhizobium plurifarium USDA 3707T
9BD (ES) (A. d)
Rhizobium huautlense USDA 4900T
11AAV (A. d)
Mesorhizobium loti LMG 4264
Rhizobium tropici group B LMG 9503T
A. rhizogenes (Agrobacterium biovar 2) LMG 150T
Rhizobium hainanense USDA 3588T
27BAV (A. m)
Rhizobium leguminosarum LMG 4260
28ABno2 (A. d)
Mesorhizobium amorphae USDA 10001T
Mesorhizobium loti USDA 3471T
20BDV (A. d)
9AD (ES) (A. d)
Rhizobium galegae LMG 6214T
18BE (ES) / Bradyrhizobium sp. (A. m)
19AH (ES) (A. m)
10BC (A. d)
10BB (A. d)
19AJ (ES) (A. m)
26BC / 26BBV (A. m)
16BAno1 (A. d)
26BAV (A. m)
31ABV (A. d)
43no1BGrootAV (A. m)
11AF (A. d)
25BCV (A. m)
25BEV (A. m)
45ACV (A. m)
40BDroogAV (A. m)
23BCV (A. m)
1AAV (A. de)
Sinorhizobium terangae USDA 4894T
13BCV (A. d)
Rhizobium sp. USDA 2947
Rhizobium giardinii bv giardinii H152T

91.6%
90.9%
67.7%
64.4%
64.3%
64.2%
63.4%
60.7%

12
SECTION II
13a
13b
13
13c
13d
13e
13f
14
15
16
SECTION III

Figure 3.3 (continued)

Figure 3.3 Detailed dendrogram based on UPGMA analysis of the correlation coefficients (*r*) between protein profiles of *Acacia* isolates and reference strains of the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. Correlation (r), where r (%) = *r* x 100, is represented on the x-axis. Reference strains are underlined in light blue. Isolates of which the closest homology were determined by 16S rDNA sequencing are underlined in light green and the homology indicated. The *Acacia* host species is indicated in parenthesis following the isolate name: *A. d* = *A. dealbata*, *A. de* = *A. decurrens* and *A. m* = *A. mearnsii*. The dendrogram was simplified by showing a single protein profile for all the isolates isolated from a single *Acacia* specimen having identical protein profiles and 94% or more similarity.

Section I

This section contained most of the slow-growing isolates and was subdivided into seven clusters (clusters 1 to 7), two of which (clusters 3 and 5) did not contain any reference strains.

Cluster 1

Cluster 1 consisted of 46 slow-growing isolates and three *Bradyrhizobium* reference strains with an overall similarity of 84.6%. Thirty one (67%) of the isolates included within this cluster were obtained from *A. dealbata*. Three distinct subclusters were distinguished, with subclusters 1a and 1b showing a similarity of 87.0%, and subcluster 1c joining them at 85.7%. Isolate 9BE (ES) joined the three subclusters at a similarity of 84.6%.

Subcluster 1a contained 31 isolates mainly isolated from *A. dealbata* and one *Bradyrhizobium* reference strain grouping in two subgroups with a similarity of 87.7%. The first subgroup contained 19 *Acacia dealbata* isolates [20BK, 20CC, 20DF (ES), 20BAV, 2AD, 20DE, 20DD, 20AA, 2AA, 2BA, 2BB, 3AA, 6AA, 4BA, 15M (ES), 4AA, 14AA, 13BD and 5AA], four *Acacia mearnsii* isolates [7BD, 7AB, 7AC, 27DFV] and the *B. japonicum* LMG 6138^T reference strain which were closely related at 90.0% similarity. Two isolates, 3AA and 6AA (isolated from *A. dealbata*), which shared 96.2% similarity, were the closest related to the *B. japonicum* LMG 6138^T reference strain at 93.3% similarity. The second subgroup contained eight isolates with 89.4% similarity: 22Cno1, 22E, 25BDV, and 25ACV from *A. mearnsii* and 33AKleinCV, 12BH (ES), 33BkleinDVno2 and 33CGrootCV from *A. dealbata*. ***Subcluster 1b*** consisted of five isolates, as well as two *Bradyrhizobium* reference strains with 88.7% similarity. Four isolates, 27DCV (*A. mearnsii*), 1BA (*A. decurrens*), 22BVno1 (*A. mearnsii*) and 30ACV (*A. mearnsii*) showed 95.5% similarity to each other and 92.7% similarity to the *B. japonicum* USDA 6^T reference strain. Isolate 28AC(ES)no2 (*A. dealbata*) joined this group at a similarity of 89.6%. The *B. liaoningense* USDA 3622^T reference strain was less closely related to this group at 88.7% similarity. ***Subcluster 1c*** consisted of nine isolates from all three *Acacia*

spp. with 85.8% similarity: 20CD (ES), 20BI, 20AB (ES), 2AC and 16AB (ES) from *A. dealbata* showing 88.1% similarity; 1AB and 35BI from *A. decurrens* showing 93.6% similarity; and 31CKleinwitCV (*A. dealbata*) and 47aAV (*A. mearnsii*), showing 94.2% similarity.

Cluster 2

Cluster 2 joined cluster 1 at 82.6% similarity and consisted of 30 isolates (mainly from *A. mearnsii*) and three reference strains with 84.9% similarity. Five subclusters could be distinguished: Subclusters 2a and 2b showed 88.7% similarity and was joined by subcluster 2c at 88.2% similarity. The remaining two subclusters both consisted of two members and joined the other three subclusters at 85.4% and 84.9% similarity, respectively.

Subcluster 2a consisted of 11 isolates showing 90.7% similarity to the *R. leguminosarum* bv *trifolii* LMG 8820^T reference strain: 24BBV, 26ADV, 26ACV, 18BD, 40AAV, 27AG, 27CBV, 27DEV (*A. mearnsii*) and 20BHno1 (*A. dealbata*) were more closely related to the reference strain at 91.1% similarity, than were 23CC and 23BE (*A. mearnsii*) at 90.7% similarity. The ten isolates of ***subcluster 2b*** showed 89.7% similarity and were all isolated from *Acacia* specimens obtained from Gauteng. The two *A. dealbata* isolates (31DsilindrDV and 31BKleinwitBV) included in this subcluster, were 94.8% or closer related to one or more of the *A. mearnsii* isolates. ***Subcluster 2c*** included one isolate [17DC (ES)] from *A. decurrens* that showed 92.4% similarity to isolates 19AG (ES) and 18AC (ES) (*A. mearnsii*). The seven isolates included within this group were all isolated from *Acacia* specimens obtained from Gauteng and clustered at 89.4% similarity. The two members (20CAVno1 and 31MuistD) of ***subcluster 2d***, clustering at 86.4% similarity, were both isolated from *A. dealbata* specimens from Gauteng. The *M. ciceri* LMG 14989^T and USDA 3383^T reference strains, comprising ***subcluster 2e***, showed 93.9% similarity.

Cluster 3

This cluster, consisting of 16 isolates with a similarity of 83.0%, joined clusters 1 and 2 at 81.3% similarity. Three subclusters could be distinguished.

Subcluster 3a consisted of ten isolates showing 87.3% similarity: 31EsilindrBV, 5BAWit, 21A, 11BD (ES) and 9BF (ES) (*A. dealbata*); 34CGrootDV and 17DA (*A. decurrens*); and 29BFV, 8AC and 18AD (ES) (*A. mearnsii*). This subcluster was joined by ***subcluster 3b*** at 84.3% similarity. Subcluster 3b contained three isolates, 32AAV (*A. mearnsii*), 48bBV (*A. mearnsii*) and 33AG (ES) (*A. dealbata*), with 86.8% similarity. ***Subcluster 3c*** showed 83.0% similarity to subclusters 3a and 3b and consisted of three isolates, 17DB (*A. decurrens*), 15G (ES) (*A. dealbata*) and 17BJ (ES) (*A. decurrens*) with 88.4% similarity.

Cluster 4

Cluster 4 joined clusters 1 to 3 at 80.4% similarity and consisted of one isolate [15P (ES) from *A. dealbata*] and the two *Azorhizobium caulinodans* LMG 6465^T and USDA 4892^T reference strains (which were 91.9% similar) with 84.4% similarity.

Cluster 5

This cluster, consisting of three isolates with a similarity of 84.2%, joined clusters 1 to 4 at 78.4% similarity: 26BD (*A. mearnsii*), 43BAVno2 (*A. mearnsii*) and 12BG (*A. dealbata*).

