Diversity of root nodule bacteria associated with *Phaseolus coccineus* and *Phaseolus vulgaris* species in South Africa

by

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SUMMARY

The association between root-nodulating bacteria and leguminous plants is advantageous due to their ability to alter atmospheric nitrogen into a useful form in a process known as biological nitrogen fixation (BNF). Research has shown that BNF is the most efficient way to supply the large amounts of nitrogen needed by plants to produce high-yielding crops. As a result, there have been numerous studies into the diversity and identity of the associated nitrogen-fixing bacterial symbionts. Recent advances in molecular microbiology together with the isolation of rhizobia from previously uninvestigated legumes have led to major revisions of rhizobial taxonomy, most notably the inclusion of bacteria from the ß-*Proteobacteria* in the genera *Burkholderia* and *Ralstonia*.

In this study, the diversity of root nodule bacteria associated with *Phaseolus coccineus* and *Phaseolus vulgaris* species in South Africa was investigated. A selection of rhizobial isolates were characterised by SDS-PAGE of whole cell proteins and rep-PCR DNA fingerprint analyses. These results were supplemented by partial 16S rDNA sequencing of a select number of isolates to confirm their identity. Where isolates displayed unexpected genus associations, partial *nodA*

sequencing was performed to determine whether these were incidental contaminants or true nodulators.

Based on 16S rDNA sequence analysis, the majority of isolates investigated were fast-growers belonging to the genus *Rhizobium*. A few isolates showed close relationship to species of the ß-*Proteobacteria* genus, *Burkholderia*. Both the SDS-PAGE analyses and the combined rep-PCR analyses were able to resolve isolates down to strain level, but the comparison of the SDS-PAGE and 16S rDNA sequencing data confirmed that bacterial discrimination using SDS-PAGE is not useful at the genus level and higher, as isolates showing affinity to *Burkholderia* were mingled with isolates showing similarity to *Rhizobium*. These isolates were separate from the *Rhizobium* isolates in the combined rep-PCR dendrogram. While there were discrepancies between results obtained from SDS-PAGE and rep-PCR analyses, results from the combined rep-PCR analysis correlated with many of the results obtained in the SDS-PAGE analysis. Both geographic location and host plant species appear to have affected the grouping of isolates. Many clusters consisted of isolates from the same location or the same host plant species in both the SDS-PAGE dendrogram as well as the combined rep-PCR dendrogram. The *nodA* sequencing demonstrated that the majority of isolates tested contain the *nodA* gene indicating that they are capable of nodulation. There was a large strain diversity observed for the isolates of this study and a number of the root-nodulating bacteria of the *Phaseolus* spp. appear to constitute several novel nodulating genotypes.

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
ClustalX	cluster analysis version X
DAF	DNA amplification fingerprinting
°C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5′-triphosphate
EDTA	ethylenediaminetetra-acetate
ERIC-PCR	enterobacterial repetitive intergentic consensus-PCR
g	grams
G + C content	mole percentage guanine and cytosine content
Ha	hectare
h	hour
ITS	internally transcribed spacer region
Kb	kilobase pairs
LMG	Laboratorium voor Microbiologie Gent Culture Collection
mg	milligrams
$mg.kg^{-1}$	milligrams per kilogram
µl	microlitre
ml	milliliter
mS/cm	millisiemans per centimetre (measure of conductivity)
MLEE	multilocus enzyme electrophoresis
mM	millimolar
MPN	most probable number
m/v	mass per volume
N_2	dinitrogen

NaOAc	sodium acetate
Nod	nodulation
NCBI	National Centre for Biotechnology Information
NFU	Nitrogen Fixation Unit
OD	optical density
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
RAPD	randomly amplified polymorphic DNA analysis
rDNA	ribosomal DNA
rep-PCR	repetitive sequence-based PCR (BOX-, ERIC-, and REP-PCR)
REP-PCR	repetitive extragenic palindromic PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
(*r*)	Pearson product moment correlation coefficient
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sp.	species
spp.	species (plural)
STB	sample treatment buffer
STM	Laboratoire des Symbioses Tropicales et Méditerranéennes
TEMED	N,N,N',N'-tetramethylethylene diamine
TY	tryptone yeast extract medium
TYB	tryptone yeast extract broth
UHQ	ultra high quality
UPGMA	unweighted pair group method of arithmetic averages
USDA	United States Department of Agriculture – ARS National Rhizobium Culture Collection
UV	ultraviolet
V	volume
v/v	volume per volume
W	watts
YMA	yeast extract mannitol agar
YMB	yeast extract mannitol broth

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CHAPTER 1

INTRODUCTION

The earth's population grows annually by 1.4% and is expected to double in the next fifty years (Graham and Vance, 2000). This increase in population necessitates a simultaneous increase in food production to maintain the dietary intake of the growing human population in an environmentally sustainable manner. This demand for higher crop production also implies a higher demand for fixed nitrogen (Graham and Vance, 2000). Chemically produced nitrogen fertilizers can provide this nitrogen, but they are expensive to produce in addition to being harmful to the environment. This damage to the environment includes changes in the global nitrogen cycle, loss of nitrous oxides to the atmosphere, acid rain, nitrate pollution of ground water and induced leaching of soil nutrients (Vilousek *et al*., 1997). An inexpensive and environmentally friendly alternative to nitrogen fertilizer is biological nitrogen fixation (BNF), which is a process whereby nitrogen gas in the atmosphere is converted into biologically useful and utilizable source of nitrogen for plants (Zahran, 1999). The majority of the world's land-based biological nitrogen fixation can be accounted for by the symbiotic nitrogen fixation relationship between leguminous plants and rhizobia. The advantages of this type of BNF have led to numerous studies investigating the diversity and identity of the associated bacterial symbionts.

Rhizobial taxonomy was originally based on the specificity of symbiotic plant range of bacterial strains and species, but it has since been found that this is an unreliable taxonomic measure (Martinez-Romero and Palacios, 1990) and a combination of phenotypic, genotypic and phylogenetic techniques should rather be used. Several new symbiotic associations with legumes have been recently discovered. Sy *et al*. (2001) described *Methylobacterium nodulans* for methylotrophic root-nodulating bacteria associated with *Crotalaria* spp. The root-nodulating strain *Burkholderia tuberum*, isolated from the South African legume *Aspalathus carnosa*, belongs to the ß-subclass of the *Proteobacteria* (Moulin *et al*., 2001; Vandamme *et al*., 2002).

The identification of these 'novel rhizobia' demonstrates that the ability of bacteria to establish a symbiosis with legumes is more widespread than previously thought.

There are thousands of species of legume, but common beans (*Phaseolus vulgaris*) are the most frequently consumed legumes worldwide. *Phaseolus* species (beans) are dominant in the staple diets of lower income groups due to their high protein content (Broughton *et al*., 2003). They are nutritious and easy to grow and while *Phaseolus* spp. are not indigenous to South Africa, they have been increasingly cultivated in South Africa on a commercial scale as well as by peasant farmers (Strijdom, 1998). Studies on the nodulation status of local legumes only began in the second half of the last century (Strijdom, 1998). A comprehensive research programme aimed at the utilization of the rhizobia-legume symbiosis was initiated at the ARC-Plant Protection Research Institute in South Africa.

The root nodules of *Phaseolus* plants were collected from areas of bean production in South Africa and indigenous rhizobia were isolated from these root nodules. The bacterial strains used in this study were characterized using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) together with combined repetitive sequence-based DNA fingerprinting. These were supplemented with partial 16S rDNA sequencing of selected isolates.

This study was conducted to broaden our understanding and knowledge of the *Phaseolus*-rhizobia association, ultimately leading to the development of better inoculant strains. The aim of this study was to determine the diversity of the root-nodulating bacteria associated with *Phaseolus* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Symbiotic nitrogen-fixing, gram-negative bacteria, collectively referred to as rhizobia, have the ability to invade the roots of leguminous plants (Spaink, 1989). The rhizobia infect the plant roots and induce the formation of plant membrane structures called nodules. These nodules usually develop on the plant roots, but may form on the stem. As the nodules are so readily identifiable, rhizobia have been studied and classified since the dawn of bacteriology (Broughton, 2003).

Rhizobia are facultative microsymbionts that can live either as members of the natural soil microbial community or symbiotically in the root nodules of the host legume. Most of these bacteria normally occur on the rhizosphere (root surface) where large numbers of rhizobia can be found as a result of the nutrients released by the plant (van Berkum and Eardley, 1998). It is these valuable root bacteria that are of interest, due to their ability to alter atmospheric nitrogen into a useful form in symbiosis with legume plants, in a process known as biological nitrogen fixation (BNF).

Legume seeds are two to three times richer in protein than cereal grains due to their ability to produce useable nitrogen in symbiosis with rhizobia. They account for approximately 20% of global food production (Broughton *et al*., 2003). Common bean (*Phaseolus vulgaris*) is one of the most important legumes grown for human consumption. Half of the grain legumes consumed worldwide are beans and in poorer countries, beans are often the primary source of protein in human diets (Broughton *et al*., 2003).

The root-nodulating bacteria were originally all placed together in the family *Rhizobiaceae* in the α-subgroup of the *Proteobacteria* (Jordan, 1984). The family consisted of the genera *Rhizobium,*

Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and the slow-growing *Bradyrhizobium.* However, improved methods for identification and classification have led to significant changes in the taxonomy of these bacteria (Young, 1996). The diversity of these symbiotic, nitrogen-fixing bacteria is far greater than previously thought. The ability to nodulate and fix nitrogen is not restricted to members of the family *Rhizobiaceae,* nor are all root-nodulating bacteria found in the α-*Proteobacteria. Rhizobium*, *Allorhizobium* and *Sinorhizobium* remain in the family *Rhizobiaceae*, while *Mesorhizobium* is grouped in the family 'Phyllobacteriaceae', and the *Bradyrhizobium* are placed in the family 'Bradyrhizobiaceae'. Bacterial genera belonging to the β-subclass of the *Proteobacteria*, such as *Burkholderia* and *Ralstonia* have been identified as root nodule-associated bacteria capable of forming successful symbiosis with their leguminous hosts (Moulin *et al*., 2001; Chen *et al*., 2001) and recently Benhizia *et al*. (2004) described symbiotic, root-nodulating bacteria found in the γ-subclass of the *Proteobacteria*. Symbiotic, root-nodulating, nitrogen-fixing bacteria will subsequently be referred to as rhizobia. This includes the traditionally defined root-nodulators, as well as the more recently described entrants to the rhizobial taxonomy, the novel nodulators.

The taxonomy of the root-nodulating bacteria has experienced a substantial series of extensions in the recent past. The development of techniques based on the microbial genotype or DNA sequence has greatly improved our understanding of the rhizobia and the application of polyphasic taxonomy has resulted in major improvements and revisions of legume root-nodulating bacteria classification.

2.2 Biological nitrogen fixation

Nitrogen is a crucial mineral element required for the sustenance of life; it is the soil nutrient element needed in greatest quantity by crops. It comprises up to 80% of the atmosphere, yet in nature very little of this nitrogen is available in mineral form and plants are unable to make use of atmospheric nitrogen. Biological nitrogen fixation is the process whereby the stable nitrogen gas in the atmosphere (N_2) is converted into ammonia (NH_4^+), a biologically useful and utilizable source of nitrogen for plants (Zahran, 1999). Biological nitrogen fixation is performed

exclusively by prokaryotes that possess the enzyme nitrogenase. Rhizobia can fix atmospheric nitrogen in symbiosis with plants.

Nitrogen fixation is second only to photosynthesis, in terms of importance for the growth and development of plants. It is estimated, on a global scale, that biological nitrogen fixation may reach 175 million metric tons of nitrogen fixed per year, approximately 90 million tonnes from agricultural areas and 50 million tonnes from undeveloped land and forests (Bezdicek and Kennedy, 1988). The transformations of nitrogen are not exclusively biological. The total nitrogen fixed biologically per year is estimated to be twice that of the total nitrogen fixation by non-biological processes (Bezdicek and Kennedy, 1988). Nitrogen in the soil originates from decomposing organic matter and from nitrogen introduced by lightning. Lightning probably accounts for approximately 10% of the world's supply of fixed nitrogen, but this is generally insufficient for extensive crop production. (Zahran, 1999).

Soil nitrogen deficiency may result in poor yields or failed crops and has traditionally been overcome by applying fertilizers, but this is expensive and harmful to the environment. The annual worldwide expenditure for nitrogen fertilizers currently exceeds 20 billion US dollars (Hardy, 1997). The production of nitrogen fertilizer requires high-energy inputs and makes use of natural gas. These fossil fuels comprise 70% - 90% of the cost of producing nitrogen fertilizers and as they become ever scarcer; their cost will continue to increase.

Environmental concerns for using nitrogen fertilizers include changes in the global nitrogen cycle, loss of nitrous oxides to the atmosphere, acid rain, nitrate pollution of ground water and induced leaching of soil nutrients. (Kinzig and Socolow, 1994; Vilousek *et al*., 1997). The input of nitrogen through biological nitrogen fixation allows for increased soil fertility, which helps to maintain soil nitrogen reserves, thus eliminating the need for nitrogenous fertilizers (Graham and Vance, 2000).

2.3 The root nodule

The nodulation process involves plant signals that activate specific bacterial genes, which leads to the production of bacterial proteins that signals plant morphogenesis. This ability involves unique properties of the host plant (Gualtieri and Bisseling, 2000). Figure 2.1 is a diagram of the process of root nodulation.

Legumes release many different compounds into the rhizosphere. Rhizobia recognise the flavonoid compounds (2-phenyl-1, 4-benzopyrone derivatives) present in the plant exudates, which induce the expression of the genes for nodulation. The rhizobial nodulation genes produce lipo-chitooligosaccharide signals or Nod-factors [Figure 2.1 (i)]. The Nod-factors elicit nodule formation in the host plant by inducing the root hair to branch, deform and curl [Figure 2.1 (ii)] preventing further cell growth. The curled root hairs trap the bacterial cells in a pocket of the host cell wall. The bacteria enter the roots at the sites where the root hair cell walls are hydrolysed and penetrate through an invagination of the plasma membrane [Figure 2.1 (iii)]. The plant host reacts by depositing new cell wall material around the lesion in the form of an inwardly growing tube, which becomes an infection thread (Vanderleyden and Van Rhijn, 1995; Hirsch and LaRue, 1997; Gage and Margolin, 2000; Gualtieri and Bisseling, 2000). The rhizobia penetrate the root tissue via infection threads. At the same time, a nodule primordium is produced through cell division in the outer or inner root cortex. Within the growing nodule, the bacteria are released from the infection threads into the host cytoplasm. Once the bacteria have entered the plant roots and infected primordial cells, they differentiate into bacteroids and the nitrogen-fixing nodules develop (Gualtieri and Bisseling, 2000) [Figure 2.1 (iv)]. The bacteria are hosted in the nodules, where they make the nitrogenase enzyme (Gualtieri and Bisseling, 2000). The modified bacteroids fix atmospheric nitrogen to ammonia, which is then used by the plant. (Somasegaran and Hoben, 1994).

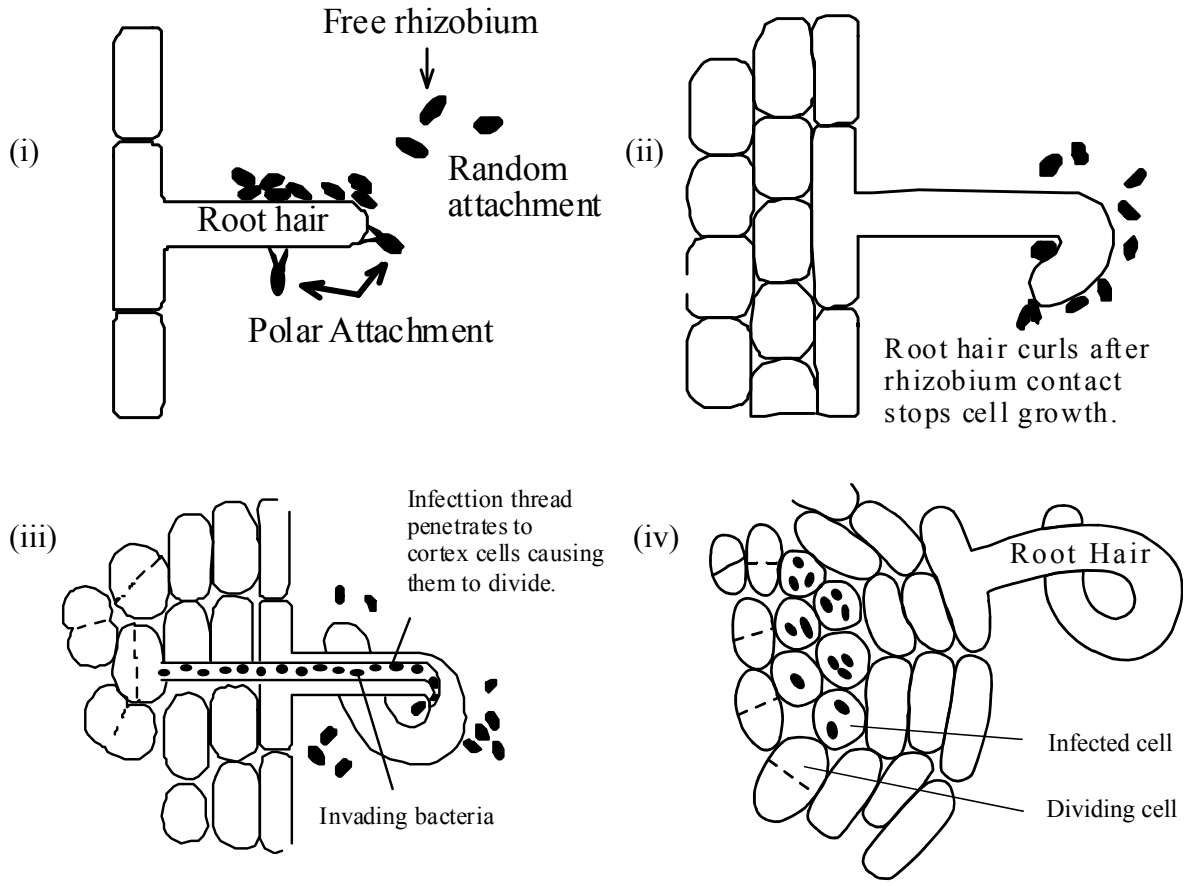


Figure 2.1 A stepwise representation of the nodulation process (Nitrogen fixation - The nodulation process, 2003). Chemical recognition between the host plant and rhizobia leads to attachment and the root hairs curl. This results in the formation of infection threads and invasion of the roots by rhizobia. Bacteria divide and convert to bacteroids and begin to form the nitrogenase enzyme, which enables the fixation of atmospheric nitrogen

2.3.1 Plant signals

Many plant genes are involved in nodule functioning (Schultze and Kondorosi, 1998). The shape of a nodule is determined by the host plant and not by the rhizobial bacteria. This supports the belief that the host plant possesses the genetic information for symbiotic infection and nodulation, while the role of the bacteria is to switch these host plant genes on (Vanderleyden and Van Rhijn, 1995).