Cluster 6

Cluster 6 joined clusters 1 to 5 at 77.7% similarity and consisted of eight isolates and two *Mesorhizobium* reference strains with a similarity of 81.8%. The *Mesorhizobium loti* LMG 6125^T reference strain and the eight isolates [14BG (ES), 14BF (ES), 14BH (ES), 14BE, 9ABV, and 12BEV from *A. dealbata*; 27CCV and 27DBV from *A. mearnsii*] formed a distinct subcluster with a similarity of 83.0%. The *Mesorhizobium mediterraneum* USDA 3392^T reference strain showed 81.8% similarity to this subcluster.

Cluster 7

Cluster 7, consisting of four isolates and *Agrobacterium*, *Allorhizobium*, *Mesorhizobium* and *Rhizobium* reference strains with 76.6% similarity, showed 76.1% similarity to clusters 1 to 6. Four subclusters could be distinguished.

Subcluster 7a consisted of two isolates (20CBV, 20BCV) from the same *A. dealbata* specimen showing 85.2% or more similarity to the *Mesorhizobium loti* LMG 4268t1 reference strain and was joined by the *Agrobacterium radiobacter* (*Agrobacterium* biovar 1) LMG 140^T reference strain at 81.2% similarity. ***Subcluster 7b*** was 79.5% similar to subcluster 7a and consisted of two isolates (32ABV and 23BAV) from two *A. mearnsii* specimens from two different locations in Gauteng and the *Rhizobium leguminosarum* bv *viciae* LMG 14904^T reference strain with 83.3% similarity. ***Subcluster 7c*** contained only the *Allorhizobium undicola* USDA 4902 and USDA 4903^T reference strains with a similarity of 96.5% and joined subclusters 7a and 7b at a similarity of 78.6%. Similarly, ***subcluster 7d*** consisted of the *Rhizobium leguminosarum* bv *viciae* USDA 2370^T and *Rhizobium leguminosarum* LMG6294t2 reference strains with a similarity of 89.0% to each other and 76.6% to the other subclusters of cluster 7.

Section II

The clusters of this section joined clusters 1 to 7 (Section I) at a similarity of 74.7%. The section was subdivided into five clusters (clusters 8 to 12) which contained *Agrobacterium*, *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* reference strains. Clusters 9 and 10 clustered at a similarity of 78.6% and joined cluster 8 at a similarity of 75.5%. Cluster 11 joined these clusters at a similarity of 73.8 % and was joined by a single isolate, 20BHno2 (Pienk) (*A. dealbata*) at 72.4% similarity and by cluster 12 at 70.9% similarity.

Cluster 8

Cluster 8 consisted of 22 isolates and an *Agrobacterium* reference strain with 76.4% similarity. Four subclusters could be distinguished: Subclusters 8a, 8b and 8c clustered at 80.2% similarity and was joined by subcluster 8d at 76.4% similarity.

Subcluster 8a consisted of ten isolates with 84.4% similarity: 36AAV and 34BKleinAV (*A. decurrens*); 10AC (ES), 3BA, 10AB, 28AC(ES)no1 and 10BD (*A. dealbata*); 42ADV, 49AV and 48cAV (*A. mearnsii*). ***Subcluster 8b***, consisting of four isolates with 82.9% similarity, was 81.8% similar to subcluster 8a. Isolates 12AD (*A. dealbata*), 48bAV (*A. mearnsii*), 39AF (*A. mearnsii*) and 37BAV (*A. mearnsii*) comprised this subcluster. The five isolates comprising ***subcluster 8c***, with a similarity of 85.3%, were all isolated from the same *A. mearnsii* specimen: 23AD(ES)no2, 23AC, 23DH (ES), 23AB and 23DEV. ***Subcluster 8d*** consisted of three isolates and an *Agrobacterium* reference strain with 80.0% similarity. Isolate 29AAV (*A. mearnsii*) showed the highest similarity to the *Agrobacterium tumefaciens* (*Agrobacterium* biovar 1) LMG 187^T reference strain at 88.0% similarity. Isolate 31CKleinwitE (*A. dealbata*) was 84.3% similar to these two strains and isolate 23CAV (*A. mearnsii*) 80.0% similar.

Cluster 9

This cluster contained 16 isolates and two *Rhizobium* reference strains with a similarity of 80.8%. Two distinct subclusters could be distinguished.

Subcluster 9a consisted of 15 isolates with 82.9% similarity: 42BAV, 42ACV, 46BAV, 50AV, 37AAV, 41BAV and 27BBV from *A. mearnsii*; 34DGrootBno1, 35ACno2 and 35ACno1 from *A. decurrens*; 15I(ES)no1, 9AE (ES), 31CKleinwitD, 10BE and 12BI (ES) from *A. dealbata*. The *Rhizobium gallicum* bv *gallicum* R602sp^T and *Rhizobium gallicum* bv *phaseoli* PhI21 reference strains were 93.0% similar and comprised ***subcluster 9b*** together with isolate 32BAV (*A. mearnsii*) with 85.1% similarity.

Cluster 10

One *Bradyrhizobium* reference strain and 28 isolates, with 79.8% similarity, comprised this cluster. Three distinct subclusters and a single associated isolate could be distinguished: Subclusters 10a and 10b were 81.4% similar, joined by the single isolate 29BEV (*A. mearnsii*) at 80.4% similarity and subcluster 10c at 79.8% similarity.

Subcluster 10a, comprising 19 isolates and one *Bradyrhizobium* reference strain with 84.9% similarity, could be divided into three subgroups. The 11 isolates of the first subgroup with 87.8% similarity, were 86.2% similar to the *Bradyrhizobium liaoningense* LMG 18230^T reference strain: 11BC, 16AA, 33BKleinDVno1 and 33AF (ES) (*A. dealbata*) and 24AA, 45AAV, 48arondAV, 43no1AKleinAV, 41AAV, 46AAV and 40ACV (*A. mearnsii*). The second subgroup, with 85.2% similarity to the first subgroup, contained six isolates: 39AAV, 44AV, 39BC, 48asilindrAV and 27BDVno1 (*A. mearnsii*); and 33BE (ES) (*A. dealbata*). Isolates 47bAV from *A. mearnsii* and 33BKleinG (ES) from *A. dealbata* comprised the third subgroup. The two members of ***subcluster 10b***, 40ABV and 40BDroogBV (with 90.2% similarity), were isolated from the same *A. mearnsii* specimen, but from different nodules. ***Subcluster 10c*** contained six isolates with 84.2% similarity: 17CB and 17CC (*A. decurrens*); 8BC and 18AE (ES) (*A. mearnsii*); and 31CKleinwitBV and 33BKleinAV (*A. dealbata*). The two isolates, 17CB and 17CC, from the same nodule of an *A. decurrens* specimen were closer related (89.3% similarity) to each other than to the other isolates in this subcluster.

Cluster 11

This cluster contained three isolates and three *Rhizobium* reference strains with a similarity of 78.0%. The three reference strains were closer related to each other (87.3% similarity) than to the isolates (79.8% similarity): *Rhizobium mongolense* USDA 1844^T and LMG 19141^T (with 92.1% similarity) were 87.3% similar to *Rhizobium etli* bv *phaseoli* LMG 17827^T. Isolates 32ACV (*A. mearnsii*) and 34AKleinCV (*A. decurrens*) were closer related to each other than to isolate 39ABV (*A. mearnsii*).

Cluster 12

Two *Sinorhizobium* reference strains and three isolates, with 72.5% similarity, comprised this cluster. The three isolates, 20BJ (Oranje), 20BAVno2 and 20BJ (Pienk), were all isolated from the same nodule of an *A. dealbata* specimen. The *Sinorhizobium medicae*

USDA 1037^T and LMG 18864 reference strains (with 91.3% similarity) were 78.0% similar to isolates 20BJ (Oranje) and 20BAVno2 (with 83.6% similarity).

Section III

The clusters of this section joined the clusters of Sections I and II at a similarity of 67.7%. The section was subdivided into eight clusters (clusters 13 to 20) which contained *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* reference strains. The single isolate 11AF (*A. dealbata*) joined cluster 13 at 64.4% similarity and was, in return, joined by cluster 14 at 64.3% similarity; by cluster 15 at 64.2% similarity; by cluster 16 at 63.4% similarity and by the single isolate 2ABV (*A. dealbata*) at 60.7% similarity. Cluster 17 joined these clusters at 59.9% similarity; cluster 18 at 57.8% similarity; cluster 19 at 55.9% similarity and cluster 20 at 55.6% similarity.

Cluster 13

This was overall the largest cluster and contained 34 isolates and 16 reference strains of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Agrobacterium* with 68.3% similarity. Six subclusters could be distinguished: Subclusters 13a and 13b (with 77.4% similarity) were joined by subcluster 13c at 74.6% similarity and by subcluster 13d at 71.3% similarity. Subcluster 13e was 69.4% similar to subclusters 13a to d and subcluster 13f 68.3%.

The 15 isolates and three *Bradyrhizobium* reference strains of ***subcluster 13a***, with 78.4% similarity, could be divided into two subgroups. The first subgroup consisted of six isolates and two *Bradyrhizobium* reference strains with 81.0% similarity. Isolates 22G (*A. mearnsii*), 20AC (ES) (*A. dealbata*) and 14AB (ES) (*A. dealbata*), which were closely related (91.3% or more similarity), were 87.8% similar to the *Bradyrhizobium* sp. LMG 8319 reference strain and isolate 30BC (*A. mearnsii*), which were 91.8% similar. The *Bradyrhizobium elkanii* USDA 76^T reference strain joined these isolates at 84.2% similarity. Isolates 35BGV (*A. decurrens*) and 2ADV (*A. dealbata*) were 85.1% similar. The nine isolates and one *Bradyrhizobium* reference strain of the second subgroup showed 81.5% similarity. Isolates 16BE (ES) (*A. dealbata*), 19AI (ES) (*A. mearnsii*), 18BF (ES)

(*A. mearnsii*), 34BKleinB (*A. decurrens*), 28ABno3 (*A. dealbata*), 18AB (*A. mearnsii*) and 19AC (*A. mearnsii*) showed 84.7% similarity. The *Bradyrhizobium elkanii* LMG 6134^T reference strain clustered with isolate 33AKleinAV (*A. dealbata*) at 90.0% similarity and with isolate 43no1BGrootBV (*A. mearnsii*) at 84.2% similarity. ***Subcluster 13b*** was divided into two distinct subgroups. The first subgroup consisted of two isolates with 77.4% similarity to subcluster 13a and was joined by the second subgroup at 77.3% similarity. Isolates 10BAV and 15O (ES), both isolated from *A. dealbata* specimens from Vandyksdrif (Mpumalanga), were 79.7% similar. The second subgroup consisted of two isolates and five *Mesorhizobium* and one *Rhizobium* reference strains with 79.3% similarity. The *Mesorhizobium huakuii* LMG 14107^T and USDA 4779^T reference strains (with 94.7% similarity) were 86.8% similar to isolate 9BD (ES) (*A. dealbata*), the *Mesorhizobium loti* LMG 6123 reference strain and the *Mesorhizobium plurifarium* USDA 3707^T reference strain (with 88.1% similarity). The *Mesorhizobium loti* LMG 6123 and *Mesorhizobium plurifarium* USDA 3707^T reference strains were 90.1% similar. This cluster of four *Mesorhizobium* reference strains and one isolate was joined by a second cluster, consisting of isolate 11AAV (*A. dealbata*) and the *Rhizobium huautlense* USDA 4900^T reference strain with 86.2% similarity, at 80.1% similarity and by the *Mesorhizobium loti* LMG 4264 reference strain at 79.3% similarity. ***Subcluster 13c*** consisted of two isolates and three *Rhizobium*, one *Agrobacterium* and one *Mesorhizobium* reference strains with 75.6% similarity. The *Rhizobium tropici* group B LMG 9503^T and *Agrobacterium rhizogenes* (*Agrobacterium* biovar 2) LMG 150^T reference strains (with 91.6% similarity) were 90.9% similar to the *Rhizobium hainanense* USDA 3588^T reference strain, 88.0% similar to isolate 27BAV (*A. mearnsii*) and 82.7% similar to the *Rhizobium leguminosarum* LMG 4260 reference strain. Isolate 28ABno2 (*A. dealbata*) and the *Mesorhizobium amorphae* USDA 10001^T reference strain clustered at 82.6% similarity. The two isolates and two reference strains of ***subcluster 13d*** clustered at 73.3% similarity. The *Mesorhizobium loti* USDA 3471^T reference strain and isolate 20BDV (*A. dealbata*) were 83.4% similar and isolate 9AD (ES) (*A. dealbata*) and the

Rhizobium galegae LMG 6214^T reference strain 75.0%. ***Subcluster 13e*** consisted of nine isolates. Seven isolates [18BE (ES), 19AH (ES), 19AJ (ES), 26BC and 26BBV (*A. mearnsii*); 10BC and 10BB (*A. dealbata*)] formed a cluster at 88.0% similarity and were 86.1% similar to isolate 16BAno1 (*A. dealbata*) and 78.4% similar to isolate 26BAV (*A. mearnsii*). The two members of ***subcluster 13f***, 31BAV (*A. dealbata*) and 43no1BGrootAV (*A. mearnsii*), showed 75.3% similarity.

Cluster 14

This cluster consisted of two isolates, 25BCV and 25BEV, from the same nodule of an *A. mearnsii* specimen, with 77.0% similarity.

Cluster 15

The two members of this cluster (45ACV and 40BDroogAV) with 69.5% similarity, were isolated from two different *A. mearnsii* specimens from Mpumalanga and Kwazulu-Natal, respectively.

Cluster 16

This cluster consisted of three isolates, one *Sinorhizobium* reference strain and two *Rhizobium* reference strains with 67.3% similarity. Isolates 23BCV (*A. mearnsii*) and 1AAV (*A. decurrens*) (with 87.0% similarity) were 70.9% similar to the *Sinorhizobium terangae* USDA 4894^T reference strain and isolate 13BCV (*A. dealbata*), which were 78.8% similar. The *Rhizobium* sp. USDA 2947 and *Rhizobium giardinii* bv *giardinii* H152^T reference strains were 75.2% similar.

Cluster 17

Cluster 17 consisted of ten isolates with 63.0% similarity. Six isolates formed a cluster at 82.6% similarity: 20BHno2 (Oranje) and 20BBV (Pienk), isolated from the same nodule of an *A. dealbata* specimen, 34DGrootBno2 (*A. decurrens*), 45BB (*A. mearnsii*), 5BBWit (*A. dealbata*) and 45BCV (*A. mearnsii*). Isolate 31AAV (*A. dealbata*) showed 72.3% similarity to this cluster and isolate 39AEV (*A. mearnsii*) 70.9% similarity. Isolates

20BBV (Oranje) and 22AV (*A. mearnsii*) with 72.5% similarity, were isolated from *Acacia* spp. collected from different locations in Gauteng with the soil pH within the 7.0 to7.9 range.

Cluster 18

Cluster 18 consisted of six isolates and one *Sinorhizobium* reference strain with 62.2% similarity. Four isolates clustered at 77.9% similarity: 11BAV and 16BC (*A. dealbata*), 26ABV (*A. mearnsii*) and 34CGrootAV (*A. decurrens*). The *Sinorhizobium meliloti* LMG 6133^T reference strain showed 68.9% similarity to this cluster. Isolates 46BCV and 46ABV, collected from different nodules of the same *A. mearnsii* specimen, were 79.6% similar.

Cluster 19

This cluster consisted of three *Sinorhizobium* reference strains. The *Sinorhizobium fredii* LMG 6219 and LMG 6217^T reference strains were 91.2% similar to each other and 66.9% similar to the *Sinorhizobium kostiense* LMG 19227^T reference strain.

Cluster 20

This cluster consisted of two isolates and two *Mesorhizobium* reference strains. The *Mesorhizobium tianshanense* USDA 3592^T and LMG 18976^T reference strains, which were 93.2% similar, showed 72.6% similarity to isolate 13AD (*A. dealbata*) and 56.8% similarity to isolate 16BD (ES) (*A. dealbata*).

The two loosely associated clusters and single loosely associated isolate

The two loosely associated clusters (clusters 21 and 22) joined the clusters of Sections I, II and III at a similarity of 43.3%. Cluster 21 contained *Agrobacterium* and *Sinorhizobium* reference strains and was joined by cluster 22 at 35.1% similarity. The single loosely associated isolate, 20BGVno1 (*A. dealbata*), joined all the isolates and reference strains at a similarity of 6.8%.