(i) Flavonoids

Flavonoids are found throughout the plant kingdom, but in nodulating plants, they specifically trigger the expression of the rhizobial genes required for nodulation. Plants discard large amounts of organic matter into the soil, most of which supports the growth of rhizosphere micro-organisms. These compounds include carbohydrates, organic acids, vitamins, amino acids and flavonoids. Rhizobia respond to these plant-derived flavonoids secreted by compatible host plants. The flavonoids are the most important feature in symbiotic interactions; without them nodulation would not be initiated (Perret *et al*., 2000).

2.3.2 Bacterial signals

(i) The nodulation genes

The plant flavonoids induce the nodulation (*nod*) genes of the bacteria. These *nod* genes are responsible for the synthesis of the nodulation factors, which are involved in the establishment of the symbiotic relationship with the legume host (Perret *et al*., 2000). The common *nod* genes (*nodABC)* are all functionally similar among rhizobial species. These genes are involved in the very early stages of symbiosis and all three genes are essential for nodulation (Triplett and Sadowsky, 1992). The common genes are involved in the formation of the lipo-chitooligosaccharide backbone, while the host-specific genes are involved in the addition of specific substitutions (Perret *et al*., 2000; Zhang *et al*., 2000). However, it has been shown that *nodA* and *nodC* are also host-specific genes, since NodC determines the length of the Nod-factor, while NodA recognises and transfers different acyl chains to the lipo-chitooligosaccharide backbone (Perret *et al*., 2000). The *nodA* gene has only been found in rhizobia and is therefore a good indication of whether an isolate is capable of nodulation (Hirsch *et al*., 2001). The *nod*D gene is the bacterial transcriptional activator, present in all rhizobia and required to induce expression of the *nod* genes (van Rhijn *et al*., 1994). The *nod* genes are organized in several operons, located on the chromosome or on large symbiotic (sym) plasmids (Triplett and Sadowsky, 1992).

Three types of nodulation loci have been identified in rhizobia: the common nod genes, host-specific nod genes (hsn) and the genotype- or cultivar-specific nod genes. (GSN or CSN). The gene cluster involved in host-specific nodulation has been named *hsnABCD*, but was previously known as *nodFEGH*. Host-specific nodulation genes are needed for the nodulation of specific host plants in different legume genera. Genotype-specific nodulation genes are bacterial sequences that allow nodulation of specific plant genotypes within a given legume species. If the plant genotype is a cultivated variety, the genes are referred to as cultivar-specific nodulation determinants. The GSN and CSN genes can affect nodulation competitiveness of an organism by inhibiting or eliminating nodulation ability (Triplett and Sadowsky, 1992).

(ii) Nod factors

The nodulation (Nod) factors are the return signals produced and secreted by the rhizobial *nod* genes in response to the plant flavonoids. The Nod factors are essential for the rhizobia to elicit the plants responses and recognition by specific legume hosts, to allow entry of the bacteria into the root (Gualtieri and Bisseling *et al*., 2000). Each rhizobial strain produces a characteristic spectrum of Nod factors that is generally unique for a given isolate (Schultze and Kondorosi, 1998). Nod factors are the signals required for the entry of rhizobia into the leguminous plants; they act like 'keys' for the invading rhizobia to the legumes root hair 'doors'. After entry of the rhizobia, additional signals, or 'keys', are necessary for later steps of the infection process (Perret *et al*., 2000).

2.3.3 Control of nodule development

The control of nodule development involves positively as well as negatively acting plant genetic factors. The legumes can sense the amount of external nitrogen and thus regulate the symbiotic process with the rhizobia (Caetano-Anollés, 1997). The plant host limits the number of nodules and this regulation might be integrated in the mechanisms that control lateral root development (Stougaard, 2000). The expression of the nod genes is under negative control, since there is an optimal Nod-factor concentration for successful nodulation

2.3.4 Nodulation of *Phaseolus* species

The common bean is a selective host plant that is nodulated by a narrow range of rhizobia. These symbionts of bean include broad host range bacteria (such as *R.tropici*) and non-specific rhizobia ineffective in nitrogen fixation, capable of nodulating *P. vulgaris* (Mhamdi *et al*., 2002). The yield of bean crops is low and bean plants generally have a low nitrogen-fixing capacity compared to other legume plants. *P. vulgaris* is sensitive to Nod factors and nodulation occurs under conditions of low induction of the *nod* genes (van Rhijn *et al*., 1994). The Nod factor oligosaccharide backbone of bean-nodulating rhizobia requires a specific attached group, the acyl chain at the non-reducing end. NodS mediated decorations are essential for invasion and subsequent nitrogen fixation in rhizobium strains that elicit nitrogen-fixing nodules on *Phaseolus vulgaris* and they produce methylated Nod factors (Laeremans and Vanderleyden, 1998).

2.4 Host specificity

In most rhizobial species, the genes that determine the plant host specificity (the genes for symbiosis) are carried on symbiotic plasmids. Rhizobia strains carry other phenotypically cryptic plasmids (Amarger *et al*., 1996). The symbiotic interaction shows a high degree of specificity. Only certain combinations of host plants and effective rhizobia are compatible with each other to form a nitrogen-fixing symbiosis. Certain rhizobial isolates are capable of forming effective nodules on some host legumes (Nod^+, Fix^+) whilst forming ineffective nodules on others (Nod^+, Fix^-). Nod^+ indicates rhizobia capable of forming nodules on certain plants and Fix^+ indicates rhizobia capable of successful fixation of atmospheric nitrogen in symbiosis with a particular host plant. Specificity is not only confined to nodulation but also extends to the ability to form effective, nitrogen-fixing nodules. Specificity among compatible partners minimizes the chance of pathogen infection and the formation of ineffective associations (Perret *et al*., 2000). Various nod gene inducers, Nod factors and polysaccharides are all involved in determining host specificity. There appears to be communication between the symbiotic partners, leading to their recognition of each other (Schultze and Kondorosi, 1998).

No strict connection can be drawn between the types of lipochito-oligosaccharides produced by the rhizobia and the plants they nodulate. The Nod factors produced by *R. etli* and *M. loti* are identical, but they have distinct and separate host ranges, *Phaseolus* spp. and *Lotus* spp. respectively. Two rhizobia that nodulate the same plant may secrete different Nod factors: *R. tropici* and *R. etli* produce different Nod factors, but both effectively nodulate *P. vulgaris*. Similarly, *B. elkanii* and *B. japonicum* have a number of common hosts, but their Nod factors vary considerably. Nod factor structure alone cannot be used to predict host range. The amounts of Nod factors released by rhizobia are important in determining the host range (Perret *et al*., 2000).

2.5 Inoculation

The symbiotic relationship between rhizobia and legumes is agriculturally important as the input of nitrogen through biological nitrogen fixation increases soil fertility, whilst decreasing the need for nitrogen fertilizers (Niemann *et al*., 1997). Where effective strains of rhizobia are not present in the soil, they can be supplied by inoculation of the seed with selected strains. Inoculation of legume seed with root-nodule bacteria can result in a large benefit-cost ratio as the cost of inoculant is only about 1% of the total cost of input (Hardy, 1997).

It is necessary to apply commercial inoculants to nitrogen deficient fields especially when the indigenous rhizobial population is limited or has a poor nitrogen-fixing ability (Barran *et al*., 1991). Availability of suitable inocula is often a limiting factor to successful cropping in many regions of the world. The number of rhizobia added to the legume seed and the number of indigenous rhizobia capable of nodulating the host influences the inoculant success, as does the specificity of the host plant and environmental conditions.

Inadequate information regarding the rhizobia indigenous to South Africa lead to the establishment of a long-term project aimed at determining the status of indigenous rhizobia and providing a culture collection of potential inoculant strains (Dagutat, 1995). Imported inoculants are often unsatisfactory, therefore it is necessary to characterise the indigenous rhizobia, as well as to establish their host ranges and factors that influence their distribution in order to provide

inoculant strains that are better adapted to local soil and environmental conditions. (Strijdom, 1998). In South Africa particularly, there is a need for better inoculant strains for beans (*Phaseolus* spp.) (I.J. Law, personal communication). Although *Rhizobium* inoculant is available in South Africa, nodulation is erratic. Two inoculants that have been used in South Africa for the inoculation for *Phaseolus* spp. are UD2 and UMR1899. UD2 is a wide-spectrum *Rhizobium* strain used to inoculate *Phaseolus* species in South Africa. However, previous inoculation experiments with UD2 gave erratic results (Strijdom, 1998).

2.6 The rhizobia-legume symbiosis

The rhizobia-legume symbiosis is of great agricultural and economic value (Niemann *et al*., 1997) and is better than the application of fertilizer nitrogen for ensuring a steady supply of nitrogen for legume-based crop production. The symbiotic relationship between legumes and rhizobia is advantageous for both the plants and the microbes. The rhizobia are protected from competition from other soil micro-organisms and are provided with nutrients, the plant supplies the carbon source for and protects the oxygen-sensitive nitrogenase enzyme of the bacterial symbiont and supplies the energy required for the biological nitrogen fixation, while the plants receive their nitrogen requirements from the bacteria (de Philip and Boistard, 1992).

Studies on the nodulation status of local legumes only really began in the second half of the last century (Strijdom, 1998). A comprehensive research programme aimed at the utilization of the rhizobia-legume symbiosis was initiated at the Plant Protection Research Institute in South Africa, where a unit for biological nitrogen fixation, the nitrogen fixation unit (NFU), was established in 1965.

Between 1964 and 1983 a systematic survey of the reported 1350 – 1400 indigenous leguminous species (100 genera) occurring in South Africa was undertaken to determine their nodulation status (Strijdom, 1998). Lists containing more than a 1000 species of plants and their nodulation status were compiled. Unfortunately the rhizobia responsible for the nodulation of the various legumes were not isolated. Such a collection of strains would have been invaluable for taxonomic studies of the nodule bacteria currently being undertaken worldwide.

The study of the indigenous rhizobia bacterial diversity is important to improve our knowledge of South African root-nodulating bacteria (Dagutat, 1995). Recent studies on rhizobia associated with leguminous plants in South Africa have included phenotypic characteristics (Dagutat, 1995; Kruger, 1998; Joubert, 2002; Le Roux, 2002), genotypic analyses (Kock, 2004) including molecular characterisation of the existing culture collection (Jaftha, 2002) and the study of phylogenetic traits (Kruger, 1998; Kock, 2004). These studies indicated that the rhizobial populations in South Africa are heterogeneous.

2.6.1 Plant symbiont: legumes

Leguminous plants are important crops as they have been used for centuries, both to supply food and for soil improvement. The use of legumes in agriculture can aid in controlling cereal crop diseases and pests and can enhance the productivity and sustainability of farming systems. The legumes are economically and ecologically significant plants; only the grasses are more agriculturally important to man than the legumes (Howieson *et al*., 2000).

2.6.1.1 The Family *Fabaceae*

The family *Fabaceae* is the third largest flowering plant family and one of the most widely distributed, occupying habitats ranging throughout the world from rain forests to arid zones. The name *Fabaceae* is the Latin name for broad bean. 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 (Doyle and Luckow, 2003).

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 approximately 20 000 species (Elkan, 1992). Molecular data shows that *Mimosoideae* and *Papilionoideae* appear to have monophyletic lineages, derived from a common ancestor, while *Caesalpinoideae* appears to have a paraphyletic lineage from several unrelated ancestors (Doyle and Luckow, 2003). Most of the genera of the subfamily *Papilionoideae* are nodulated (97% of those examined). This sub-family is the most diverse and the most economically important (Somasegaran and Hoben, 1994; van Berkum and Eardly, 1998). More

than 90% of the genera of the subfamily *Mimosoideae* are capable of forming symbiosis with rhizobia, while in the Caesalpinoideae mostly non-nodulating genera occur. Vanderleyden and Van Rhjin (1995) argued that since legumes are so diverse in morphology and ecology, symbiosis is not an adaptation to a specialised ecological niche, but is more dependent on a genetic peculiarity of legumes.

2.6.1.2 The genus *Phaseolus*

Beans (*Phaseolus* spp.) are one of the most ancient crops of the world. *Phaseolus* is the Latin name for bean, meaning 'small boat' referring to the pods of the plants. They are dominant in the staple diets of lower income groups in the Americas, Africa and Asia, together with maize. Beans are extremely diverse crops in terms of their uses, cultivation methods, and their morphological variability. Their value was realised as early as 2 300 years ago when Theophrastus (370 – 285 B.C.) wrote "...the bean best reinvigorates the soil. Beans are not a burdensome crop to the ground: they even seem to manure it, because the plant is of loose growth and rots easily" (Van Kessel and Reeves, 2000). The diversity of beans is also displayed in the range of environments to which they have adapted. They are found from sea level up to 3,000 metres above sea level and are cultivated in monoculture, in associations, or in rotations.

The earth's population increases annually by 1.4% and is expected to reach 8.3 billion by the year 2025 (Graham and Vance, 2000). Large increases in crop production will be needed to maintain the dietary intake of the growing human population without causing harm to humans or endangering the environment. Legumes are vital in agriculture due to their symbiosis with rhizobial bacteria and the resulting biological nitrogen fixation. Effectively, this nitrogen fixation amounts to internal fertilization and is the main reason that legumes are richer in proteins than all other plants (Broughton *et al*., 2003).

Beans are consumed as mature grain, as immature seed and also as a vegetable. Their pods can be obtained in as little as two months, and rotations are possible with other crops during short growing seasons as they are adapted to many niches, as well as inter-planting with other species until the main crop can be harvested. On subsistence farms, bean plants are maintained and

continually harvested for approximately six months as an assurance. Over the past twenty years, beans have also been increasingly cultivated on a commercial scale (Broughton *et al*., 2003). In South Africa, approximately 50 000 hectares is used for bean cultivation (Kipe-Nolt and Giller, 1993). The average amount of nitrogen fixed by common bean is approximately 50 Kg of nitrogen per hectare per year (Hardarson *et al*., 1993), which is relatively low compared to that of other legumes, such as Lucerne (250 $Kg.N.Ha^{-1}$ per year) or Leucaena (325 $Kg.N.Ha.^{-1}$ per year).

The diet of subsistence level farmers in Africa, Asia and Latin America often contains sufficient carbohydrates (through maize, rice and wheat), but is poor in proteins. Dietary proteins can take the form of animal products (eggs, milk, and meat), but these are scarce and are more likely to be sold as a source of income rather than consumed. Therefore their dietary proteins are usually derived from legumes. Thousands of legume species exist, but more of the Common beans (*Phaseolus vulgaris*) are being eaten worldwide than any other legume. Although not indigenous to South Africa, the widespread cultivation of beans in South Africa has led to established rhizobial populations (Strijdom, 1998).

2.6.2 Microbial symbiont: The rhizobia

The rhizobia are beneficial nodule-forming bacteria that are abundant in the soil. Rhizobia are gram negative, rod-shaped cells. They do not form endospores and are motile by two to six peritrichous flagella, or a single polar flagellum, for *Mesorhizobium* (Bergey's Manual Trust, 2001). These organisms are aerobic, chemo-organotrophs and are relatively easy to culture. They grow well in the presence of simple carbohydrates and amino compounds (Young *et al*., 2001).

The nomenclature of rhizobial species was originally based on the belief that a natural classification could be based on the specificity of symbiotic plant range of bacterial strains and species. It has since been found that the genes for nodulation, nitrogen fixation and host specificity are characteristics of strains carrying particular transmissible symbiotic (Sym) plasmids, and therefore are unreliable taxonomic measures (Martinez-Romero and Palacios, 1990). The use of both phenotypic (symbiotic, physiological and morphological) and

phylogenetic traits is minimal requirement for the description of new rhizobial species and genera (Graham *et al*., 1991).