Cluster 21

The 12 isolates and four *Sinorhizobium* and one *Agrobacterium* reference strains comprising this cluster clustered at 47.3% similarity. Four subclusters could be distinguished: Subclusters 21a and 21b showed 71.2% similarity and was joined by subcluster 21c at 67.6% similarity. Isolate 34CGrootBV (*A. decurrens*) showed 58.5% similarity to subclusters 21a to c and isolate 42AAV (*A. mearnsii*) showed 51.1% similarity. Subcluster 21d joined at 47.3% similarity.

Subcluster 21a consisted of one isolate and the four *Sinorhizobium* and one *Agrobacterium* reference strains. The *Sinorhizobium arboris* USDA 4878^T and LMG 14919^T reference strains were 94.5% similar to each other; 89.4% similar to the *Sinorhizobium saheli* USDA 4893^T reference strain; 85.9% similar to isolate 12BDV (*A. dealbata*); 81.4% similar to the *Sinorhizobium meliloti* USDA 1002^T reference strain and 80.5% similar to the *Agrobacterium rubi* LMG 17935^T reference strain. ***Subcluster 21b*** consisted of four isolates with 76.6% similarity: 16BA(ES)no2 (*A. dealbata*) and 18BG(ES) (*A. mearnsii*) were the closest related at 95.1% similarity and showed 89.0% similarity to 29ACV (*A. mearnsii*). Isolate 28AAV (*A. dealbata*) was the fourth member of this subcluster. The two members of ***subcluster 21c***, 35ABV (*A. decurrens*) and 31DsilindrBV (*A. dealbata*), clustered at 78.8% similarity. ***Subcluster 21d*** consisted of three isolates: isolates 46BBV (*A. mearnsii*) and 17CA (*A. decurrens*) were 64.8% similar to each other and 60.6% similar to isolate 42ABV (*A. mearnsii*).

Cluster 22

The two members of cluster 22, 15FV (*A. dealbata*) and 34AKleinDV (*A. decurrens*), clustered at 53.4% similarity.

3.3.3 16S rDNA sequencing of selected putative rhizobial strains

The partial 16S rDNA sequences of twenty indigenous *Acacia* isolates were compared to those of members of the rhizobial genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, as well as the genera *Agrobacterium* and

Phyllobacterium available from the GenBank database. A tree showing the phylogenetic positions of the indigenous isolates is shown in Fig. 3.4. Fifteen of the sequenced isolates (75%) grouped within the *Bradyrhizobium* branch of the phylogenetic tree. Two of these isolates [18AB and 18BE (ES)] were isolated from the same *A. mearnsii* specimen and exhibited closest homology to the species *B. elkanii*. The remaining thirteen isolates, obtained from all three the *Acacia* spp., were closely homologous to *B. japonicum* and *B. liaoningense*. Isolate 10BAV from *A. dealbata* fell within the *Mesorhizobium* branch, homologous to *M. plurifarium*. Three isolates fell within the *Agrobacterium-Allorhizobium-Rhizobium* branch of the tree. Within the cluster containing two isolates, as well as *R. etli*, *R. leguminosarum* bv *viciae* and *R. leguminosarum* bv *trifolii*, isolate 20BCV (*A. dealbata*) was resolved on a separate branch, whilst isolate 23BAV (*A. mearnsii*) was closely homologous to *R. leguminosarum* bv *trifolii*. Isolate 46ABV (*A. mearnsii*) was resolved in the tight cluster containing *R. tropici* group B and *A. rhizogenes*, with closest homology to the latter. Isolate 20BGVno1 (*A. dealbata*) was resolved on a separate branch, with no close homology to any of the above genera. This isolate was also only 6.8% similar to all of the indigenous isolates and reference strains in the SDS-PAGE analysis.

A search of the NCBI's Nucleotide BLAST database, Standard nucleotide-nucleotide BLAST/blastn, revealed the partial sequence of isolate 20BGVno1 to be closely homologous to the 16S rRNA gene sequences of species of *Xanthomonas* (Class: *Gammaproteobacteria*, Order: *Xanthomonadales*, Family: *Xanthomonadaceae*) (Bergey's Manual Trust, 2001), with closest homology to *X. campestris*. A phylogenetic tree that included partial 16S rRNA gene sequences of species of *Xanthomonas* in addition to the sequences included in the above phylogenetic tree (Fig. 3.4), confirmed the close homology of isolate 20BGVno1 to the genus *Xanthomonas*, as well to the species *X. campestris* (data not shown). Members of this genus are plant pathogens, causing various diseases on a variety of plants (including members of the family *Leguminosae*) (Bradbury, 1984). Xanthomonads may also survive in many ways, including with perennial hosts.

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2%

Sinorhizobium saheli
Sinorhizobium arboris
Sinorhizobium meliloti
Sinorhizobium medicae
Sinorhizobium xinjiangense
91.7
Sinorhizobium fredii
27.4
Sinorhizobium kostiense
Sinorhizobium terangae
Rhizobium giardinii
44.7
100.0
Phyllobacterium rubiacearum
Phyllobacterium myrsinacearum
Mesorhizobium tianshanense
75.6
Mesorhizobium loti
53.2
Mesorhizobium ciceri
Mesorhizobium huakuii
Mesorhizobium amorphae
Mesorhizobium chacoense
98.3
Mesorhizobium mediterraneum
Mesorhizobium plurifarium
99.9
10BAV (*Acacia dealbata*)
99.8
Agrobacterium vitis
Allorhizobium undicola
41.9
Agrobacterium larrymoorei
99.8
Agrobacterium radiobacter
Agrobacterium tumefaciens (*Agrobacterium* bv 1)
Agrobacterium rubi
Rhizobium huautlense
43.6
Rhizobium galegae
Rhizobium yanglingense
67.5
Rhizobium mongolense
Rhizobium gallicum
50.1
20BCV (*Acacia dealbata*)
50.6
Rhizobium etli
Rhizobium leguminosarum bv viciae
100.0
95.0
Rhizobium leguminosarum bv trifolii
23BAV (*Acacia mearnsii*)
Rhizobium hainanense
100.0
Rhizobium tropici IIB
99.6
Agrobacterium rhizogenes
93.4
46ABV (*Acacia mearnsii*)
20BGVno1
(*Acacia dealbata*)
Azorhizobium caulinodans
57.3
100.0
Bradyrhizobium elkanii
18AB (*Acacia mearnsii*)
18BE (ES) (*Acacia mearnsii*)
45BCV (*Acacia mearnsii*)
16BD (ES) (*Acacia dealbata*)
17DB (*Acacia decurrens*)
35BI (*Acacia decurrens*)
10AB (*Acacia dealbata*)
Bradyrhizobium japonicum
Bradyrhizobium liaoningense
27BBV (*Acacia mearnsii*)
29BBV (*Acacia mearnsii*)
15P (ES) (*Acacia dealbata*)
32AAV (*Acacia mearnsii*)
20BI (*Acacia dealbata*)
4BA (*Acacia dealbata*)
23DH (ES) (*Acacia mearnsii*)
40AAV (*Acacia mearnsii*)

Figure 3.4 Phylogenetic tree of selected *Acacia* isolates and some related bacteria in the *Alpha-Proteobacteria*. The tree was constructed by the neighbour-joining method from 16S rDNA sequences. Bootstrap probabilities are indicated at the branching points. The scale bar represents percentage nucleotide difference. The *Acacia* isolates are indicated in green and their host species are indicated in parenthesis.

CHAPTER 4

CHAPTER 4
DISCUSSION

The association between indigenous rhizobial isolates and exotic *Acacia* spp. was determined in this study by isolating putative rhizobial strains from the exotic species *A. mearnsii*, *A. dealbata* and *A. decurrens*.

In several instances more than one rhizobial type were isolated from the same nodule, which is in accordance with previous reports indicating that diverse rhizobia were isolated from the same leguminous host and even the same nodule (Dagutat, 1995; Dreyfus and Dommergues, 1981; Kruger, 1998).

Several species have been described for isolates from *Acacia* spp.. De Lajudie *et al.* (1994, 1998b) described two species, *S. terangae* and *M. plurifarium*, for strains isolated from several *Acacia* spp. in Senegal (West Africa). Two other species in the genus *Sinorhizobium*, *S. arboris* and *S. kostiense*, were also described for fast-growing isolates from *A. senegal* and *Prosopis chilensis* in Sudan and Northern Kenya (Nick *et al.*, 1999b). The remainder of the isolates from *A. senegal* and *Prosopis chilensis* belonged to the genus *Sinorhizobium* (Haukka *et al.*, 1996; Nick *et al.*, 1999b). In general, *Acacia* spp. in African countries are nodulated by members of the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* (Dreyfus and Dommergues, 1981; De Lajudie *et al.*, 1994, 1998b; Dupuy and Dreyfus, 1992; Dupuy *et al.*, 1994; Haukka *et al.*, 1996; Nick *et al.*, 1999b; Odee *et al.*, 1997; Zhang *et al.*, 1991). *Agrobacterium* biovar 1 strains have also been isolated from root nodules of *Acacia* spp. in Africa (De Lajudie *et al.*, 1999). In South Africa the symbionts of *Acacia* spp. are diverse, belonging to the genera *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* (Dagutat, 1995; Kruger, 1998).

In this study, the indigenous isolates associated with the exotic *Acacia* species were predominantly members of the genus *Bradyrhizobium* (clusters 1, 2a, 2b, 3b, 3c, 8a, 8c,

10a, 13a, 13e), whilst others showed close similarity to the genera *Agrobacterium* (clusters 8d, 13c), *Mesorhizobium* (clusters 7a, 13b) and *Rhizobium* (clusters 2a, 7b, 9b, 13b, 13c). Only one isolate with close similarity to *Sinorhizobium* (cluster 21a) was found, whilst none of the isolates showed any similarity to *Allorhizobium* (cluster 7c) and *Azorhizobium* (cluster 4). Earlier South African studies also indicated a predominance of *Bradyrhizobium* strains among tree legume symbionts, as well as the absence of indigenous strains similar to *Azorhizobium* (Dagutat, 1995; Kruger, 1998).

No correlation was evident between the protein electrophoretic groupings and the *Acacia* host species, geographic origin or soil pH. However, Australian authors (Lafay and Burdon, 1998, 2001; Marsudi *et al.*, 1999) have suggested that *Bradyrhizobium* species may be the most common species in acid or near-neutral soil. In this study, as well as previous South African studies (Dagutat, 1995; Kruger, 1998), *Bradyrhizobium* strains were predominant among the isolates. The upper limit of the soil pH range from which the isolates were isolated in this study was near neutral pH, i.e. pH 7.64. Thus, the correlation between the distribution of *Bradyrhizobium* strains and the soil pH may be the same as in Australia and should be investigated in future studies.

Both fast- and slow-growing strains were isolated from *A. dealbata*, *A. mearnsii* and *A. decurrens*, although few fast-growing strains (less than 30%) were isolated from *A. decurrens*. The same nodulation responses were indicated for *A. dealbata* and *A. mearnsii* in southeastern Australia (Lafay and Burdon, 2001). Dreyfus and Dommergues (1981), however, indicated that African *A. mearnsii* is nodulated by slow-growing strains only (something not found in clusters 2a, 7b, 8d, 9b, 13c).

The higher similarity observed between the slow-growing isolates compared with the fast-growing isolates was the direct result of the lower quality (less distinct and sharp bands) of the protein profiles of the former (Dagutat, 1995; Dupuy *et al.*, 1994; Kruger, 1998; Moreira *et al.*, 1993). Dupuy *et al.* (1994) indicated that numerical analyses in which *r* values are used for profiles of this type of low quality are influenced more by the specific selection of strains. AFLP fingerprinting, direct sequencing of the IGS region

and IGS ARDRA may be more suitable methods for the initial screening of bradyrhizobial strains (Doignon-Bourcier *et al.*, 2000; Willems *et al.*, 2000, 2001b, 2001c).

Different rhizobial species or groups were separated well below the similarity level of 91% in the SDS-PAGE analysis, with the exception of *R. tropici* group B and *A. rhizogenes* (*Agrobacterium* biovar 2) which had a similarity of 91.6% (cluster 13c). ARDRA analysis of the 16S rRNA genes of the genus *Rhizobium* and other genera indicated that the variation observed between the 16S rRNA gene sequences was sufficient to permit the identification of the individual species, except for *R. tropici* and *A. rhizogenes* (Laguerre *et al.*, 1994). These findings are also consistent with phylogenetic data based on 16S rRNA gene sequencing showing that *R. tropici* group A, *R. tropici* group B and *A. rhizogenes* are intermixed in a tight cluster (Amarger *et al.*, 1997; Chen *et al.*, 1997; De Lajudie *et al.*, 1994, 1998a, 1998b; Dupuy *et al.*, 1994; Sawada *et al.*, 1993; Van Berkum *et al.*, 1998; Velázquez *et al.*, 2001; Wang *et al.*, 1998; Willems and Collins, 1993; Yanagi and Yamasato, 1993; Young *et al.*, 2001).

Therefore, strains that clustered in groups having 92% or more similarity was considered as having potential species status, whilst strains sharing 94% or more similarity were considered as being identical (based upon the accepted 94% limit for reproducibility). Two isolates from *A. dealbata* in cluster 1a, as well as three isolates from *A. mearnsii* and one isolate from *A. decurrens* in cluster 1b were considered to belong to the species *B. japonicum*. In several instances isolates from different specimens of the same *Acacia* sp. shared 94% or more similarity, for example: (i) Isolates 3AA and 6AA from different *A. dealbata* specimens from Gauteng shared 96.2% similarity (cluster 1a); (ii) Isolates 19AG (ES) and 18AC (ES) from different *A. mearnsii* specimens from Evaton, Gauteng, shared 94.2% similarity (cluster 2c); and (iii) Isolates 34DGrootBno1 and 35ACno2 from different *A. decurrens* specimens from Centurion, Gauteng, shared 97.4% similarity (cluster 9a). Isolates from different *Acacia* spp. were also 94% or more similar: (i) Isolates 31CKleinwitCV (*A. dealbata*) and 47aAV (*A. mearnsii*), which were isolated from specimens that were collected from different regions in South Africa, shared 94.2%

similarity (cluster 1c); (ii) Isolates 31BKleinwitBV and 31DsilindrDV, isolated from a single *A. dealbata* specimen, were 94.8% or closer related to one or more of the *A. mearnsii* isolates in cluster 2b (the isolates in this cluster originated from three different locations in Gauteng); (iii) Isolates 42BAV (*A. mearnsii*) and 34DGrootBno1 (*A. decurrens*), originating from different regions in South Africa, shared 100% similarity (cluster 9a); and (iv) Isolates 16BA(ES)no2 (*A. dealbata*) and 18BG(ES) (*A. mearnsii*) also originated from different regions in South Africa and shared 95.1% similarity (cluster 21b).

Clusters 12 and 14 consisted of isolates that were isolated from the same nodule of *A. dealbata* and *A. mearnsii* specimens, respectively. Two of the isolates in cluster 12 were closely related to each other, but were relatively unrelated to *S. medicae* and the third isolate. The two isolates of cluster 14 were relatively unrelated. In contrast, clusters 7a, 8c and 10b included isolates that were closely related even though they were isolated from different nodules of the same *Acacia* specimen, respectively.

The two type strains of each of nine species (*A. caulinodans*, *B. elkanii*, *B. japonicum*, *B. liaoningense*, *M. loti*, *M. tianshanense*, *R. leguminosarum* bv *viciae*, *R. mongolense* and *S. meliloti*) obtained from the Laboratorium voor Microbiologie Gent Culture Collection (State University Gent, Belgium) and the United States Department of Agriculture-ARS National Rhizobium Culture Collection (USA), respectively, did not share 94% or more similarity in the SDS-PAGE analysis as expected and in some instances were recovered in different clusters. The different reference strains of *M. loti* (LMG 4264, LMG 4268t1, LMG 6123, LMG 6125^T and USDA 3471^T), *R. leguminosarum* (LMG 4260, LMG 6294t2, LMG 8820^T, LMG 14904^T and LMG 2370^T), *S. fredii* (LMG 6217^T and LMG 6219) and *S. medicae* (LMG 18864 and USDA 1037^T), respectively, were also either recovered in different clusters or shared less than 92% similarity. These strains will have to be characterized further using genotypic methods in order to explain their relationships in the SDS-PAGE analysis. Possible explanations for the relationships between the *M. loti* type and reference strains, as well as between the *S. meliloti* type strains are given later in the text.