2.6.3 The current taxonomy of the root-nodulating bacteria

Jordan (1984) originally included all known rhizobia in the family *Rhizobiaceae* in the α-2-subgroup of the *Proteobacteria.* At the time of their description, the genera *Rhizobium* (Jordan, 1984), *Bradyrhizobium* (Jordan, 1982), *Azorhizobium* (Dreyfus *et al*., 1988), *Sinorhizobium* (Chen *et al*., 1988; de Lajudie *et al*., 1994), *Mesorhizobium* (Jarvis *et al*., 1997), and *Allorhizobium* (de Lajudie *et al*., 1998a) were originally all included in the family *Rhizobiaceae*. Also included in the *Rhizobiaceae*, was the genus *Agrobacterium*, plant pathogens that are closely related to the genus *Rhizobium* (van Berkum and Eardly, 1998). The *Agrobacterium* do not form nitrogen fixing symbiotic root nodules and therefore are not rhizobia. Figure 2.2 shows the relatedness of these genera of the α-*Proteobacteria* based on 16S rDNA sequences.

The taxonomy of the rhizobia has changed significantly with the development of new techniques and the study of more diverse legumes. In terms of phylogenetic relationships from 16S rDNA analyses, the family *Rhizobiaceae* is considered to be relatively closely related to the families *Bartonellaceae, Brucellaceae* and *Phyllobacteriaceae* in the α-*Proteobacteria*. These families include pathogens and parasites of both animals and man (*Brucellaceae* and *Bartonellaceae)* [Young *et al*., 2001].

According to the new Bergey's Manual (Bergey's Manual Trust, 2001), rhizobia are now included in several different families. *Rhizobium*, *Allorhizobium* and *Sinorhizobium* are placed in the family *Rhizobiaceae*. *Mesorhizobium* is grouped in the family 'Phyllobacteriaceae', *Bradyrhizobium* are placed in the family 'Bradyrhizobiaceae', while *Azorhizobium* and the genus *Devosia* in which a newly nodulating species have been described belong to the family *Hyphomicrobiaceae* and the other nodulating species of the α-*Proteobacteria* belong to the genus *Methylobacterium* in the family 'Methylobacteriaceae'. However, Gaunt *et al*. (2001) found little support for this in their study of the α-*Proteobacteria*.

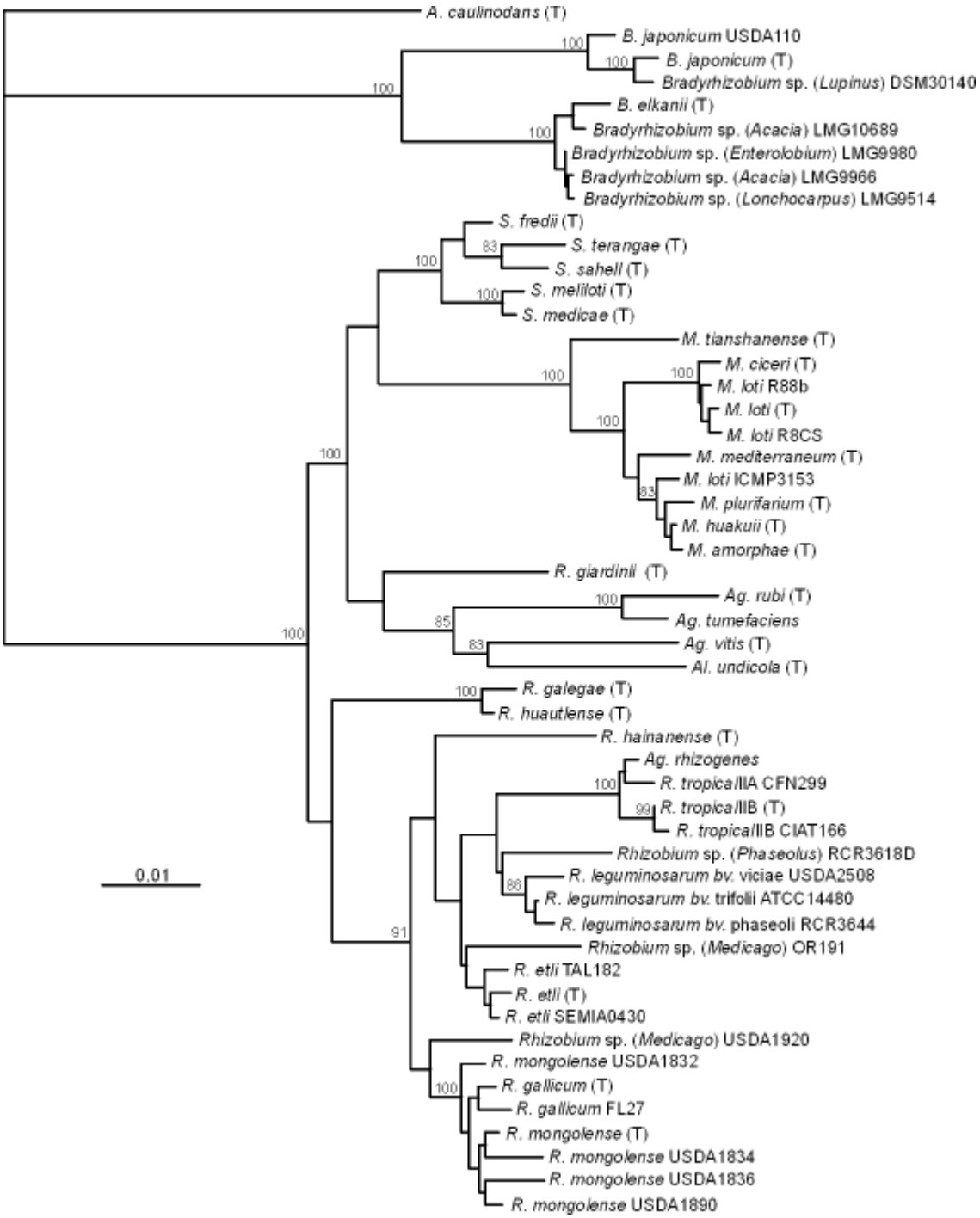


Figure 2.2 Neighbour-joining tree showing the phylogenetic relationships of rhizobia as determined by their 16S rRNA gene sequence. Branch lengths were proportional to the estimated number of nucleotide substitutions and bootstrap probability values were determined from 1 000 resamplings (Laguerre *et al*., 2001). The recently described novel genera including *Burkholderia*, *Ralstonia* and *Methylobacteria* were not included. *A. Azorhizobium*; *Ag. Agrobacterium*; *Al. Allorhizobium*; *B. Bradyrhizobium*; *M. Mesorhizobium*; *R. Rhizobium*; *S. Sinorhizobium*; (T) type strain.

Recently, it became clear that the ability to nodulate and fix nitrogen is not restricted to the α-*Proteobacteria,* but that several species in the β-*Proteobacteria* acquired the ability as well. Two genera involved, *Burkholderia* and *Ralstonia* belong to the families *Burkholderiaceae* and *Ralstoniaceae* respectively, in the order 'Burkholderiales' (Bergey's Manual Trust, 2001). Benhizia *et al*. (2004) found bacteria in the root nodules of Mediterranean wild legumes that appear to belong to the γ- *Proteobacteria.*

An overview of the currently accepted genera is included, with a focus on the symbionts associated with bean in section 2.6.4 and a section on the novel nodulators in section 2.6.6. Table 2.1 is a comprehensive summary listing the species names, host plants and references of the current root-nodule nitrogen fixers.

Table 2.1 Rhizobia and their principle legume host

Species	Host legume(s)	Reference
Rhizobium		Frank, 1889
R. leguminosarum [TS]		Jordan, 1984
• bv. viciae	*Pisum, Vicia, Lathyrus, Lens,*	Jordan, 1984
• bv. trifolii	*Trifolium* spp.	Jordan, 1984
• bv. phaseoli	*Phaseolus vulgaris*	Jordan, 1984
R. galegae		Lindström, 1989
• bv. officinalis	*Galega officinalis*	Radeva *et al.*, 2001
• bv. orientalis	*G.orientalis*	Radeva *et al.*, 2001
R. tropici	*Phaseolus vulgaris,* *Leucaena spp.*	Martinez-Romero *et al*., 1991
R. etli		
• bv. phaseoli	*Phaseolus vulgaris*	Segovia *et al*., 1993
• bv. mimosae	*Phaseolus vulgaris, Mimosa affinis*, *Leucaena leucocephala*	Wang *et al*., 1999a
R. gallicum	*Phaseolus vulgaris*	Amarger *et al*., 1997
• bv. gallicum	*Phaseolus spp., Onobrychis viciifolia, Macroptilium artropurpureum, Leucaena leucocephala*	Amarger *et al*., 1997

Table 2.1 (continued)

Species	Host legume(s)	Reference
R. gallicum	*Phaseolus vulgaris*	Amarger *et al*., 1997
• bv. phaseoli	*Phaseolus spp., M. artropurpureum*	Amarger *et al*., 1997
R. giardinii		Amarger *et al*., 1997
• bv. giardinii	*L. leucocephala, M. artropurpureum Phaseolus spp.*	Amarger *et al*., 1997
• bv. phaseoli	*Phaseolus spp.*	Amarger *et al*., 1997
R. hainanense	*Desmodium sinuatum*	Chen *et al*., 1997
R. huautlense	*Sesbania herbacea*, *Leucaena leucocephala*	Wang *et al*., 1998
R. mongolense	*Medicago ruthenica*, *Phaseolus vulgaris*	van Berkum *et al*., 1998
R. yanglingense	*Coronilla varia*, *Gueldenstaedtia, mulitiflora, Amphicarpaea triserma*	Tan *et al*., 2001
R. indigoferae	*Indigofera* spp., *Kummerowia stipulacea*	Wei *et al.*, 2002
<u>*Bradyrhizobium*</u>		
B. japonicum[TS]	*Glycine* spp., *Macroptilium artropurpureum*	Jordan, 1982
B. elkanii	*Glycine max* (Soybean)	Kuykendall *et al*., 1992
B. liaoningense	*Glycine max, G.soja*	Xu *et al*., 1995
B. yuanmingense	*Lespedeza cuneata*	Yao *et al.*, 2002
<u>*Sinorhizobium*</u>		
S. fredii [TS]	*Glycine max, G.soja, Vigna unguiculat, Cajunus cajam*	Scholla and Elkan, 1984; Chen *et al.*, 1988
S. meliloti	*Medicago, Melilotus, Trigonella*	Jordan, 1984

Table 2.1 (continued)

Species	Host legume(s)	Reference
S. xinjiangense	*Cajanus cajun, Glycine max, G. soja, Vigna unguiculata*	Chen *et al.*, 1988
S. saheli	*Acacia seyal, Sesbania* spp.	de Lajudie *et al.,* 1994
• bv. acaciae	*Acacia* spp.	Boivin and Giraud, 1999
• bv. sesbaniae	*Sesbania* spp.	Boivin and Giraud, 1999
S. terangae	*Acacia* spp., *Sesbania* spp.,	de Lajudie *et al.,* 1994
• bv. acaciae	*Acacia* spp.	Lortet *et al.,* 1996
• bv. sesbaniae	*Sesbania* spp.	Lortet *et al.,* 1996
S. arboris	*Acacia senegal, Prosopis chilensis*	Nick *et al*., 1999
S. medicae	*Medicago polymorpha* and other annual *Medicago* spp.	Rome *et al.*, 1996
S. kostiense	*Acacia senegal, Prosopis chilensis*	Nick *et al.*, 1999
S. morelense	*Leucaena leucocephala*	Wang *et al.* 2002
S. kummerowiae	*Kummerowia stipulacea, Indigofera* spp.	Wei *et al.* 2002
S. americanum♦	*Acacia* spp., *Phaseolus vulgaris, Leucaena leucocephala*	Toledo *et al.*, 2003
<u>*Mesorhizobium*</u>		
M. loti [TS]	*Lotus* spp., *Lupinus densiflorus, Anthyllis vulneraria*	Jarvis *et al*., 1982
M. huakuii	*Astragalus sinicus*	Chen *et al*., 1991
M. ciceri	*Cicer arietinum* (chickpea)	Nour *et al*., 1994
M. mediterraneum	*Cicer arietinum*	Nour *et al*., 1995
M. tianshaneense	*Glycine max, Glycyrrhiza* spp., *Caragona polourensis, Halimodendron holodendron*	Chen *et al*., 1995
M. amorphae	*Amorphae fruticosa*	Wang *et al*., 1999b

Table 2.1 (continued)

Species	Host legume(s)	Reference
M. plurifarium	*Acacia* spp., *Neptunia oleracea*, *Leucaena leucocephala*	de Lajudie *et al*., 1998b
M. chacoense	*Prosopis alba, P.chilensis, P.flexuosa*	Velásquez *et al*., 2001
Azorhizobium		
A. caulinodans [TS]	*Sesbania rostrata*	Dreyfus *et al*., 1988
Allorhizobium		
A. undicola [TS]	*Neptunia natans*, *Acacia* spp., *Lotus arabicus*, *Medicago sativa*	de Lajudie *et al*., 1998a
Novel nodulators		
Methylobacterium nodulans	*Crotalaria* spp.	Sy *et al*., 2001
Burkholderia vietnamensis	*Oryzae* spp.	Gillis *et al*., 1995
Burkholderia tuberum	*Aspalathus carnosa*	Moulin *et al.,* 2001; Vandamme *et al*., 2002
Burkholderia phymatum	*Machaerium lunatum*	Vandamme *et al*., 2002
Ralstonia taiwanensis	*Mimosa* spp.	Chen *et al*., 2001; Chen *et al*., 2003
Devosia neptuniae	*Neptunia natans*	Rivas *et al*., 2003
Blastobacter denitrificans	*Aeschynomene indica*	van Berkum and Eardley, 2002

[TS] Type species
♦ Species name has not yet been validated

(i) The genus *Rhizobium*

The genus *Rhizobium* is phylogenetically heterogeneous and contains 11 accepted species. The genus *Rhizobium* was the first named (Frank, 1889). The name *Rhizobium* means 'root living' in

Latin. For many years this genus was a 'hold-all' that included all known rhizobacteria. In 1982, Jordan revised the symbiotic nitrogen-fixing bacteria and classified them into two genera based on clustering analyses of phenotypic characteristics, DNA-DNA re-association data, as well as other data. The fast growing-producing strains were kept in *Rhizobium* and slow-growing, alkali-producing strains were allocated to *Bradyrhizobium*. Later, this genus was again divided between the *Rhizobium* and the genera *Sinorhizobium* (de Lajudie *et al*., 1994) and *Mesorhizobium* (Jarvis *et al*., 1997*)*. Three species within the genus *Rhizobium* were amalgamated into one: *R. phaseoli, R. trifolii* and *R. leguminosarum* were placed together to form three biovars in *R.leguminosarum,* named to distinguish their plant affinities: biovar *viciae* nodulates *Vicia* 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 G + C content of the DNA of this genus is 59 – 64 mol%. The type species is *R. leguminosarum*.

(ii) The genus *Bradyrhizobium*

Jordan described *Bradyrhizobium* in 1984 as slow-growing bacteria that are clearly different from other legume symbionts, as they grow slowly and produce an alkaline reaction. This is an extremely heterogeneous group of symbionts. Scientific investigation of legume symbionts often focuses on the lineages of other rhizobia rather than on *Bradyrhizobia*, because of their slow growth. Due to this, the taxonomic relationships have not advanced and are likely to still change significantly (van Berkum and Eardley, 1998).

The rRNA cistrons of *Rhizobium* and *Bradyrhizobium* are relatively dissimilar to each other, whilst the rRNA cistrons of *Rhizobium* and the genera *Mesorhizobium* and *Sinorhizobium* resemble each other greatly (van Berkum and Eardley, 1998). Jarvis *et al.* (1986) proposed a photosynthetic ancestry for *Bradyrhizobium*, because of the close relationship of their rRNA cistrons to those of the photosynthetic *Rhodopseudomonas palustris*. Conclusions made by van Berkum *et al*. (1995) from the analysis of bradyrhizobial isolates show agreement with this suggestion. The G + C content of this genus is 61 – 65 mol%. The genus *Bradyrhizobium* currently contains four species and type species is *B. japonicum*.

(iii) The genus *Azorhizobium*

This genus contains the fast-growing, specific, stem- and root-nodulating strains from *Sesbania rostrata* (Dreyfus *et al*., 1988). Isolates from the stem-nodules were shown to be different from other *Rhizobiaceae* in their ability to grow with dinitrogen as the sole nitrogen source. Molecular systematic data shows that this genus is clearly distinct from other genera in the *Rhizobiaceae*. The G+C content of this genus is 66.5 mol%. Only one species has been described, *A.caulinodans* (Dreyfus *et al*., 1988).

(iv) The genus *Sinorhizobium*

Chen *et al.* (1988) described the genus *Sinorhizobium* for fast-growing soybean bacteria from China. The results of numerical taxonomy, DNA-DNA hybridization, serological analysis data, G + C content, soluble protein patterns, and bacteriophage typing showed that the new genus differed adequately from *Bradyrhizobium*, *Rhizobium* and *Azorhizobium* to warrant the description of the new genus. This genus exhibits fast growth and acid production. In 1986, Jarvis reported that the reference strain used to identify rhizobia nodulating *Medicago* and *Trigonella* spp. was closely related to fast-growing soybean isolates. In 1988, Chen *et al.* proposed that these fast-growing soybean isolates from China, belong to a genus separate from the *Rhizobium*. This proposal was eventually supported by a distinct separation of the fast-growing soybean rhizobia from the slow-growing soybean rhizobia. The new genus was given the name *Sinorhizobium*. It contains ten validated species including *S. fredii*, previously *R. fredii* (Scholla and Elkan, 1984). The species *S. americanum* was described by Toledo *et al*. in 2003, but the name has not as yet been included in the list of bacterial names with nomenclature standing (The International Committee on Systematic of prokaryotes, 2004). The strains of the genus *Sinorhizobium* do not nodulate a wide host range and exhibit host specificity (Chen *et al*., 1988; de Lajudie *et al*., 1994). The type species of this genus is *S.fredii*. The G + C content of the genus is 57 – 66 mol%.