B. liaoningense USDA 3622^T clustered in cluster 1 together with *B. japonicum* LMG 6138^T and USDA 6^T (showing 88.7% similarity to the latter), whilst *B. liaoningense* LMG 18230^T was recovered in cluster 10a in the SDS-PAGE analysis. *B. elkanii* USDA 76^T and LMG 6134^T were recovered in cluster 13a. DNA-DNA hybridization analyses of the three *Bradyrhizobium* species indicated that *B. liaoningense* is genotypically highly related to *B. japonicum*, whereas *B. elkanii* is more distantly related to these two species (Willems *et al.*, 2001a). The genospecies representing the former two species were recovered in the same subgeneric group, being more closely related to each other (>40% DNA hybridization) than to the genospecies representing *B. elkanii* (<40% DNA hybridization), which was recovered in a separate subgeneric group (Willems *et al.*, 2001b, 2001c).

Although *R. leguminosarum* bv *trifolii* was originally described for strains isolated from *Trifolium* spp. and *R. leguminosarum* bv *viciae* for symbionts of *Vicia*, *Pisum*, *Lathyrus* and *Lens* spp. (Jordan, 1984), indigenous rhizobial isolates from, among others, *Acacia* spp. showed affinity to these biovars in previous South African studies (Dagutat, 1995; Kruger, 1998). In this study several isolates from *A. mearnsii*, as well as an isolate from *A. dealbata*, showed close similarity to *R. leguminosarum* bv *trifolii* (cluster 2a), whilst isolates from *A. mearnsii* showed similarity to *R. leguminosarum* bv *viciae* (cluster 7b) in the SDS-PAGE analysis. The close similarity of the indigenous isolates to these two biovars of *R. leguminosarum* was confirmed by the 16S rDNA sequencing analysis. *Acacia* isolates from Australia are also similar to *R. leguminosarum* (Lafay and Burdon, 1998; Marsudi *et al.*, 1999).

One of the isolates from *A. mearnsii* included in cluster 2a belongs to the *Bradyrhizobium* lineage as determined by 16S rDNA sequencing. This isolate, as well as several other isolates from *A. mearnsii* and a single isolate from *A. dealbata*, were 91.1% similar to *R. leguminosarum* bv *trifolii* in the SDS-PAGE analysis. Another isolate from *A. mearnsii*, also belonging to the *Bradyrhizobium* lineage as determined by 16S rDNA sequencing, was included in cluster 2b and was 88.7% similar to *R. leguminosarum* bv *trifolii*. These are high percentages of similarity between strains of different genera. Therefore, the

relationships between the strains of clusters 2a and 2b will have to be resolved using additional genotypic methods. Similarly, the relationships between the two *A. dealbata* isolates and *M. loti* included in cluster 7a will have to be resolved: one isolate, which was 85.2% similar to *M. loti* and the other isolate, belonged to the *Rhizobium* lineage as determined by 16S rDNA sequencing.

A. caulinodans very specifically nodulates the stems and roots of *Sesbania rostrata* (Dreyfus *et al.*, 1988). The bradyrhizobial stem nodule isolates of another legume genus, *Aeschynomene*, produce bacteriochlorophyll and perform photosynthesis. A single isolate from *A. dealbata*, belonging to the *Bradyrhizobium* lineage as determined by 16S rDNA sequencing, exhibited relative close similarity to *A. caulinodans* in the SDS-PAGE analysis (cluster 4). No isolates were related to this genus in the 16S rDNA sequencing analysis. The absence of indigenous strains similar to *Azorhizobium* was also indicated in previous South African studies (Dagutat, 1995; Kruger, 1998).

Our SDS-PAGE results indicated a very high degree of relatedness between *M. loti* LMG 6123 and *M. plurifarium* USDA 3707^{T}, as well as a high degree of relatedness between these two species and *M. huakuii* LMG 14107^{T} and USDA 4779^{T} (cluster 13b). The other *M. loti* reference strains were dispersed throughout the dendrogram with strain LMG 6125^{T} (showing a high degree of relatedness to *M. mediterraneum* USDA 3392^{T}) in cluster 6, strain LMG 4268t1 in cluster 7a, and strains LMG 4264 and USDA 3471^{T} in cluster 13b and 13d, respectively. De Lajudie *et al.* (1998b) indicated that *M. loti* LMG 6123, *M. huakuii* and *M. plurifarium* formed a tight cluster in a phylogenetic analysis of 16S rRNA gene sequences. *M. loti* LMG 6125^{T} was recovered in a separate rRNA gene subcluster together with *M. ciceri* and showed 23% total DNA hybridization with *M. loti* LMG 6123. Based on these data and the fact that *M. loti* reference strains were recovered in different positions in the dendrogram obtained from SDS-PAGE of whole cell proteins, these authors suggested that this species needs further examination to define different groups and to secure its taxonomic status. Several other studies indicating separate positions of *M. loti* reference strains in SDS-PAGE analyses (De Lajudie *et al.*, 1998a, 1998b; Dupuy *et al.*, 1994; Kruger, 1998; Moreira *et al.*, 1993), low DNA

homology between rhizobia classified as *M. loti* (Sullivan *et al.*, 1996) and divergent 16S rRNA genes (De Lajudie *et al.*, 1998b; Laguerre *et al.*, 1994, 1997; Sullivan *et al.*, 1996) support this opinion.

Strains of *Agrobacterium* biovar 1 LMG 140^T and LMG 187^T exhibited diverse protein patterns and were recovered in different positions in clusters 7a and 8d, respectively, in the SDS-PAGE dendrogram. Similar observations were made previously (De Lajudie *et al.*, 1998a, 1998b). Indigenous isolates from *A. mearnsii* and *A. dealbata* exhibited similarity to *Agrobacterium* biovar 1 LMG 187^T. These isolates, especially isolate 29AAV (*A. mearnsii*) which was more than 85% similar to the reference strain, will be characterized further in future studies (especially with regard to their nodulation status).

Although one of the isolates from *A. mearnsii* included in cluster 7b in the SDS-PAGE analysis was less than 85% similar to *R. leguminosarum* bv *viciae*, this isolate belonged to the *Rhizobium* lineage, with closest homology to *R. leguminosarum* bv *trifolii* according to the 16S rDNA sequencing analysis. Therefore the relationships within this cluster will also have to be resolved using different techniques. Similarly, an isolate from *A. dealbata* that was homologous to *M. plurifarium* in the 16S rDNA sequencing analysis, exhibited less than 85% similarity to *M. plurifarium* in the SDS-PAGE analysis (cluster 13b).

Allorhizobium undicola was described for the strains that effectively nodulate *Neptunia natans*, a stem-nodulated tropical legume found in waterlogged areas of Senegal (De Lajudie *et al.*, 1998a). *M. plurifarium* strains, isolated from *Acacia* spp., can nodulate *N. natans* (De Lajudie *et al.*, 1998b). However, no indigenous isolates showed similarity to *A. undicola* in the SDS-PAGE (cluster 7c) or 16S rDNA sequencing analyses.

Several studies indicated that *R. etli* strains are not restricted to the Americas and that these strains are able to nodulate host legumes other than common bean (Amarger *et al.*, 1997; Dagutat, 1995; Diouf *et al.*, 2000). In the study by Dagutat (1995) *R. etli* strains were isolated from the tree legume *Acacia melanoxylon*. Although none of the indigenous

strains characterized in the present study showed close similarity to *R. etli* bv *phaseoli* in the SDS-PAGE analysis (cluster 11), a single isolate from *A. dealbata* did exhibit close similarity to this species in the 16S rDNA sequencing analysis.

A single indigenous isolate from *A. dealbata* exhibited close similarity to *M. loti* and *M. plurifarium* in the SDS-PAGE analysis (cluster 13b). *M. loti* (Jarvis *et al.*, 1982, 1997) forms effective nodules on *Lotus*, *Lupinus* and/or *Anthyllis* spp., whereas *M. plurifarium* (De Lajudie *et al.*, 1998b) was described for strains isolated from several *Acacia* spp. in Senegal (West Africa). In the 16S rDNA sequencing analysis none of the sequenced indigenous isolates exhibited similarity to *M. loti*, whereas a different single isolate from *A. dealbata* was closely related to *M. plurifarium*.

In this study the species *R. hainanense* exhibited close similarity to *R. tropici* group B and *A. rhizogenes* (*Agrobacterium* biovar 2) in both the SDS-PAGE (cluster 13c) and 16S rDNA sequencing analyses. Two different indigenous isolates from *A. mearnsii* also exhibited similarity to this cluster of rhizobial species in the SDS-PAGE analysis and 16S rDNA sequencing analysis, respectively. Isolates related to *R. tropici* were isolated from *A. mearnsii* and *A. dealbata* in southeastern Australia (Lafay and Burdon, 2001).

S. meliloti strains could be subdivided into two subgroups (A1 and A2) based on MLEE (Eardly *et al.*, 1990) and PCR (De Bruijn, 1992) data. Although both *S. meliloti* strains LMG 6133 and USDA 1002 are derivatives of the type strain ATCC 9930 belonging to subgroup A1, these strains clustered in separate positions in the SDS-PAGE dendrogram in this study. However, in the study by Dagutat (1995) these strains also exhibited low affinity to each other, compelling the author to question the status of strain USDA 1002 as a representative of *S. meliloti*. In contrast to previous studies (Dagutat, 1995; Kruger, 1998), none of the indigenous isolates showed similarity to *S. meliloti* (clusters 18 and 21a).

Based on their protein profiles alone, some of the isolates are clearly differentiated and cannot be assigned to any of the previously recognized species. The taxonomic position

and identity of these isolates that clustered in groups linked to known species or in distinct groups with no specific relationship should be clarified by further genotypic studies. Some of these isolates may represent new species within the rhizobial group or may belong to taxa outside this group (for example isolate 20BGVno1).

The 16S rDNA sequencing analysis gave results comparable to those of the SDS-PAGE analysis: Most of the sequenced isolates were resolved within the bradyrhizobial branch of the phylogenetic tree, whilst a few were resolved within the branches containing the fast-growing rhizobial genera. The relationships within the *Bradyrhizobium* branch containing *B. japonicum* and *B. liaoningense* will have to be resolved using RFLP analysis. It was proposed that all species of *Agrobacterium*, as well as *Allorhizobium undicola*, be included in the genus *Rhizobium* (Young *et al.*, 2001) and therefore the specific relationships within the *Agrobacterium-Allorhizobium-Rhizobium* branch of the phylogenetic tree should be resolved using additional techniques. Isolate 20BGVno1 (*A. dealbata*), belonging to the *Xanthomonas* lineage, may have infected the *Acacia* specimen from which it was isolated (though no *Acacia* spp. are reported as hosts for these pathogens), or may have been in a state of survival in the plant tissue. Originally, all bacteria considered capable of nodulating legumes were included within the α-subdivision of *Proteobacteria*. However, bacteria belonging to the genus *Burkholderia* within the β-subdivision of *Proteobacteria* are also capable of nodulating leguminous plants (M. M. Kock, unpublished data; M. Lindeque, unpublished data; Moulin *et al.*, 2001). Therefore, the possibility of isolate 20BGVno1 being capable of nodulating legumes should not be ignored and should be investigated in future studies.

The comparison of the SDS-PAGE and 16S rDNA sequencing data confirmed that identification above the species level is not possible based on SDS-PAGE data alone: clusters containing potential *Bradyrhizobium* strains can be found among clusters containing only *Rhizobium* species (Dupuy *et al.*, 1994) and in some clusters a high similarity was observed between strains belonging to different genera (clusters 2a, 2b and 7a). SDS-PAGE is therefore only useful for the initial grouping of large numbers of highly related strains into clusters having potential species status. Genotypic data,

including chromosomal DNA-DNA hybridizations, DNA-rRNA hybridizations and rDNA sequencing, are necessary to determine the taxonomic status of the different clusters (Dupuy *et al.*, 1994; Moreira *et al.*, 1993).

Although both fast- and slow-growing strains were isolated from the three studied *Acacia* spp., they exhibited a predominant association with the slow-growing strains of the genus *Bradyrhizobium*. Ideally, plants used to trap rhizobia for application in the inoculant industry should be promiscuous and nodulated by a wide range of rhizobial strains. Therefore the legume species *A. mearnsii*, *A. dealbata* and *A. decurrens* would not play a significant role to trap rhizobia for application in the inoculant industry in South Africa, but nonetheless should not be ignored as trap plants.

The establishment of successful plantations of *A. mearnsii*, an important commercial tree in South Africa, is often limited by marginal growing conditions (Yobo, 1998). The collection of strains obtained in this study would provide an invaluable source of potential inoculant strains for *A. mearnsii* which might be better adapted to the local climatic and soil conditions. However, these strains will also be tested with regard to their symbiotic effectiveness with agriculturally important legumes. In South Africa there is especially a need for better inoculant strains for beans (*Phaseolus* spp.) (Ian Law, personal communication), leading to collaborations between SA and INRA-Laboratoire de Microbiologie des Sols (France), as well as between SA and the group of Monique Gillis (State University Gent, Belgium). Presently strains of *R. leguminosarum* bv *trifolii* are used as inoculants for clover (*Trifolium* spp.), *R. leguminosarum* bv *viciae* strains as inoculants for peas (*Pisum* spp.) and vetch (*Vicia* spp.), *R. leguminosarum* bv *phaseoli* strains as inoculants for beans (*Phaseolus* spp.) and *B. japonicum* strains as inoculants for soy bean (*Glycine max*). *B. liaoningense*, *B. elkanii* and *S. fredii* also nodulate soy bean and may be considered as inoculant strains for this legume species. The same applies to *R. etli* bv *phaseoli*, *R. gallicum*, *R. giardinii* and *R. tropici*, which may be considered as inoculant strains for bean. In the SDS-PAGE analysis several strains exhibited 90% or more similarity to the *B. japonicum* (clusters 1a and 1b), *B. elkanii* (cluster 13a) and *R. leguminosarum* bv *trifolii* (cluster 2a) reference strains. These strains will receive special

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consideration when screening the collection for potential inoculant strains for soybean and clover.

CHAPTER 5

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CHAPTER 5
CONCLUDING REMARKS

SDS-PAGE analysis of whole cell proteins is a useful tool for the initial grouping of a large collection of closely related strains into groups having potential species status and provides differentiation at intraspecies level. SDS-PAGE data should not, however, be used alone, but should rather form part of a polyphasic approach in order to make useful conclusions regarding the strains investigated. SDS-PAGE data should especially be supplemented by genotypic data to determine the exact taxonomic status of the different SDS-PAGE clusters above the species level.

More than one rhizobial type can occupy the same nodule.

Both fast-and slow-growing rhizobial strains were isolated from *A. mearnsii*, *A. dealbata* and *A. decurrens*.

The majority of the isolates associated with the exotic Australian *Acacia* spp. were slow-growing strains belonging to the genus *Bradyrhizobium*. Some of the other isolates exhibited close relationships to the genera *Agrobacterium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*.

Isolates related to the genera *Agrobacterium* and *Xanthomonas* should especially be investigated regarding their nodulation status in order to determine whether microorganisms other than rhizobia may nodulate *Acacia* spp.

The taxonomic position and identity of the isolates that were clearly differentiated and could not be assigned to any of the previously recognised species based on the protein data alone should be clarified by further genotypic studies.

The legume species *A. mearnsii*, *A. dealbata* and *A. decurrens* would not play a significant role to trap rhizobia for application in the inoculant industry in South Africa.

From the large collection of indigenous rhizobia obtained in this study, there may be some strains that can play an important role to improve nitrogen fixation in South Africa, especially with regard to the commercial cultivation of *A. mearnsii*.

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APPENDIX

APPENDIX

The three Australian *Acacia* species, *A. mearnsii*, *A. dealbata* and *A. decurrens*, are commonly known in South Africa as black wattles (or tan wattles), silver wattles and green wattles, respectively. These *Acacia* spp. are sometimes confused, but in most cases they can be distinguished by the colour of their leaves and branchlets, as well as in the details of their leaves. Therefore it was thought appropriate to include an appendix containing descriptive information of each.