(v) The genus Mesorhizobium

The genus *Mesorhizobium* was proposed by Jarvis *et al*. (1997) to include strains that are intermediate in both their phylogenetic position and their growth rate; the prefix 'meso' means 'middle'. *Mesorhizobium* generally grow faster than *Bradyrhizobium*, but slower than either *Rhizobium* or *Sinorhizobium.* This genus shares acid production with *Rhizobium* and *Sinorhizobium*. Strains from this genus generally form nitrogen-fixing nodules on the roots of a restricted range of leguminous plants. Cross-inoculation between the strains of one species of this genus and the plant hosts associated with another species is not known. Other distinguishing characteristics include the location of their symbiotic genes, integrated into the genome instead of being located on plasmids. *Mesorhizobium* have polar or sub-polar flagellation, as opposed to the peritrichous flagella of other fast-growing genera (van Berkum and Eardley, 1998). The G + C content is 59 – 64 mol %. There are eight recognised species in this genus; the type species is *M. loti* (Jarvis *et al*., 1982).

(vi) The genus *Allorhizobium*

The name *Allorhizobium*, meaning 'other rhizobium', was proposed by de Lajudie *et al*. (1998a) for a genus comprising the nitrogen-fixing isolates from the aquatic plant *Neptunia natans*. The G+C content of the DNA is 60.1 mol%. *Allorhizobium* is considered an artificial genus, justified on the basis of the anomalous state of *Agrobacterium* nomenclature (Young *et al*., 2001). The only species in this genus is *Allorhizobium undicola* (de Lajudie *et al*., 1998a).

2.6.4. Bean symbionts

The symbionts of bean are genetically heterogeneous; a diverse range of bacteria including broad host range bacteria and non-specific rhizobia ineffective in nitrogen fixation can nodulate *P.vulgaris* (Mhamdi *et al*., 2002). However, *P.vulgaris* plants are very selective of the microsymbiont to which they form a symbiotic association (Aguilar *et al*., 2001).

(i) *Rhizobium tropici*

Two major groups were recognised within the species *Rhizobium leguminosarum* bv *phaseoli,* namely Type I and Type II. Martínez-Romero *et al*. (1991) described *Rhizobium tropici* for the strains previously known as *R. leguminosarum* bv. phaseoli Type II. Two distinct subgroups, Type IIA and Type IIB were reported. The subgroups differ in phenotypic and genotypic characteristics (Martínez-Romero *et al*., 1991). Strains from *R. tropici* form effective nodules on *Leucaena leucocephala*, *L. esculenta* and *Phaseolus vulgaris* (Martínez-Romero *et al*., 1991). Van Berkum *et al*. (1998) isolated strains belonging to *R. tropici* from *Medicago ruthenica*, which it nodulates effectively.

(ii) *Rhizobium etli*

Rhizobium etli was described by Segovia *et al*. (1993) for the former *R. leguminosarum* bv. phaseoli type I strains. They included one biovar in the species, namely *R. etli* bv. phaseoli, which nodulates and fix nitrogen only with *Phaseolus vulgaris*. Non-symbiotic strains were also included in the species (Segovia *et al*., 1993). It has since been found that *R. etli* strains are not restricted to the Americas and they can nodulate hosts other than common bean (Amarger *et al*., 1997; Dagutat and Steyn, 1995). In a study on indigenous South African legumes, Dagutat (1995) isolated putative *R. etli* strains from nodules of *Desmodium*, *Melolobium*, *Indigofera*, *Acacia melanoxylon* and *Chamaecrista stricta* plants.

(iii) *Rhizobium gallicum*

Amarger *et al*. (1997) described *Rhizobium gallicum* for isolates from the nodules of *Phaseolus vulgaris* grown in France. Two biovars, *R. gallicum* bv. gallicum and *R. gallicum* bv. phaseoli were included in the species. *R. gallicum* bv. gallicum strains nodulate the following legumes: *Leucaena leucocephala*, *Macroptilium atropurpureum*, *Phaseolus* spp. and *Onobrychis viciifolia* and fix nitrogen with *Phaseolus vulgaris*. The strains of *R. gallicum* bv. phaseoli nodulate *Macroptilium atropurpureum* after one month and effectively nodulates *Phaseolus* spp.

(iv) *Rhizobium giardinii*

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

R. giardinii bv. giardinii nodulates *Leucaena leucocephala*, *Macroptilium atropurpureum* and *Phaseolus* spp. The strains are weakly efficient in nitrogen fixation with *P. vulgaris*. The strains of *R. giardinii* bv. phaseoli nodulate *Phaseolus* spp. and form nodules on *Macroptilium atropurpureum* after a month or more. The nitrogen fixation level of the strains with *Phaseolus vulgaris* is low (Amarger *et al*., 1997).

Laguerre *et al*. (1997) reported the isolation of *R. gallicum* strains in Canada from *Onobrychis viciifolia* and *Oxytropis riparia*. Mhamdi *et al*. (2002) reported the isolation of *R. gallicum* bv. gallicum from beans in Tunisia and Silva *et al*. (2003) isolated *R. gallicum* bv. gallicum isolates from *P. vulgaris* and *P. coccineus* in Mexico. These results indicate that several hosts and geographic distributions exist for *R. gallicum*.

2.6.5 Proposed reclassification of the rhizobia

Recently, Young *et al*. (2001) proposed the emended description of the genus *Rhizobium* to include the genera *Allorhizobium, Agrobacterium* (genus of plant pathogens closely related to *Rhizobium* but incapable of forming nitrogen fixing symbiosis) and *Rhizobium.* This proposal was based on high 16S rDNA sequence similarity values and no clear phenotypic differences between the three genera (Young *et al*., 2001). Evidence for their separation based on phylogenetic differentiation depends on the choice of algorithm and sequences included in the analysis. Young and colleagues (2001) argued that no consistency existed in phylogenetic differentiation between these three genera and that they should encompass the single genus *Rhizobium*. Broughton (2003) stated, with reference to the inclusion of *Agrobacterium* in the genus *Rhizobium,* that it is important for a bacterial name to reflect the reality of its symbiotic or pathogenic nature, even if the true nature (symbiotic or pathogenic) of the organism is not

reflected in its 16S rDNA sequence. The International Committee on Systematics of Prokaryotes (2004) declared that the latest proposal of a new name does not mean that it has preference over the older name. The committee suggested that individual experts decide which name they want to use. Broughton (2003) suggested that authors should wait for the sequence data of other conserved genes to become available before new changes are made to the taxonomic groupings of rhizobia. This opinion was shared by Van Berkum *et al*. (2003).

2.6.6 Novel root-nodulating species

Several novel species of root-nodulating bacteria have been described recently. *Methylobacterium nodulans* was described for facultative methylotrophic unpigmented isolates from *Crotalaria* spp. (Sy *et al*., 2001). Chen *et al*. (2001) described the species *Ralstonia taiwanensis*, a species in the β-*Proteobacteria* capable of legume nodulation. Moulin *et al*. (2001) reported the existence of *Burkholderia* spp. which nodulate legumes effectively. *Devosia neptuniae* is another novel species which nodulates legumes and fixes nitrogen (Rivas *et al*., 2003). *Blastobacter denitrificans* was described by van Berkum and Eardley (2002) for nodulation of a water plant. Benhizia *et al*. (2004) recently found evidence of root nodule bacteria that belong to the gamma sub-class of the *Proteobacteria*.

(i) *Burkholderia* species

The genus *Burkholderia* comprises 19 species, some being common soil and rhizosphere inhabitants whilst others are plant and human pathogens. Several species, previously placed within the *Pseudomonas* group, have been transferred to the genus *Burkholderia*. These bacteria are found in the β-subclass of the *Proteobacteria* (Gillis *et al*., 1995). Several nitrogen-fixing bacterial strains associated with rice roots have been isolated from rice growing in Vietnam. These strains have been shown to be phenotypically, as well as genotypically, very similar to *B.cepacia,* which has been isolated from cystic fibrosis patients (Balandreau *et al*., 2001). In 2002, Vandamme *et al*. proposed two new *Burkholderia* species capable of effectively nodulating legumes and in 2003 three new species of nitrogen-fixers were added: *Burkholderia tuberum* and *Burkholderia phymatum*. *B. brasilensis*, *B. kururiensis*, and *B. tropicalis* (Marin *et al*., 2003).

(ii) *Methylobacterium nodulans*

Methylobacterium nodulans was described for facultative methylotrophic unpigmented isolates from *Crotalaria* spp. (Sy *et al*., 2001). The symbiosis between the bacteria and the legume species is very specific. In 2002, Jaftha *et al*. reported pigmented methylotrophic bacteria closely related to *Methylobacterium nodulans* (98% 16S rDNA sequence similarity) isolated in South Africa from *Lotononis bainesii*.

(iii) *Devosia neptuniae*

The species *Devosia neptuniae* was reported by Rivas *et al*. (2003) for root-nodulating isolates from *Neptunia natans*. The *nodD* and *nifH* genes of the isolates are similar to that of *R. tropici*, which suggest that the symbiotic genes were transferred from *R. tropici* to *Devosia neptuniae*. In 2003, Rivas *et al*. conducted a polyphasic approach to characterise the new species and to formally describe the new species, *Devosia neptuniae*.

(iv) *Ralstonia taiwanensis*

Chen and co-workers revealed that isolates obtained from *Mimosa* root nodules corresponded to members of the *Ralstonia* genus (Chen *et al*., 2001). These isolates were shown to possess *nif* genes and to effectively nodulate *Mimosa* species. This was the first report of members of the β-subclass of the *Proteobacteria* as nodulating symbionts of leguminous hosts. Chen *et al*. (2001) described the novel species, *Ralstonia taiwanensis* found in the β-*Proteobacteria.* All isolates effectively nodulate *Mimosa diplotricha* and *Mimosa pudica*.

(v) *Blastobacter denitrificans*

Van Berkum and Eardly (2002) described *Blastobacter denitrificans*, a nitrogen fixing bacterium isolated from the water plant *Aeschynomene indica*. *Blastobacter* spp. are freshwater bacteria. Comparative analyses of ribosomal 16S rRNA gene and internally transcribed spacer region

sequences indicated that *B. denitrificans* is a member of the α-subdivision of the *Proteobacteria*.

2.7 Methods used for the characterisation of bacteria

Rhizobia were previously classified based on plant infection, but after it became apparent that the genes for nodulation, nitrogen fixation and host specificity are located on transmissible plasmids, this method became obsolete as the only tool for taxonomic purposes. Graham *et al*. (1991) proposed the use of both phylogenetic and phenotypic (symbiotic, cultural, morphological and physiological) traits as minimal criteria for the description of new rhizobia species and genera. The description should be based on a large number of strains, chosen from different geographical origins focusing on the origin of the host legume. Any new species should be supported by phenotypic differences, enabling the non-taxonomist to identify the new species (Graham *et al*., 1991).

Polyphasic taxonomy (Colwell, 1970) aims to incorporate different types of data and information (phenotypic, genotypic and phylogenetic) of micro-organisms to essentially indicate a consensus type of taxonomy. This approach allows more reliable and better resolution of the interrelationship among micro-organisms (Vandamme *et al*., 1996). Phenotypic methods include techniques not directly involving the genetic material (DNA or RNA) of the organism of concern, such as proteins and their function or other expressed features (Vandamme *et al*., 1996). Genotypic analysis corresponds to all methods aimed at the investigation of nucleic acids (DNA or RNA) within the cell. The genetic diversity of isolates can be revealed with the use of molecular methods, such as AFLP and rep-PCR fingerprinting. Phylogenetic position can be determined by use of partial or full length sequencing of the 16S rRNA gene. Phylogenetic relationships are determined making use of genotypic information from the ribosomal RNA genes.

2.7.1 Protein electrophoresis

Phenotypic methods include techniques not directly involving the genetic material (DNA or RNA) of the organism of concern. Classical phenotypic traits, substrate utilization tests and

whole-cell protein analysis are some of the phenotypic techniques engaged as part of a polyphasic approach. Morphological, physiological and biochemical features are the classical phenotypic features, which can be used to supply descriptive information of a species (Vandamme *et al*., 1996). The complete DNA base sequence is considered an absolute reference standard for the estimation of relationships between bacteria, but cellular proteins also form an information source for the identification and differentiation of bacteria. Methods utilizing cellular proteins will be discussed.

Protein electrophoretic profiles were originally used to investigate the chemical composition of cells, but it was realised that the protein profiles obtained could be used to compare large numbers of strains for their relatedness. Cellular proteins form an information source for the identification and differentiation of bacteria and are valuable, as techniques such as MLEE and SDS-PAGE are extremely sensitive and can resolve intraspecies relationships (Priest and Austin, 1993; Vandamme *et al*., 1996). The most commonly used phenotypic electrophoretic techniques for comparative analysis of a large number of strains are sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and multilocus enzyme electrophoresis (MLEE).

(i) SDS-PAGE of whole-cell proteins

One of the most widely applied phenotypic tools in bacterial systematics is SDS-PAGE analysis of whole-cellular proteins. Patterns obtained from samples prepared under denaturing conditions such as 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. Separation of these proteins by gel electrophoresis produces a complex banding pattern called a protein electrophoregram. Each bacterial strain, when grown under identical conditions, produces a constant electropherogram, which is reproducible and represents a fingerprint that can be used for comparison (Kersters, 1985).

Whole-cell protein analysis is a standardized and reliable method to group large numbers of closely related isolates. SDS-PAGE can differentiate at and below species level (Vandamme *et al*., 1996), and once standardized, is a robust method that allows the construction of large

databases of strains from numerous bacterial species. The same level of discrimination than that obtained by DNA-DNA hybridization could be achieved by comparison of electrophoretic groupings of whole-cellular protein extracts when comparing organisms and this technique is more affordable and rapid than DNA-DNA hybridization (Kersters and de Ley, 1975; Kersters, 1985; Priest and Austin, 1993; Vauterin *et al*., 1993). However, the discriminatory level of protein electrophoresis 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).

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). Yao *et al*. (2002) used SDS-PAGE of whole-cell proteins to characterise rhizobial isolates from *Lespedeza*. They found that the clusters obtained from the protein analysis, numerical taxonomic analysis and DNA-DNA hybridization data were in agreement, supporting their description of the *Bradyrhizobium* species, *B. yuanmingense* (Yao *et al*., 2002). This electrophoretic technique is more time-consuming than other fingerprinting techniques and requires a large amount of cells, as well as a large number of experimental steps. Each of the experimental steps may introduce some experimental error; therefore, standardization is absolutely necessary (Kersters, 1985; Vauterin *et al*., 1993).

(ii) Multilocus enzyme electrophoresis (MLEE)

Multilocus enzyme electrophoresis (MLEE) is a high-resolution phenotypic method that can determine the amount of genetic recombination between members of a population (Schloter *et al*., 2000). The electrophoretic properties of several enzymes are compared, usually focusing on enzymes that are common to a group. The method involves the electrophoretic separation of non-denatured proteins, followed by specific staining, which results in a colour reaction that reveals the enzymatic activity. 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). The migration of the proteins is a function of its molecular mass, its electrophoretic charge and the conformation of the molecule. Mutations are reflected by differential migration of the individual enzymes in the electrophoretic field (Selander *et al*., 1986). Wang and colleagues (1999b) used MLEE as one of the techniques to characterise rhizobia, which led to the description of *Mesorhizobium amorphae*.

Enzyme electrophoresis is an indirect measure for genomic diversity, as enzyme variants resulting from post-translational modifications are unfortunately given an equal value for genetic divergence as isoenzymes generated by different alleles. Another restriction of this method is that only a limited number of representative enzymes can be assessed by this method (Selander *et al*., 1986; Vauterin *et al*., 1993).

2.7.1.1 Comparison of the protein electrophoretic techniques

The utility of protein gel electrophoresis in bacterial systematics is well established (Kersters, 1985; Vauterin *et al*., 1993). Both SDS-PAGE and MLEE are excellent tools to study population genetics. (Priest and Austin, 1993; Vandamme *et al*., 1996; Vauterin *et al*., 1993). However, the number of loci analysed by MLEE directly affects the number of electrophoretic types recognized in a sample; if only a few loci are analysed, the full extent of genotypic variation in a population may be underestimated. Whole-cell protein electrophoresis allows identification of strains based on the overall genetic background of the cell, whereas only a limited number of representative enzymes can be compared with MLEE (Vauterin *et al*., 1993). When the sample is prepared under non-denaturing conditions, as it is for MLEE, the proteins retain their enzymatic activity and can be detected on the basis of this activity. However, the ribosomal, membrane and nucleic acid-bound proteins are usually not included in the electrophoretic analysis. Patterns obtained from samples prepared under denaturing conditions, most commonly by treatment of cells with sodium dodecyl sulphate (SDS), the majority of the cellular proteins are included, including ribosome- and membrane-bound proteins. As the molecular weight of homologous proteins is more conserved than their net charge or isoelectric point, SDS electrophoretic patterns should theoretically detect broader taxonomic relationships. (Kersters, 1985; Vauterin *et al*., 1993). A

disadvantage of using SDS-PAGE for rhizobia is the production of exopolysaccharides, which may cause distortion of the electropherogram

SDS-PAGE of whole-cell proteins gives highly discriminatory results, especially at the intra-subspecific level. The comparison of 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 the majority of the cell's chromosome (Kersters, 1985; Priest and Austin, 1993; Vauterin *et al*., 1993).

2.7.2 DNA fingerprinting techniques

The development of molecular microbiology has changed the process of characterising, comparing and identifying bacteria. DNA fingerprinting techniques are a means of visualizing DNA polymorphisms between samples as they involve the display of a set of DNA fragments from a specific DNA sample.

DNA fingerprints may be used as a tool for determining the identity of a specific DNA sample or to assess the relatedness between samples. Rapid DNA typing methods such as the techniques that target the whole genome (RAPD, ribotyping, AFLP, PFGE and rep-PCR) and individual genes (PCR-RFLP, ARDRA and sequencing) enable the differentiation between species and strains of the same species (Stackebrandt *et al*., 2002)

The choice of fingerprinting technique is dependant both on the organism to be studied and the application of that study. The criteria for evaluating which DNA fingerprinting technique to use include typeability, reproducibility, discriminatory power and ease of interpretation. Typeability is the ability of a method to provide readable results for each isolate analysed. Reproducibility measures the ability of a technique to yield the same result when replicate assays are performed on the same isolate. Discriminatory powers are the ability of a typing method to distinguish different strains. Ease of performance includes both performance of the technique and interpretation of the results (Coenye *et al*., 2002). An identification technique should be insensitive to previous manipulation of the strains and the technical difficulty, cost, and time to

obtain a result must also be evaluated in assessing the value of a particular typing method (Olive and Bean, 1999).

2.7.2.1 Locus-specific DNA fingerprinting techniques

(i) PCR-based locus-specific RFLP

Restriction fragment length polymorphism (RFLP) makes use of restriction endonucleases that cut the DNA at specific sites, generating numerous smaller DNA fragments that result in an illegible smear when viewed by gel electrophoresis. To overcome this problem, the PCR-RFLP technique is used; oligonucleotide primers are used to first amplify specific regions of the genome, combined with RFLP restriction enzyme digestion of these amplification products to generate fingerprint patterns (van Berkum and Eardley, 1998). The discriminatory power of locus-specific PCR is generally not as good as that of other methods, due primarily to the limited region of the genome that can be examined.

(ii) Amplified rDNA restriction analysis (ARDRA)

Amplified rDNA restriction analysis (ARDRA) is the name of the technique when PCR-RFLP targets the ribosomal DNA (16S or 23S rDNA or parts of both genes, with or without the intergenic spacer region [ITS]). The rDNA is amplified by using universal primers located in the conserved regions of the rRNA genes and then digested with a combination of restriction enzymes.

ARDRA is a rapid method, less demanding than direct sequencing or hybridization with specific probes. It is able to distinguish between closely related species (Vaneechoutte *et al*., 1993; Vandamme *et al*., 1996). Koeleman *et al*. (1998) found ARDRA to be less suited to species identification than RAPD analysis. ARDRA has been applied to the differentiation of many bacterial genera, including several species of rhizobia; *Rhizobium galegae* (Terefework *et al*., 1998) and rhizobia nodulating Acacia trees in Morocco (Khbaya *et al*., 1998). Mhamdi *et al*.

(2002) used 16S RFLP to determine the identity of rhizobia nodulating *Phaseolus vulgaris* in Tunisian soils.

2.7.2.2 DNA fingerprinting techniques utilizing the whole genome

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

Short arbitrary sequences are used as primers in the generation of genomic fingerprints for samples where little is known of the target sequences to be amplified. Arbitrarily primed PCR fingerprinting (AP-PCR) makes use of primers of 20 bases (Welsh and McClelland, 1990). Randomly amplified polymorphic DNA (RAPD) analysis has random primers of 9 – 10 bases (Williams *et al.,* 1990), while DNA amplification fingerprinting (DAF) can use primers as short as 5 bases, but typically 8 – 10 bases (Caetano-Anollés *et al*., 1991). AP-PCR fingerprinting and RAPD both involve the amplification of random DNA segments by PCR, using single primers of arbitrary nucleotide sequences in order to reveal differences as DNA polymorphisms or fingerprints. The annealing temperatures are low, allowing the random primers to hybridize. The resulting DNA fingerprints are determined by the nucleotide sequence of the primer and the nature of the DNA template. These techniques are generally applicable to a wide variety of organisms as a result of the universal primers and are species or sub-species specific (Welsh and McClelland, 1990; Williams *et al*., 1990). DAF differs from AP-PCR and RAPD in its reaction conditions, 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).

Imperfect hybridization occurs between the primer and target site, as the primers are not directed to a specific locus. Therefore, the amplification process is extremely sensitive to slight changes in the annealing temperature and this can lead to variability in the banding patterns. These techniques need to be optimized and used with consistency to ensure reproducibility within laboratories and standardization, which is usually low (Olive and Bean, 1999). Differences in equipment, enzymes and PCR conditions may lead to variation that can interfere with the amplification fragments generated. All three methods use random distribution throughout the

genome and require low quantities of purified template DNA (Caetano-Anollés *et al*., 1991). RAPD has been used successfully to distinguish bacterial strains among diverse species including *Heliobacter pylori* (Akopyanz *et al*., 1992), and *Staphylococcus aureus* (Saulnier *et al*., 1993).

(ii) Pulsed-field gel electrophoresis (PFGE)

For PFGE, bacterial isolates are grown either in broth or on solid media, combined with molten agarose and poured into small moulds. The results are agarose plugs containing the whole bacteria. The embedded bacteria are subjected to *in situ* detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme. The digested bacterial plugs are then inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of very large molecular length DNA fragments ranging from 10 to 800 kb (Olive and Bean, 1999). The gels are stained with a fluorescent dye such as ethidium bromide and then the electrophoretic patterns can be visualized. Gel results can be photographed, and the data can be stored and data analysis can be accomplished by using any of a number of commercially available software packages available.

PFGE is highly discriminatory, with moderate running and set-up costs. One of the factors that has limited the use of PFGE however, is the time involved in completing the analysis. While the procedural steps are straightforward, the time needed to complete the procedure can be two to three days. This can reduce the laboratory's ability to analyze large numbers of samples (Olive and Bean, 1999). PFGE has been used for the typing of *Pseudomonas aeruginosa* (Grundmann *et al*., 1995) and *Staphylococcus aureus* (Saulnier *et al*., 1993).

(iii) Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a selective restriction fragment amplification technique, which produces highly complex DNA profiles. The technique comprises three steps: digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site-specific adaptors to all restriction fragments; selective amplification of some

of these fragments with two PCR primers, which have corresponding adaptor and restriction site-specific sequences; and electrophoretic separation on a gel matrix (Vos *et al*., 1995). The restriction fragments are selected by performing restriction using two restriction enzymes, ideally a rare and a frequent cutter. This would yield DNA fragments with two different types of sticky ends to which adapters are ligated to provide primer-binding sites for amplification (Vaneechoutte, 1996). The selective amplification is achieved by using two different primers consisting of the same sequence as the adaptors with extra selective nucleotide(s) 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. This results in an array of fragments, which are detected by denaturating polyacrylamide gel electrophoresis. The choice of restriction enzymes, the number and base composition of the selective nucleotides in the primers and the complexity of the genomic DNA determine the number and size of DNA fragments. These fragments are either group-specific or strain specific (Vandamme *et al*., 1996; Vos *et al*., 1995).

AFLP is a reliable and powerful high resolution DNA fingerprinting technique for DNA of any origin or complexity and could be used for identification and typing purposes of highly related bacterial strains without prior knowledge of the nucleotide sequence. It is useful as a rapid screening technique for large collections of bacterial isolates as it yields reproducible results that are comparable to DNA-DNA hybridization. The method does, however, involve high set-up costs, with a moderate cost per test. It also requires purified high molecular weight DNA. The banding patterns produced are very complex and similar sized fragments may not be homologous (Olive and Bean, 1999). Gao *et al*. (2001) used AFLP to look at the genetic diversity of rhizobia isolated from *Astralgus*.

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

Enterobacteria contain families of short interspersed repetitive elements. These include the repetitive extragenic palindromic (REP) element, the enterobacterial repetitive intergenic consensus (ERIC) sequence and the BOX element. The function of these elements is unknown, but it has been suggested that they are involved in stabilizing mRNA, translational coupling

between genes homologous recombination and the binding of DNA polymerase. The repetitive extragenic palindromic (REP) sequences are 38-bp long, consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem. (Stern *et al*., 1984). The REP elements are palindromic and are able to form stem-loops. The enterobacterial repetitive intergenic consensus (ERIC) sequences are a second set of DNA sequences which have been successfully used for DNA typing. ERIC sequences are 126-bp elements, which contain a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome

The REP and ERIC elements do not show significant homology to each other. They contain highly conserved central inverted repeats and are normally found in intergenic regions that are transcribed, but not translated. The relative position of these elements in the genome of a given bacterial isolate is conserved in closely related strains and is distinct in diverse genera. By using consensus primers for these elements in PCR amplification, the regions between neighbouring repetitive elements, which are up to 5Kb from each other, are amplified to generate DNA fingerprints that are species and strain specific (Versalovic *et al*., 1991). These PCR reactions result in 5 – 30 PCR fragments per genome, ranging in size from 200 bp to 6 Kb.

Fingerprints generated by rep-PCR allow the recognition of strains between laboratories. REP- and ERIC-PCR analyses yield similar levels of discrimination (Laguerre *et al*., 1997). Both procedures are extremely rapid, they 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 REP and ERIC sequences are the most commonly used targets for DNA typing, but another repetitive element, the BOX sequence, has been found in *Streptococcus pneumoniae.* BOX elements are located within intergenic regions and can also form stem-loop structures due to their dyad symmetry. They are mosaic repetitive elements composed of various combinations of three subunit sequences referred to as boxA, boxB, and boxC. The three-subunit sequences have molecular lengths of 59, 45, and 50 nucleotides, respectively. These BOX elements have no sequence relationship to either REP or ERIC sequences (Martin *et al*., 1992). While initially

thought to be unique to *S. pneumoniae,* BOX elements have now been found in a number of 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. Vinuesa *et al*. (1998) first reported the use of the combined BOX-, ERIC and REP patterns to maximize strain discrimination and to obtain more phylogenetically coherent groups. Laguerre *et al*. (1996) found that DNA banding patterns of the same isolate could differ depending on the supplier of primers, batches of primers, different *Taq* enzymes and for the type of thermal cycler used. However, they also concluded that these techniques could be used to rapidly type a large number of strains under well-standardied conditions to improve the reproducibility of results.

Repetitive sequence-based PCR can 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). The technique is easy to perform and can be applied to large or small numbers of isolates. Vinuesa *et al*. (1998) used the combined BOX-, ERIC and REP patterns in their study of *Bradyrhizobium* isolates. Aguilar *et al*. (2001) used REP- and ERIC-PCR to determine the intraspecies diversity of *R. etli* strains which nodulate *P. vulgaris* in Northwest Argentina. Laguerre *et al*. (1997) used rep-PCR to determine the genetic variability within rhizobial isolates. De Bruijn (1992) used rep-PCR to type *Rhizobium meliloti* and other soil bacteria and found the method to be sensitive and extremely powerful. Rademaker *et al*. (2000) concluded that the combined analysis of BOX-, ERIC- and REP-PCR fingerprints of the genus *Xanthomonas* correlated well with DNA-DNA homology studies.

2.7.2.3 Comparison of the DNA fingerprinting techniques

Although a particular typing method may have high discriminatory power and good reproducibility, the complexity of the method and interpretation of results as well as the costs involved in setting up and using the method may be beyond the capabilities of the laboratory. The choice of a molecular typing method will therefore depend upon the needs, skill level, and

resources of the laboratory. Methods such as locus-specific PCR, RAPD analysis, and rep-PCR are similar in their procedures and are generally the easiest to implement.

Vila *et al*. (1996) found that RAPD assays are more discriminating than 16S RFLP or 16S-23S RFLP, but less discriminating than rep-PCR. RAPD assays were also found to be less versatile than rep-PCR, as RAPD PCR reactions need to be optimized and rep-PCR reactions do not (Niemann *et al*., 1997). Repetitive sequence-based PCR shows better discriminatory power than plasmid profiling or restriction analysis of the 16S rRNA gene or the 16S – 23S spacer region. Studies have shown rep-PCR to be superior to other typing methods such as multilocus enzyme electrophoresis (Woods *et al*., 1992) and ribotyping (Snelling *et al*., 1996; Vila *et al*., 1996). Methods that cover the entire genome, such as rep-PCR, RAPD and AFLP are more informative than locus specific techniques, such as PCR-based locus-specific RFLP or ARDRA. While PFGE appears to be time-consuming, it too is not difficult to implement. The level of difficulty of a technique therefore needs to be taken into consideration, along with cost and time. One other criterion is the ability of a method to allow analysis of large numbers of samples. For high throughput, simple techniques with high discriminatory power and low cost, such as rep-PCR, may be most suitable. For sub typing bacteria, the laboratory must weigh the fact that the discriminatory power of a technique such as rep-PCR is sufficiently high to yield excellent sub-typing results for a fraction of the setup costs as well as lower costs per test (Olive and Bean, 1999).

2.8 DNA sequencing in bacterial systematics

DNA sequencing is a method of determining the nucleotide sequence of a segment of DNA. It is both expensive and impractical to sequence large regions of the chromosome; therefore sequencing must be directed at a very small region of the chromosome of an organism. For comparative purposes, the DNA targeted by sequencing must be variable enough to differentiate between strains (Olive and Bean, 1999). Both the 16S rRNA gene, and to a lesser extent the 23S rRNA gene, have been used to identify new species of organisms; however they show limited variability between strains of a bacterial species. The 16S-23S intergenic spacer region (ITS) has also been used for identification with variable results (Barry *et al*., 1991) and Stackebrandt *et al*.

(2002) have reported the use of other protein-encoding housekeeping genes in conjunction with 16S sequencing and DNA-DNA hybridisation to delineate species.
Sequencing of the symbiotic genes (nitrogen fixing genes and nodulation genes) has been used in the identification and association of rhizobia. Nodulation trees constructed from Nod sequences have been reported to be more related to the host plant taxonomy than phylogenetic trees constructed from 16S rDNA (Guo *et al*., 1999).

(i) Sequencing of the16S rRNA gene

The ribosomal RNA genes can be used as indicators of relatedness as they have a conserved function, which makes evolutionary studies possible. The rRNA molecules are functionally stable, since they play an important role in protein synthesis. All organisms, except viruses, have rRNA molecules. Different regions of the rRNA molecules have different evolutionary rates. They consist of both conserved regions, for comparing distantly related organisms and variable regions, used for comparing or grouping of more closely related organisms (Woese, 1987).

Partial sequences should not be used to draw phylogenetic conclusions, but they can be used to identify and assign organisms to phylogenetic groups. The use of full-length 16S rDNA sequences is vital to reconstruct phylogenetic trees (Ludwig *et al*., 1998). In the case of the phylogenetic use of 16S data, it is important to include both related and unrelated reference organisms in the analysis (Vandamme *et al*., 1996). It is better to target the conserved region as information will be lost when comparing only the hypervariable regions, since a substantial amount of difference is not concentrated in these regions (Stackebrandt and Goebel, 1994).

Evaluation of rhizobial genotypic diversity requires a higher level of taxonomic resolution than can be achieved by 16S rDNA sequencing (Fox *et al*., 1992). The method is unable to resolve the position of closely related strains of a species (Leblond-Bourget *et al*., 1996), as there is limited variability between strains of a bacterial species. The use of 16S rDNA sequencing enables the determination of the phylogenetic position of isolates, but recently diverged species cannot be differentiated, as recent evolutionary events are not detected with 16S rDNA. It is clear that phylogenetic trees based on 16S rDNA sequence data should be used with caution, but the trees can still give a basic idea of the taxonomic position of an isolate.

CHAPTER 3

CHARACTERISATION OF PUTATIVE RHIZOBIAL ISOLATES WITH SODIUM DODECYL-SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND PARTIAL 16S rDNA SEQUENCING

3.1 INTRODUCTION

The symbiotic association between legumes and the gram-negative bacteria, collectively called rhizobia, is an agriculturally important association. The gram-negative rhizobia belong to several genera in the α-*Proteobacteria*, namely *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium*. New species of nodulating bacteria have been described in recent years. Both *Methylobacterium nodulans* (Sy *et al*., 2001) and *Devosiae neptuniae* (Rivas *et al*., 2003) within the α-*Proteobacteria* are capable of nodulation. The ability to nodulate was thought to be exclusive to the α-*Proteobacteria*, but species in the β-*Proteobacteria* (*Ralstonia* and *Burkholderia* species) capable of root-nodulation have been described (Chen *et al*., 2001; Vandamme *et al*., 2002).

Previous studies on rhizobia associated with leguminous plants in South Africa have included phenotypic characterisation in the form of sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [Dagutat, 1995; Kruger, 1998; Joubert, 2002; Le Roux, 2002]. Whole-cell protein analysis is a standardized and reliable method to group large numbers of closely related isolates. The whole-cell protein profiles obtained from SDS-PAGE correlate with total DNA sequence homology (Kersters, 1985; Priest and Austin, 1993; Vauterin *et al*., 1993). Chen (1991) used SDS-PAGE in conjunction with G + C content and DNA homology studies to study root-nodulating isolates. Yao and colleagues (2002) used SDS-PAGE of whole-cell proteins to characterise rhizobial isolates. Unfortunately, SDS-PAGE of whole-cell proteins requires large amounts of cell material and it is more time-consuming than other fingerprinting techniques (Vauterin *et al*., 1993). The growth conditions, including incubation time and temperature must be kept constant as deviation in these conditions result in variable electrophoretic patterns. Another disadvantage of SDS-PAGE is the production of exopolysaccharides by rhizobia, which may cause distortion of the electropherogram.