A.1 *Acacia mearnsii* De Wild (Black wattle)

The general assumption is that *A. mearnsii*, which is indigenous to south-eastern Australia, was introduced in South Africa in 1864. However, records indicate that this species occurred in the Cape Town Botanical Gardens as early as 1858. It was originally introduced to Natal for its shade and as firewood, but by 1880 it was cultivated in commercial plantations for its bark. From these plantations it spread to disturbed areas, such as overgrazed and burnt areas. The black wattle is a widespread plant invader in South Africa, especially near watercourses, and also invades grassland, forest gaps and roadsides. It grows well in deep, well-drained soil in regions with a high rainfall, but may also grow in shallower soil types if enough water is available. Thus, although this species is commercially very important, it is an undesirable aggressive invader when occurring in regions other than commercial plantations and should not be planted without great deliberation. (Henderson, 1995; Poynton, 1973, 1975; Stirton, 1978)

Black wattles are unarmed, evergreen trees growing five to ten metres high, but may grow as high as 15 to 30 m. Branchlets are shallowly ridged and slightly hairy with characteristic yellow, redbrown or golden, hairy growth tips. The leaves are dark olive green, finely hairy and bipinnate with crowded, short (1.5 to 4 mm) leaflets (Figure A.1). In addition to the several raised glands on the upper surface of the leaf mid-rib at the junctions of pinnae pairs, the leaf of the black wattle also has one or more glands between

the junction of each pinnae pair (Figure A.1). The trees flower between August and November, up to December, carrying strongly scented, pale yellow or cream flowers in globular flowerheads that are grouped together at the tips of the branches. The dark brown pods are flat, finely hairy and usually markedly constricted between the seeds (Figure A.1). The seeds mature within approximately 14 months, but may take up to 15 months. (Henderson, 1995; Henderson and Musil, 1987; Moll and Scott, 1981; Poynton, 1973, 1975; Stirton, 1978)

A.2 *Acacia dealbata* Link (Silver wattle)

A. dealbata originates from Australia and is cultivated for shelter, shade and firewood. However, this species is also considered a declared weed and dangerous plant invader when occurring in areas other than commercial plantations and invades grassland, roadsides and watercourses. (Henderson, 1995)

Silver wattles are unarmed, evergreen trees that usually grow five to ten metres high, but may grow as high as 15 m. Branchlets are shallowly ridged, often tinged grey or purple with growth tips that are hairy, initially yellow turning greyish or white. The leaves are silver-grey to light/dull green, finely hairy and bipinnate (Figure A.1). The crowded leaflets are short (two to five and a half millimetres) and a raised gland occurs at each junction of pinnae pairs on the mid-rib of the leaf (Figure A.1). The trees flower between July and August, carrying pale to bright yellow flowers in globular flowerheads that are grouped together at the tips of the branches. The greyish or purplish-brown pods are flat and not or only slightly constricted between the seeds (Figure A.1). (Henderson, 1995; Henderson and Musil, 1987)

A.3 *Acacia decurrens* (Wendl.) Willd. (Green wattle)

A. decurrens is indigenous to East Australia and was introduced to South Africa for its bark. Today, however, it is cultivated only for shelter, shade and as ornaments, mainly in cool, moderately humid regions. This species also invades grassland, roadsides and

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watercourses when occurring in areas other than commercial plantations. (Henderson, 1995; Poynton, 1973, 1975)

Green wattles are unarmed, evergreen trees growing five to ten metres high, but may grow as high as 15 to 30 m. These trees are devoid of hairs or powdery bloom. Branchlets are green and prominently angled with wing-like ridges (Figure A.1). The leaves are bright green, devoid of hairs, bipinnate and feathery (Figure A.1). *A. decurrens* is readily distinguished from *A. mearnsii* and *A. dealbata* by its longer (six to 15 mm), slender and more openly arranged bright green leaflets. A single raised gland occurs at each junction of pinnae pairs on the upper surface of the mid-rib of the leaf (Figure A.1). The trees flower between July and August, carrying bright yellow flowers in globular flowerheads, which are grouped together at the tips or axils of the branches. The dark brown pods are flat, devoid of hairs, slightly constricted and approximately seven to ten centimeters in length (Figure A.1). The seeds mature within approximately four to five months. (Henderson, 1995; Henderson and Musil, 1987; Poynton, 1973, 1975)

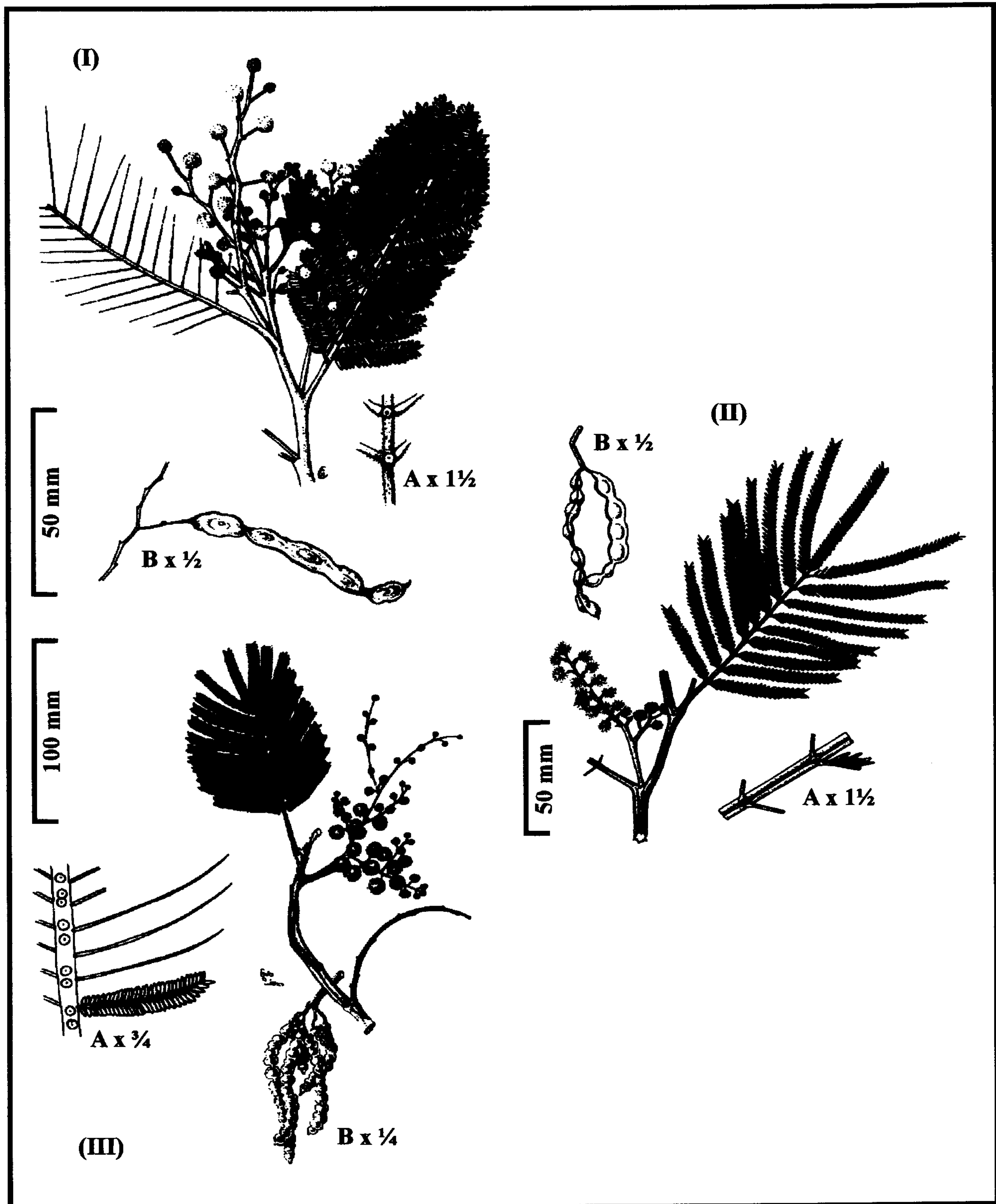

Figure A.1 (I) *Acacia dealbata* Link. (II) *Acacia decurrens* (Wendl.) Willd. (III) *Acacia mearnsii* De Wild. A: Sketch indicating position(s) of gland(s) on mid-rib of leaf. B: Pod(s). A scale is given for the main drawing. Dissections have the magnification marked on them. (Henderson, 1995)

A.4 References

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