The aim of this study was to investigate the diversity of root nodule-associated bacteria of *Phaseolus* spp. in South Africa with SDS-PAGE analysis of whole-cell proteins. *Phaseolus* species (beans) are not indigenous to South Africa, but the widespread cultivation of beans in South Africa has led to established rhizobial populations (Strijdom, 1998). Rhizosphere soil samples and the root nodules of *Phaseolus* plants were collected both from major bean production fields and from areas with a history of bean cultivation by subsistence farmers in South Africa. The bacterial strains used in this study were isolated from these root nodules. Type and/or reference strains of the genera *Azorhizobium*, *Bradyrhizobium, Burkholderia*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* were included in the SDS-PAGE analysis. The emergence of nodulating members of the β-*Proteobacteria*, *Burkholderia*, obtained from South Africa (Moulin *et al*., 2001), led to the inclusion of *Burkholderia* reference strains in this study. Representative isolates were chosen for further genomic characterisation by partially sequencing the conserved 16S rDNA regions as an additional technique to confirm the results obtained from SDS-PAGE analysis.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains used and maintenance

Bacterial strains analysed in this study were obtained from the rhizobial collection of the ARC-Plant Protection Research Institute (Private Bag X134, Pretoria 0001, South Africa), courtesy of Dr Ian Law (Table 3.1). These isolates were previously obtained from the root nodules of two *Phaseolus* species, namely *P. coccineus* and *P. vulgaris*. The inoculant strains UD2 and UMR 1899 were also obtained from the ARC-Plant Protection Research Institute. Figure 3.1 indicates the five geographic locations where the root nodule-bacteria were collected (Delmas, Mlondozi, Jacobsdal, Bethlehem and Ermelo).

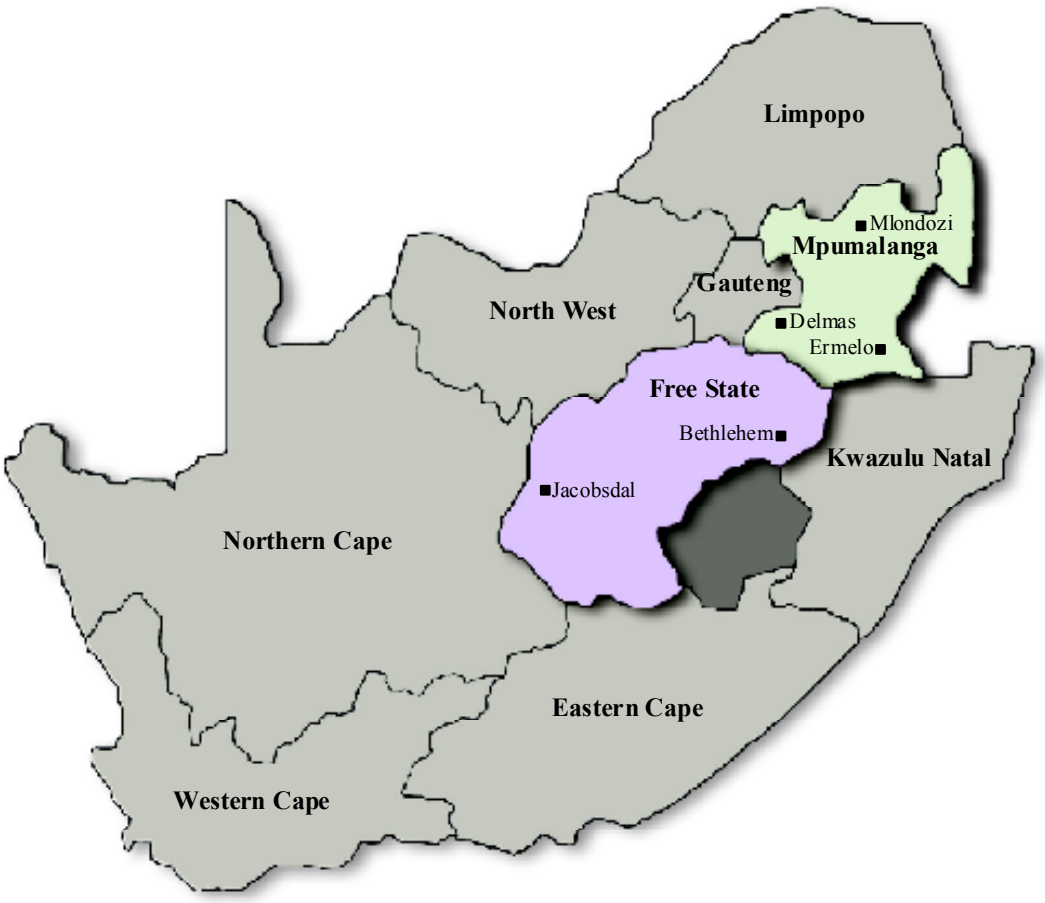


Figure 3.1 Map of South Africa indicating the geographic location of the bacterial isolates used in this study. The provincial names are indicated in bold.

The strains were maintained on yeast extract mannitol agar (YMA) slants containing: 1% (m/v) mannitol (UniVar), 0.5% (m/v) K_2HPO_4 (Merck), 0.02% (m/v) $MgSO_4.7H_2O$ (Merck), 0.01% (m/v) NaCl (NT Chemicals), 0.04% (m/v) yeast extract (Biolab) and 1.5% (m/v) bacteriological agar (Biolab) supplemented with 1% (v/v) of a 0.25% (m/v) Congo red stock solution (Merck). Plates were incubated for 5 to 7 days at 28°C and examined for growth. Cultures were purified by at least three consecutive streakings of single colonies on the same medium. To confirm purity of isolates, living and Gram-stained cells were checked by microscopy. Cultures were grown on YMA plates containing Bromothymol blue [0.5% (v/v) of a 0.5% (m/v) stock (Merck)] to determine whether they were fast-growing or slow-growing rhizobia (Somasegaran and Hoben, 1994). For long-term storage, cultures were grown in yeast extract mannitol broth (YMB) at 28°C for approximately five days with rigorous shaking. The turbid culture suspensions were subsequently mixed at a 1:1 ratio with sterile 50% (v/v) glycerol in sterile cryotubes and duplicates of each stored at both -20°C and -70°C.

Table 3.1 List of putative rhizobial isolates from *Phaseolus* species included in this study

Isolate no.	Host species	Locality	Isolate no.	Host species	Locality
PCD1	*P. coccineus*	Delmas	KD15	*P. vulgaris*	Delmas
PCD2	*P. coccineus*	Delmas	KD16	*P. vulgaris*	Delmas
PCD3	*P. coccineus*	Delmas	KD17	*P. vulgaris*	Delmas
PCD5	*P. coccineus*	Delmas	KD18	*P. vulgaris*	Delmas
PCD6	*P. coccineus*	Delmas	KD19	*P. vulgaris*	Delmas
PCD7	*P. coccineus*	Delmas	KD20	*P. vulgaris*	Delmas
PCD9	*P. coccineus*	Delmas	KD21	*P. vulgaris*	Delmas
PCD10	*P. coccineus*	Delmas	KM1	*P. vulgaris*	Mlondozi
PCM1	*P. coccineus*	Mlondozi	KM2	*P. vulgaris*	Mlondozi
PCM2	*P. coccineus*	Mlondozi	KM3	*P. vulgaris*	Mlondozi
PCM4	*P. coccineus*	Mlondozi	KM4	*P. vulgaris*	Mlondozi
PCM5	*P. coccineus*	Mlondozi	KM5	*P. vulgaris*	Mlondozi
PCM6	*P. coccineus*	Mlondozi	KM6	*P. vulgaris*	Mlondozi
PCM7	*P. coccineus*	Mlondozi	KM7	*P. vulgaris*	Mlondozi
PCM8	*P. coccineus*	Mlondozi	KM8	*P. vulgaris*	Mlondozi
PCM9	*P. coccineus*	Mlondozi	KM9	*P. vulgaris*	Mlondozi
PCJ1	*P. coccineus*	Jacobsdal	KM10	*P. vulgaris*	Mlondozi
PCJ2	*P. coccineus*	Jacobsdal	KM11	*P. vulgaris*	Mlondozi
PCJ3	*P. coccineus*	Jacobsdal	KM12	*P. vulgaris*	Mlondozi
PCJ4	*P. coccineus*	Jacobsdal	KM13	*P. vulgaris*	Mlondozi
PCJ5	*P. coccineus*	Jacobsdal	KM14	*P. vulgaris*	Mlondozi
PCJ7	*P. coccineus*	Jacobsdal	KM15	*P. vulgaris*	Mlondozi
PCJ8	*P. coccineus*	Jacobsdal	KM17	*P. vulgaris*	Mlondozi
PCJ9	*P. coccineus*	Jacobsdal	KM18	*P. vulgaris*	Mlondozi
PCJ10	*P. coccineus*	Jacobsdal	KM19	*P. vulgaris*	Mlondozi
PCB1	*P. coccineus*	Bethlehem	KM21	*P. vulgaris*	Mlondozi
PCB2	*P. coccineus*	Bethlehem	KM22	*P. vulgaris*	Mlondozi
PCB3	*P. coccineus*	Bethlehem	KJ1	*P. vulgaris*	Jacobsdal
PCB4	*P. coccineus*	Bethlehem	KJ2	*P. vulgaris*	Jacobsdal
PCB5	*P. coccineus*	Bethlehem	KJ3	*P. vulgaris*	Jacobsdal
PCB6	*P. coccineus*	Bethlehem	KJ5	*P. vulgaris*	Jacobsdal
PCB7	*P. coccineus*	Bethlehem	KJ6	*P. vulgaris*	Jacobsdal
PCB8	*P. coccineus*	Bethlehem	KJ7	*P. vulgaris*	Jacobsdal
PCB9	*P. coccineus*	Bethlehem	KJ8	*P. vulgaris*	Jacobsdal
PCB10	*P. coccineus*	Bethlehem	KJ9	*P. vulgaris*	Jacobsdal

Table 3.1 (continued)

Isolate no.	Host species	Locality	Isolate no.	Host species	Locality
PCE1	*P. coccineus*	Ermelo	KJ10	*P. vulgaris*	Jacobsdal
PCE2	*P. coccineus*	Ermelo	KJ11	*P. vulgaris*	Jacobsdal
PCE3	*P. coccineus*	Ermelo	KJ12	*P. vulgaris*	Jacobsdal
PCE4	*P. coccineus*	Ermelo	KJ13	*P. vulgaris*	Jacobsdal
PCE5	*P. coccineus*	Ermelo	KJ14	*P. vulgaris*	Jacobsdal
PCE6	*P. coccineus*	Ermelo	KJ15	*P. vulgaris*	Jacobsdal
PCE7	*P. coccineus*	Ermelo	KJ16	*P. vulgaris*	Jacobsdal
PCE8	*P. coccineus*	Ermelo	KJ17	*P. vulgaris*	Jacobsdal
PCE9	*P. coccineus*	Ermelo	KJ18	*P. vulgaris*	Jacobsdal
PCE10	*P. coccineus*	Ermelo	KJ19	*P. vulgaris*	Jacobsdal
KD1	*P. vulgaris*	Delmas	KJ20	*P. vulgaris*	Jacobsdal
KD2	*P. vulgaris*	Delmas	KB1	*P. vulgaris*	Bethlehem
KD3	*P. vulgaris*	Delmas	KB2	*P. vulgaris*	Bethlehem
KD4	*P. vulgaris*	Delmas	KB3	*P. vulgaris*	Bethlehem
KD5	*P. vulgaris*	Delmas	KB5	*P. vulgaris*	Bethlehem
KD6	*P. vulgaris*	Delmas	KB7	*P. vulgaris*	Bethlehem
KD7	*P. vulgaris*	Delmas	KB8	*P. vulgaris*	Bethlehem
KD8	*P. vulgaris*	Delmas	KB11	*P. vulgaris*	Bethlehem
KD9	*P. vulgaris*	Delmas	KB12	*P. vulgaris*	Bethlehem
KD10	*P. vulgaris*	Delmas	KB13	*P. vulgaris*	Bethlehem
KD11	*P. vulgaris*	Delmas	KB15	*P. vulgaris*	Bethlehem
KD12	*P. vulgaris*	Delmas	KB18	*P. vulgaris*	Bethlehem
KD13	*P. vulgaris*	Delmas	KB19	*P. vulgaris*	Bethlehem
KD14	*P. vulgaris*	Delmas	KB20	*P. vulgaris*	Bethlehem

The isolate names were compiled as follows: the host plant of origin was indicated by either PC for *P.coccineus* or K for *P.vulgaris*. The location was indicated by D for Delmas, M for Mlondozi, J for Jacobsdal, B for Bethlehem or E for Ermelo. The numerals are arbitrary and indicate the order that the bacteria were isolated in.

3.2.2 Rhizosphere soil properties

Soil samples were collected from the rhizosphere in the areas of bean production where the isolates were obtained. These samples were analysed by the ARC-Plant Protection Research Institute. Physico-chemical properties of the rhizosphere soils were analysed and rhizobial numbers were determined by most probable number (MPN) plant infection count (Somasegaran and Hoben, 1994).

3.2.3 SDS-PAGE of whole-cell proteins

(i) Bacterial strains and references used

In this study, a total of 118 putative rhizobial isolates from two *Phaseolus* species were included in the SDS-PAGE analyses (Table 3.1). Reference cultures of the different rhizobial genera were obtained from the bacterial culture collections of the Laboratorium voor Microbiologie (LMG), State University Gent, Belgium and the United States Department of Agriculture-ARS (USDA) *Rhizobium* Culture Collection, Maryland, USA and from Laboratoire des Symbioses Tropicales et Méditerranéennes (STM), Montpellier, France. A *Burkholderia* strain was donated by M.M. Kock, University of Pretoria (Kock, 2004). These reference strains represented various genera within the *Rhizobiaceae* and the genus *Burkholderia* and were used in the SDS-PAGE analysis (Table 3.2). The protein extracts of the reference strains were prepared as in Section 3.2.3 (ii) for the bacterial isolates.

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

Reference strain	Strain number	Host plant
Azorhizobium caulinodans	LMG 6465^{T}	*Sesbania rostrata*
Bradyrhizobium elkanii	LMG 6134 ^{T}	*Glycine max*
Bradyrhizobium japonicum	LMG 6138^{T}	*Glycine max*
Mesorhizobium mediterraneum	USDA 3392^{T}	*Cicer arietinum*
Rhizobium etli bv. phaseoli	LMG 17827^{T}	*Phaseolus vulgaris*
Rhizobium etli	USDA 9032	*Phaseolus vulgaris*
Rhizobium hainanense	USDA 3588 ^{T}	*Desmodium sinuatum*
Rhizobium huautlense	USDA 4900^{T}	*Sesbania herbacea*
R. leguminosarum bv. trifolii	LMG 8820 ^{T}	*Trifolium repens*
Rhizobium mongolense	LMG 19141 ^{T}	*Medicago ruthenica*
Rhizobium tropici	USDA 9030 ^{T}	*Phaseolus vulgaris*
Sinorhizobium fredii	LMG 6217 ^{T}	*Glycine max*
Burkholderia phymatum	STM 815 ^{T} ✧	*Aspalathus* spp.
Burkholderia tuberum	UCT36*	*Cyclopia galioides*

Table 3.2 (continued)

T	Type strain
LMG	Laboratorium voor Microbiologie Gent Culture Collection
USDA	United States Department of Agriculture-ARS National Rhizobium Culture Collection
STM	Laboratoire des Symbioses Tropicales et Méditerranéennes,
✧	The type strain for *Burkholderia phymatum* was donated by C. Boivin-Masson, 34398 Montpellier Cedex 5, France.
*	The strain for *Burkholderia tuberum* was a gift from Dr M. M. Kock, University of Pretoria.

(ii) Preparation of whole-cell protein extracts

Whole-cell protein extracts were prepared as described by Dagutat (1995), except that the strains were grown in tryptone yeast extract broth (TYB) [0.5% (m/v) tryptone (Difco), 0.3% (m/v) yeast extract (Biolab), 0.13% (m/v) $CaCl_2.6H_2O$ (UniLab), 1.5% (m/v) bacteriological agar (Biolab)] instead of yeast extract beef medium. Cultures were incubated at 28°C for 5 days with rigorous shaking in TYB and 5 ml of this suspension was used as inoculum for 50 ml of TYB. The inoculated TYB was incubated at 28°C with rigorous shaking, for 5 days. Cells were harvested by centrifugation at 10 000 rpm for 10 minutes. Resultant cell pellets were washed twice with 0.2 M sodium phosphate buffer (pH 6.88). The cells were resuspended in 1 ml 0.2 M sodium phosphate buffer (pH 6.88), transferred to Eppendorf tubes and centrifuged at 12 000 rpm for 10 minutes. The supernatants were discarded and the pellet size was determined. The cell pellets were suspended in 30 – 400 µl mixture of sample treatment buffer (STB) [0.5 M Tris-HCl pH 6.5, 5% (v/v) 2-β-mercaptoethanol (BDH), 10% (v/v) glycerol (Merck), 2% (m/v) SDS (Univar)] depending on pellet size. They were heated to 95°C for 3 minutes. Cell pellets were disrupted with a Cole-Parmer ultrasonic homogenizer (Series 4710) at 50% maximum output (40 Watt) for 15 seconds and the same volume of the STB-SDS mixture was again added, mixed and centrifuged at 12 000 rpm for 10 minutes. The supernatants were transferred to sterile Eppendorf tubes and frozen overnight at -20°C. Samples containing excess slime were heated to 95°C for 30 minutes, cooled to room temperature and centrifuged at 12 000 rpm for 10 minutes. The supernatants were transferred to clean Eppendorf tubes and stored at -20°C.

(iii) Polyacrylamide gel electrophoresis

The method of Laemmli (1970), as modified by Kiredjian *et al*. (1986) was used. A monomer solution containing 29.2% (m/v) acrylamide (BDH Electran) and 0.8% (m/v) N^1-N^1-bismethyleneacrylamide (BDH Electran) was used to prepare 1.5 mm thick gels comprising 5% stacking gels (0.5 M Tris-HCl pH 6.68, conductivity 31.0 mS/cm) and 12% separation gels (1.5 M Tris-HCl pH 8.87, conductivity 16.02 mS/cm). Electrophoresis was conducted in a BioRad Protean II gel apparatus with a constant current of 5 Watts through the stacking gel and 10 Watts through the separation gel. Gels were stained in Coomassie Blue solution [15% (v/v) of a 2% (m/v) Coomassie Brilliant Blue R stock solution (ICN Biomedicals), 50% (v/v) methanol and 10% (v/v) acetic acid]. Gels were destained overnight in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid.

(iv) Analysis of the protein profiles

The protein electrophoretic patterns were scanned using a flat bed scanner (Labcsan v 3.01, BioRad). Analyses of the data were performed using the GelCompar II computer programme (Applied Maths, Kortrijk, Belgium). Normalized densitometric traces were grouped and the similarities between all organisms were calculated 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).

Protein samples of *Psychrobacter immobilis* (LMG 1125) were included in each gel (six tracks per gel) to ensure reproducibility (Reber *et al.*, 1979). One *P. immobilis* selected in the program, acted as standard and was used to compare the reproducibility of references on successive gels where a correlation (*r*) of 94% (where r = r x 100%) was considered as reproducible (Personal communication, Dr M. Oosthuisen). The SDS-PAGE gels were normalized in Gel Compar II (Applied Maths, Kortrijk, Belgium) by aligning the six *P. immobilis* profiles in each gel.

Bar graphs were constructed to compare the ratio of host of origin and geographic location of all of the isolates; the distribution of isolates from various geographical regions within each cluster in the SDS-PAGE dendrogram and the distribution of isolates from different host plant species within each cluster in the SDS-PAGE dendrogram.

3.2.4 Partial 16S rDNA sequencing of selected putative rhizobial strains

(i) Bacterial strains used

From the SDS-PAGE results, 32 isolates were selected and subjected to 16S rDNA sequencing (Table 3 .3).

Table 3.3 List of isolates selected for partial 16S rDNA sequencing

Isolate					
KD1	KB1	PCE1	KM2	KJ2	PCJ1
KD4	KB2	PCE4	KM8	KJ3	PCJ4
KD7	KB5	PCE6	KM10	KJ13	PCJ9
KD11	KB8	PCE7	KM17	KJ14	
KD13	KB11	PCD7	PCM6	PCB5	
KD14	KB13	PCD9	PCM9		

(ii) Extraction of genomic DNA

Isolates were incubated on tryptone yeast extract medium agar (TY) slants [0.5% (m/v) tryptone, 0.3% (m/v) yeast extract, 0.065% (m/v) $CaCl_2$ and 1.5% (m/v) bacteriological agar] at 28°C for 5 days. TY reduces slime formation by the rhizobia. Cells were harvested by adding sterile distilled water and gently shaking until cells were suspended. Genomic DNA was extracted using the modified proteinase K (Roche Molecular Biochemicals) method (Laguerre *et al*., 1997) as previously described by Lemanceau *et al*. (1995). Cell suspensions were transferred to sterile plastic tubes. The optical density (OD) of cell suspensions was measured at 620 nm using water as a blank. To calculate the number of cells needed for further use, the following formula was used: V (µl) = $^{0.2}/_{OD260}$ x 1000 (Lemanceau *et al*., 1995). This results in a DNA concentration of approximately 50 µg/ml. The appropriate volume of cells (100 µl to 1 000 µl) was transferred to a clean Eppendorf tube and centrifuged at 12 000 x *g* for 5 minutes, the supernatant discarded and the pellet blotted dry. The cell pellet was resuspended in 100 µl of ultra-high quality (UHQ) 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. The proteinase

K was inactivated by heating the suspension of 10 minutes at 96°C. The cell lysates were stored at –20°C until further use.

(iii) Amplification of the 16S rRNA gene

Amplification of the 16S rRNA gene of selected strains was performed with the primers fD1 and rP2 as described by Weisburg *et al*. (1991); however the linker sequences of the primers were not included in the primer synthesis. These shorter primers were thus designated fD1SHRT and rP2SHRT (Table 3.4). Amplification was carried out in 50 µl reaction volumes containing 5µl of the cell lysate, 50 pmol of each primer (fD1SHRT and rP2SHRT), 250 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Super Therm GOLD Taq DNA polymerase (Southern Cross Biotechnologies).

Amplification was conducted in an Eppendorf Mastercycler gradient thermocycler 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 running an aliquot (5 µl) of each reaction on a 1% (m/v) horizontal agarose gel (Promega) containing 10 mg/ml ethidium bromide in 1 x TAE buffer (40 mM Tris-HCl, pH 8.0; 20 mM NaOAc and 1 mM EDTA, pH 8.5) to determine size, concentration and purity. Molecular marker VI (Roche Molecular Biochemicals) was included as standard marker on every gel.

Table 3.4 Primers used for 16S rDNA PCR amplification

Primer❖	Sequence (5′ to 3′)	Target gene	Reference
fD1SHRT	AGA GTT GAT CCT GGC TCA G	16S rRNA gene	Weisburg *et al*., 1991
rP2SHRT	ACG GCT ACC TTG TTA CGA CTT	16S rRNA gene	Weisburg *et al*., 1991

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

The amplification products of the 16S rDNA were purified, to remove any trace enzymes or unincorporated dNTPs as these can negatively influence the sequencing reactions. The PCR

products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) as described by the manufacturer. An aliquot (1 µl) of each purified 16S PCR product was run on a 1% (m/v) horizontal agarose gels (Promega). The molecular marker VI (Roche Molecular Biochemicals) was included on each gel.

(iv) Partial 16S rDNA sequencing

Sequencing was carried out in 10 µl reaction volumes: approximately 50 ng purified 16S rDNA template, 2 µl BigDye™ Terminator RR Mix (PE Applied Biosystems) and 10 pmol of the primer rP2SHRT (Weisburg *et al*., 1991). The sequencing reaction was carried out in an Eppendorf Mastercycler gradient thermocycler using the following thermal profile: an initial denaturation at 96°C for 5 seconds followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 55°C for 5 seconds and extension at 60°C for 4 minutes.

Sequencing reaction products were precipitated with ice-cold absolute ethanol and 3 M NaOAc on ice for 10 minutes. The precipitate was collected through centrifugation for 30 minutes and the supernatant discarded, followed by two successive wash steps with 70% (v/v) ethanol. The precipitate was then vacumn-dried and stored at -20°C. For electrophoresis, the purified products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer, prepared by combining deionized formamide and 25 mM EDTA (pH 8.0) containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran. Sequencing samples were submitted to the sequencing facility of the University of Pretoria for electrophoresis on the Perkin-Elmer ABI 377 Automated Sequencer. Homology searches were performed on each consensus sequence using the BLAST programme from the GenBank database of the National Centre for Biotechnology (NCBI) [www.ncbi.nlm.nih.gov/BLAST/].

(v) Phylogenetic analysis of the partial 16S rDNA sequences

The 16S rDNA sequences of relevant rhizobial reference strains, including members of the α- and β-*Proteobacteria*, were obtained from NCBI Nucleotide GenBank database [www.ncbi.nlm.nih.gov/Genbank/] (Table 3.5). The sequencing gels were analysed and sequences edited with the ABI Prism Sequencing Analysis 3.1 and the ABI Prism Sequencing Navigator 1.0.1 computer programmes (Perkin Elmer Applied Biosystems). The ClustalX

programme (Thompson *et al*., 1997) was used to align the edited sequences and the reference sequences obtained from GenBank. A distance matrix was constructed by pair-wise alignment of the sequences and the neighbour-joining method (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the distance matrix using PAUP* 4.0 (Swofford, 2000). Branch lengths were proportional to the estimated divergence along each branch. Confidence levels of the phylogenies were estimated with the bootstrap method (Felsenstein, 1985).

Table 3.5 Reference sequences obtained from GenBank[⌘] included in the phylogenetic analysis.

Reference strain	Strain number	Host plant or relevant characteristics	GenBank Accession number
Azorhizobium caulinodans	LMG 6465^T	*Sesbania rostrata*	X67221
Bradyrhizobium elkanii	USDA 76^T	*Glycine max*	U35000
Bradyrhizobium japonicum	LMG 6138^T	*Glycine max*	X66024
Bradyrhizobium liaoningense	LMG 18230	*Glycine max*	AJ250813
Burkholderia phymatum	STM 815^T	*Machaerium lunatum*	AJ302312
Burkholderia tropicalis	Ppe8^T	Plant-associated N_2-fixer	AY391283
Burkholderia tuberum	STM 678^T	*Aspalathus carnosa*	AJ302311
Burkholderia unamae	TR3.4	Sugarcane	AY391283
Mesorhizobium amorphae	ICMP 11726	*Clianthus puniceus*	AY491078
Mesorhizobium ciceri	SEMIA 396	*Cicer arietinum*	AY904731
Mesorhizobium huakuii	IAM 14158	*Astragalus sinicus*	D12797
Mesorhizobium mediterraneum	UPM-Ca36^T	*Cicer arietinum*	L38825
Mesorhizobium plurifarium	LMG 7854	*Leucaena leucocephala*	X68391
Rhizobium etli	CFN 42^T	*Phaseolus vulgaris*	U28916
Rhizobium gallicum	R602sp^T	*Phaseolus vulgaris*	U86343
Rhizobium hainanensis	I66^T	*Desmodium sinuatum*	U71078
Rhizobium huautlense	USDA 4900^T	*Sesbania herbacae*	AF025852
Rhizobium leguminosarum	ICMP 14642	*Trifolium* spp	AY491062
Rhizobium leguminosarum	LMG 8820	*Phaseolus vulgaris*	X67227

Table 3.5 (continued)

Reference strain	Strain number	Host plant or relevant characteristics	GenBank Accession number
Rhizobium mongolense	USDA 1844^{T}	*Medicago ruthenica*	U89817
Rhizobium tropici	LMG 9517 ^{T}	*Phaseolus vulgaris*	X67234
Sinorhizobium fredii	LMG 6217^{T}	*Glycine max*	X67231
Sinorhizobium meliloti	SEMIA 135	*Medicago sativa*	AY904728
Sinorhizobium morelense	LMG 20571	*Leucaena leucocephala*	AJ420776
Sinorhizobium saheli	LMG 7837	*Acacia seyal*	X68390
Sinorhizobium terangae	LMG 7834^{T}	*Sesbania sp.*	X68388
Sinorhizobium xinjiangense	IAM 14142	*Vigna unguiculata*	D12796

⌘	GenBank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi.nlm.nih.gov/Genbank/]
^{T}	Type strain
CFN	Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico
IAM	Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan
LMG	BCCM™/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium
STM	Laboratoire des Symbioses Tropicales et Méditerranéennes, Montpellier, France
UPM	Universidad Politécnica Madrid, Spain
USDA	United States Department of Agriculture-ARS National Rhizobium Culture Collection

3.3 RESULTS

Putative rhizobial isolates were collected from five localities in the Free State and Mpumalanga regions of South Africa. They were obtained from two species of bean, 45 isolates from *P. coccineus*, and 73 from *P. vulgaris*. Figure 3.2 shows the occurrence of isolates from each location. The isolates were grown on YMA plates containing bromothymol blue to determine whether any of the isolates were slow-growing *Bradyrhizobia*. Medium- and fast-growing rhizobia produce an acid reaction; whereas the slow-growers produce an alkaline reaction (a pH indicator changes the colour blue for alkalinity or yellow for acidity). None of the isolates appeared to produce an alkaline reaction while the *Bradyrhizobium* reference strains did produce an alkaline reaction as expected.

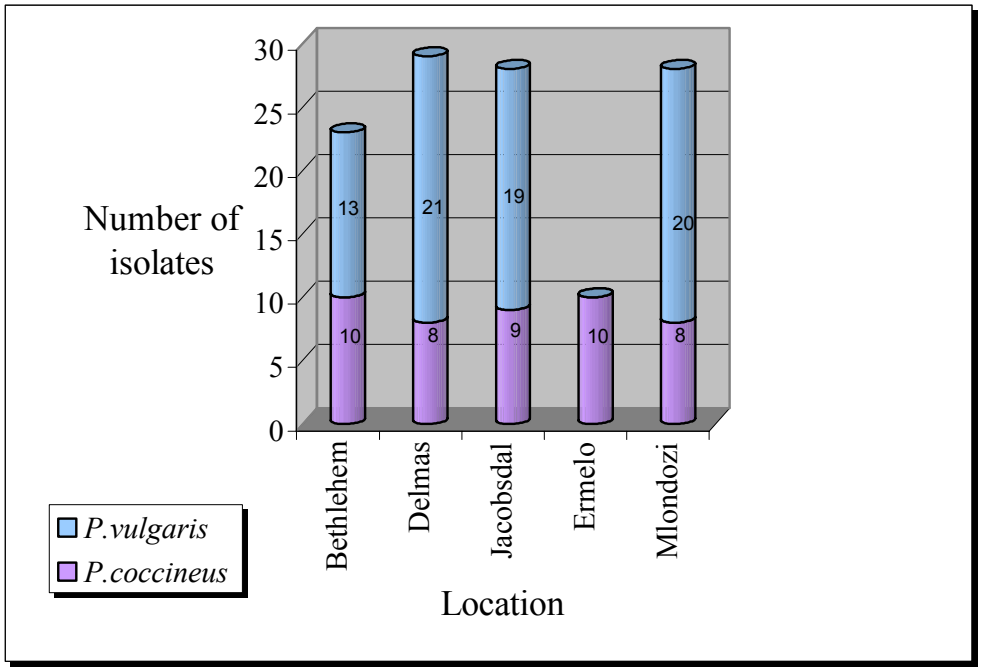


Figure 3.2 Ratio of isolates from *P. vulgaris* and *P. coccineus* obtained from the various geographic locations as indicated.

3.3.1 Rhizosphere soil properties

The rhizosphere soil composition from four of the five areas of bean production was determined (Table 3.6), but data was not available for Mlondozi. The pH range for the four rhizosphere soils was slightly acidic (pH 5.42 – 6.09). The soil compositions varied; both Jacobsdal and Ermelo soils had high amounts of sand (89% and 81% respectively), with little clay (8% and 14% respectively) and tiny amounts of silt. The Bethlehem and Delmas soils shared similar soil compositions, with a lower concentration of sand approximately 60% sand and 30% clay. The number of rhizobia in the soils varied from relatively high numbers in Delmas (5.7×10^5 cells.g^{-1}), to very low numbers in Ermelo (62.0 cells.g^{-1}). The rhizosphere soil from Ermelo was also very nutrient poor, with low concentrations of the essential elements. The concentration of total nitrogen available in the rhizosphere soil samples varied from very high values in Bethlehem (909 mg.kg^{-1}) to low values in Ermelo (319 mg.kg^{-1}). Bethlehem also had the highest concentration of fixed nitrogen (25 mg.kg^{-1}), while Ermelo again had the lowest (3 mg.kg^{-1}).

Table 3.6 Properties of rhizosphere soils

Rhizosphere soil	MPN (cells.g^{-1})	Sand (%)	Silt (%)	Clay (%)	pH (H_2O)	Total N (mg.kg^{-1})	N$^{\#}$	P	K	Ca	Mg	Na
Bethlehem	6.5 x 10^4	59	13	28	5.42	909	25	22	185	943	188	13
Delmas	5.7 x 10^5	62	8	30	5.73	751	14	18	285	713	223	8
Jacobsdal	6.0 x 10^3	89	3	8	6.09	345	19	26	253	623	188	13
Ermelo	62.0	81	5	14	5.81	319	3	8	50	310	48	5

$^{\#}$ NH_4+NO_3

3.3.2 SDS-PAGE of whole-cell proteins

In this study, the protein profiles of 118 putative rhizobial isolates obtained from two *Phaseolus* spp were characterised by SDS-PAGE. Reference strains of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* were included, as well as representatives from the genera *Burkholderia* (Figure 3.3). Several isolates clustered with reference strains of the genus *Rhizobium*. There were also isolates clustering with *Burkholderia* reference strains, but there was no significant homology with the reference strains of the genera *Azorhizobium* or *Bradyrhizobium* and only one isolate was found clustered together with the *Mesorhizobium* – *Sinorhizobium* grouping. Based on the correlation of 94% between gels using the *P. immobilis* standard, isolates sharing 94% or greater similarity were considered as representing the same bacterial strain.

The isolates and reference strains grouped into two major sections (arbitrarily defined) that could be further divided into 12 clusters and four loosely associated strains (Figure 3.3). The separation into two sections is to facilitate discussion of the results and does not imply a closer relationship between isolates in the sections.

Section I

This section was the largest; comprising eight clusters (1 – 8) with a total of 109 isolates containing representatives from all five localities. This section also contained the seven

Rhizobium reference strains, the two *Burkholderia* reference strains and the two inoculant strains.

Cluster 1

This cluster contained 19 isolates. It was further divided into six smaller groupings. All of the isolates in this cluster were obtained from *P. vulgaris*. Sub-cluster 1a contained seven isolates sharing 83.5% similarity. The four isolates in sub-cluster 1b were all obtained from Jacobsdal. Sub-cluster 1c, 1d and 1f contained three isolates each. All three isolates in sub-cluster 1d originated from Jacobsdal. Sub-cluster 1e contained the isolates KM22 and KJ10.

Cluster 2

There were eight isolates found in this cluster, which was sub-divided into two sub-clusters. Sub-cluster 2a contained five isolates, of which three originated from *P. coccineus* plants in Ermelo. The three isolates in sub-cluster 2b were obtained from the roots of *P. vulgaris* plants.

Cluster 3

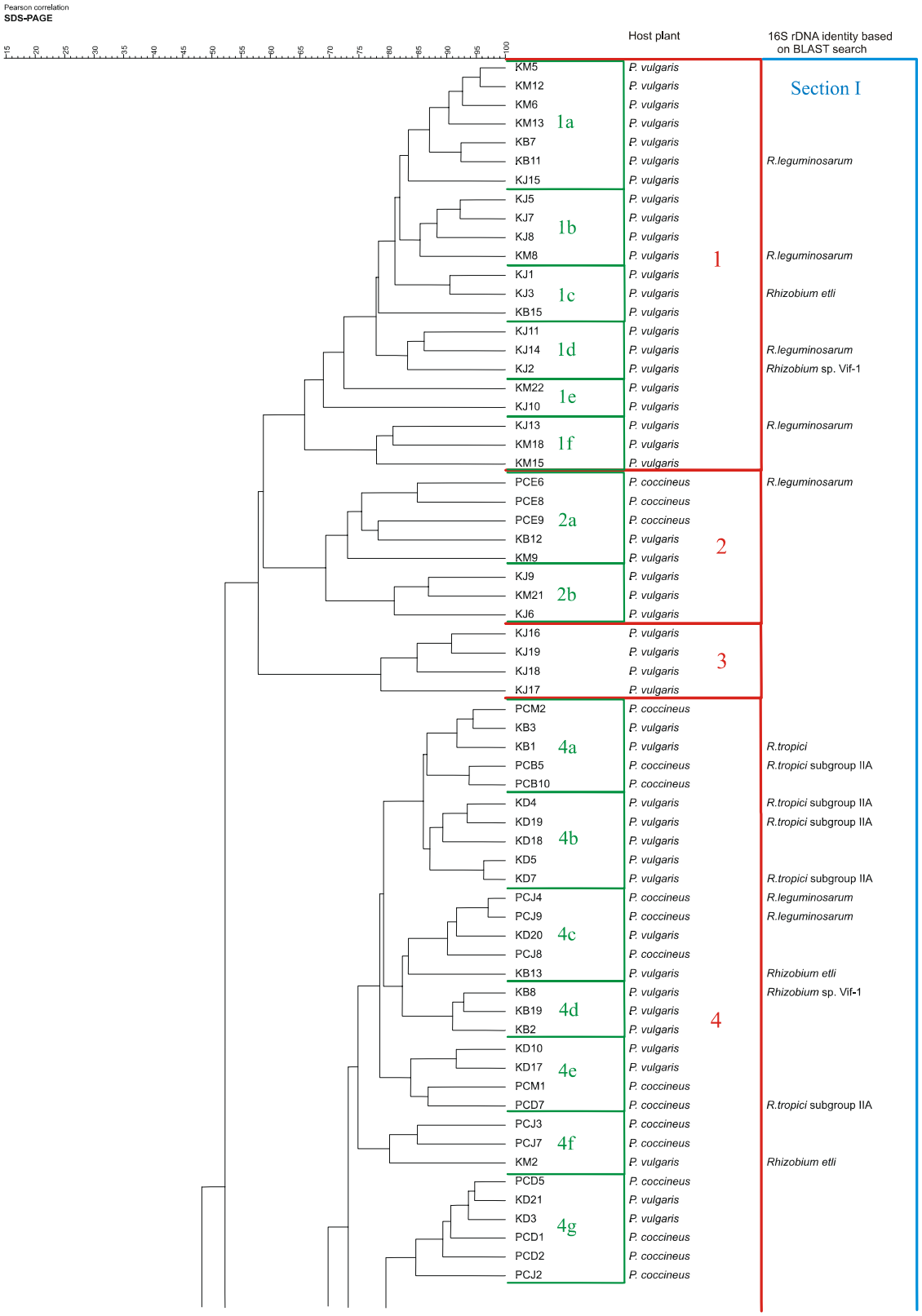
All four isolates in this grouping were obtained from the root nodules of *P. vulgaris* plants, found in Jacobsdal. KJ16 and KJ19 showed the highest similarity (90.8%), with the overall similarity of the cluster being 78.8%.

Cluster 4

Cluster 4 was the largest; it contained 42 isolates and two reference strains, ranging in similarity from 68.5% to 96.3%. The cluster contained representatives from four of the localities (excluding Ermelo) and was divided into ten sub-clusters (a – j).

Sub-cluster 4a: There were five isolates in this grouping. Four isolates originated from the Bethlehem area; KB1 and KB3 were isolated from *P. vulgaris* plants, whilst PCB5 and PCB10 were isolated from *P. coccineus* plants.

Sub-cluster 4b: This sub-cluster contained five isolates that were all obtained from *P. vulgaris* plants that originated in Delmas. The isolates KD5 and KD7 share 96.8% similarity.


Pearson correlation
SDS-PAGE
Host plant
16S rDNA identity based on BLAST search
Section I
KM5 P. vulgaris
KM12 P. vulgaris
KM6 P. vulgaris
KM13 1a P. vulgaris
KB7 P. vulgaris
KB11 P. vulgaris R.leguminosarum
KJ15 P. vulgaris
KJ5 P. vulgaris
KJ7 P. vulgaris
KJ8 1b P. vulgaris
KM8 P. vulgaris 1 R.leguminosarum
KJ1 P. vulgaris
KJ3 1c P. vulgaris Rhizobium etli
KB15 P. vulgaris
KJ11 P. vulgaris
KJ14 1d P. vulgaris R.leguminosarum
KJ2 P. vulgaris Rhizobium sp. Vif-1
KM22 P. vulgaris
KJ10 1e P. vulgaris
KJ13 P. vulgaris R.leguminosarum
KM18 1f P. vulgaris
KM15 P. vulgaris
PCE6 P. coccineus R.leguminosarum
PCE8 P. coccineus
PCE9 2a P. coccineus
KB12 P. vulgaris 2
KM9 P. vulgaris
KJ9 P. vulgaris
KM21 2b P. vulgaris
KJ6 P. vulgaris
KJ16 P. vulgaris
KJ19 P. vulgaris 3
KJ18 P. vulgaris
KJ17 P. vulgaris
PCM2 P. coccineus
KB3 P. vulgaris
KB1 4a P. vulgaris R.tropici
PCB5 P. coccineus R.tropici subgroup IIA
PCB10 P. coccineus
KD4 P. vulgaris R.tropici subgroup IIA
KD19 P. vulgaris R.tropici subgroup IIA
KD18 4b P. vulgaris
KD5 P. vulgaris
KD7 P. vulgaris R.tropici subgroup IIA
PCJ4 P. coccineus R.leguminosarum
PCJ9 P. coccineus R.leguminosarum
KD20 4c P. vulgaris
PCJ8 P. coccineus
KB13 P. vulgaris Rhizobium etli
KB8 P. vulgaris Rhizobium sp. Vif-1
KB19 4d P. vulgaris 4
KB2 P. vulgaris
KD10 P. vulgaris
KD17 4e P. vulgaris
PCM1 P. coccineus
PCD7 P. coccineus R.tropici subgroup IIA
PCJ3 P. coccineus
PCJ7 4f P. coccineus
KM2 P. vulgaris Rhizobium etli
PCD5 P. coccineus
KD21 P. vulgaris
KD3 4g P. vulgaris
PCD1 P. coccineus
PCD2 P. coccineus
PCJ2 P. coccineus

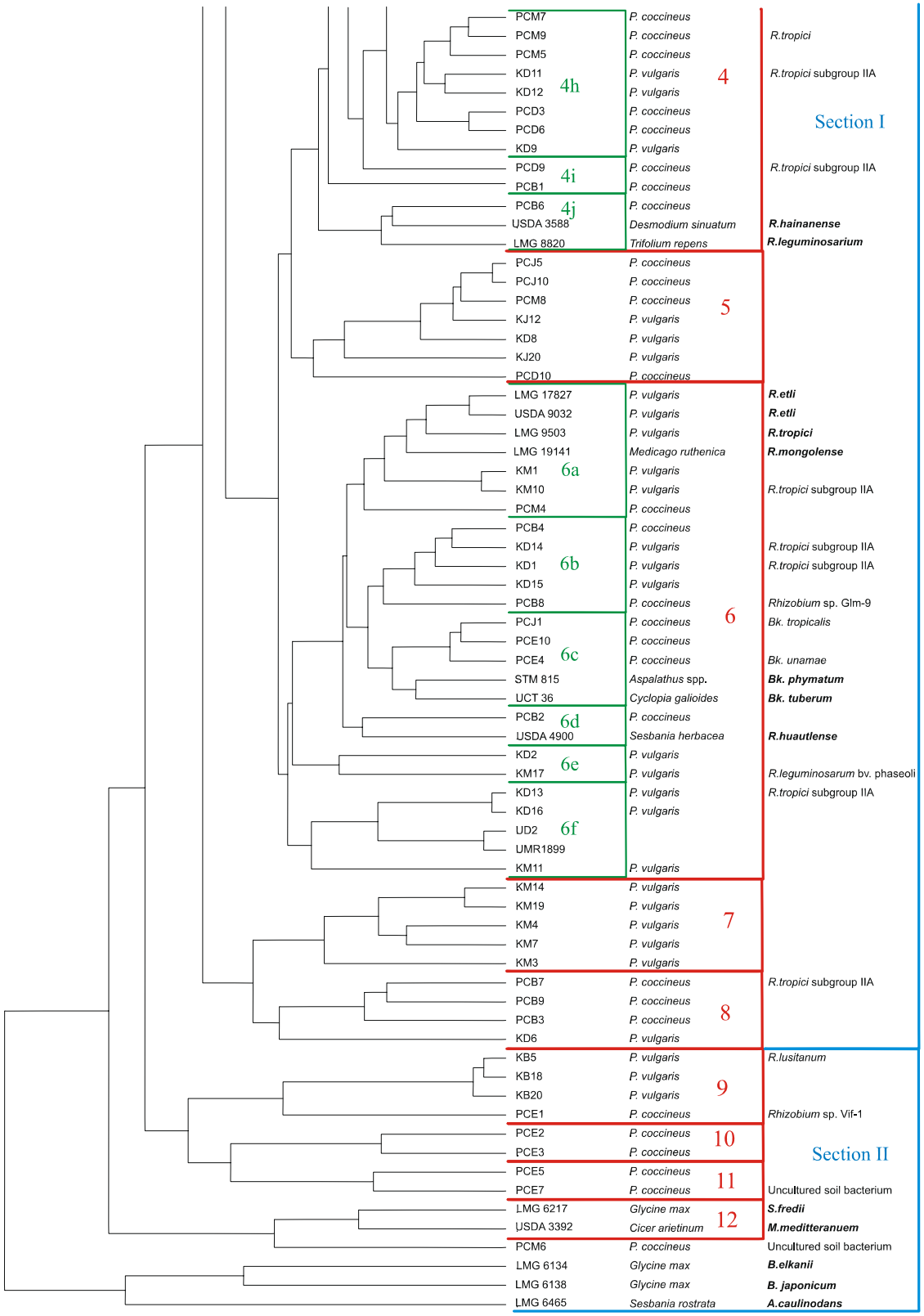


Figure 3.3 Dendrogram based on the normalized electrophoretic protein profiles of isolates and reference strains. Isolates were clustered using the UPGMA method and the Pearson correlation coefficient. The levels of similarity are expressed as percentages on the x-axis. The bacterial isolates are indicated by isolate number followed by host plant species name. The reference strains are indicated in bold.

A. Azorhizobium, *B. Bradyrhizobium*, *Bk. Burkholderia*, *M. Mesorhizobium*, *R. Rhizobium*, *S. Sinorhizobium*

Sub-cluster 4c: Contained five isolates; three of the isolates originated in Jacobsdal from *P. coccineus* plants. The isolates PCJ4 and PCJ9 shared 97.0% similarity.

Sub-cluster 4d: Three isolates that originated in Bethlehem from *P. vulgaris* plants formed sub-cluster 4d. These isolates (KB8, KB19 and KB2) share 89.8% similarity.

Sub-cluster 4e: Contained four isolates grouping at 83.9% similarity. Three isolates originated in Delmas (PCD7, KD10 and KD17) and two of those were isolated from *P. vulgaris* plants.

Sub-cluster 4f: This sub-cluster contained three isolates that shared 80.2% similarity. The isolates PCJ3 and PCJ7 were both obtained from *P. coccineus* plants in Jacobsdal.

Sub-cluster 4g: Contained six isolates, five of which originated in Delmas. This sub-cluster contained PCD5 and KD21, which shared 94.7% similarity.

Sub-cluster 4h: This sub-cluster was comprised of eight isolates, with PCM7 and PCM9 sharing 93.6% similarity and the isolates PCD3 and PCD6 sharing 93.7% similarity; these four isolates were all symbionts of *P. coccineus*.

Sub-group 4i: There were only two isolates in this sub-cluster, both originating from *P. coccineus* plants. The isolates PCD9 and PCB1 joined the sub-cluster 4h at 75.7% and 69.8% respectively.

Sub-group 4j: This grouping contained three representatives, the isolate PCB6 and the reference strains *R. hainanense* (USDA 3588) and *R. leguminosarum* (LMG 8820) joined at 78.8% similarity.

Cluster 5

There were seven isolates in this cluster ranging in similarity from 67.2% to 97.7% similarity between the isolates PCJ5 and PCJ10.

Cluster 6

This cluster contained seventeen isolates, seven reference strains (including the two *Burkholderia* reference strain) and the two inoculant strains UD2 and UMR1899.

Sub-cluster 6a: Contained four reference strains and three isolates. The two reference strains for *R. etli* (LMG17827 and USDA 9032) shared 94.0% homology. They were joined by the reference strains *R. tropici* (LMG 9503) and *R. mongolense* (LMG 19141). Three isolates (KM1, KM10 and PCM4) joined these reference strains at 75.8% homology. The isolates KM1 and KM10 shared 95.6% similarity with each other.

Sub-cluster 6b: Five isolates were found in sub-cluster 6b. Two of these were from the Bethlehem area, isolated from *P. coccineus* plants, while the other three were from *P. vulgaris* plants in the Delmas area. They grouped at an overall similarity of 79.1%.

Sub-cluster 6c: This sub-cluster contained three isolates that grouped together at 90.5%. They joined the two novel β–*Proteobacteria* reference strains, *Burkholderia phymatum* (STM 815) and *Burkholderia tuberum* (UCT 36) that shared 80.6% similarity. The overall similarity for this sub-cluster was 80.6%.

Sub-cluster 6d: Only two strains were found in this sub-cluster; isolate PCB2 and the reference strain *R. huautlaense* (USDA 4900) shared 75.5% similarity.

Sub-cluster 6e: Two isolates formed this sub-cluster; KD2 and KM17 shared 71.6% similarity.

Sub-cluster 6f: Contained three isolates and the two inoculant strains, UD2 and UMR1899. These inoculant strains were 96.2% similar to each other and the isolate KD13 showed 97.6% similarity to KD16. These two groupings were joined by KM11 at 66.9%.

Cluster 7

All five isolates in cluster 7 were isolated from *P. vulgaris* plants in Mlondozi. They grouped at 69.1% similarity, while the isolates KM14 and KM19 shared 92.9% homology.

Cluster 8

Three of the four isolates in cluster 8 were symbionts of *P. coccineus* and were obtained from Bethlehem (PCB7, PCB9 and PCB3). They shared 75.9% similarity and were joined at 61.4% by KD6.

Section II

There were four clusters in this section (9 – 12) and four loosely associated strains. This section was extremely heterogenous as it contained reference strains of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium*.

Cluster 9

This cluster contained four isolates. Three of these showed very high homology to each other, grouping at 94.4%. KB5, KB18 and KB20 were all isolated from the roots of *P. vulgaris* plants in Bethlehem. They were joined by PCE1 at 62.1% similarity.

Cluster 10

Both isolates in cluster 10 were obtained in Ermelo from *P. coccineus* plants. PCE2 and PCE3 showed 78.8% homology to each other,

Cluster 11

These two isolates were also obtained in Ermelo from *P. coccineus* plants. PCE5 and PCE7 share 77.4% homology. This cluster was joined to cluster 10 with 53.1% homology.

Cluster 12

Cluster 20 contained two reference strains, *S. fredii* (LMG6217) and *M. meditteraneum* (USDA 3392) representing two different genera sharing 74.8% similarity.

The loosely associated strains

Branching points consisting of only one isolate having a similarity of 60.0% or less to any of the other branching points within the dendrogram were considered as loosely associated. The isolate PCM6 was loosely associated with cluster 12, joining at 59.5%. The reference strain *Bradyrhizobium elkanii* (LMG 6134) and *B. japonicum* (LMG 6138) showed only 55.4% similarity to each other. They were joined by *A. caulinodans* (LMG 6465) at 35.0% and these strains attached to the rest of the dendrogram at 32.4% similarity.

Graphs revealing the distribution of isolates within the different clusters throughout the dendrogram were constructed. In Figure 3.4 the distribution of isolates from the five locations is represented, while the distribution of isolates from each host plant is displayed in figure 3.5.

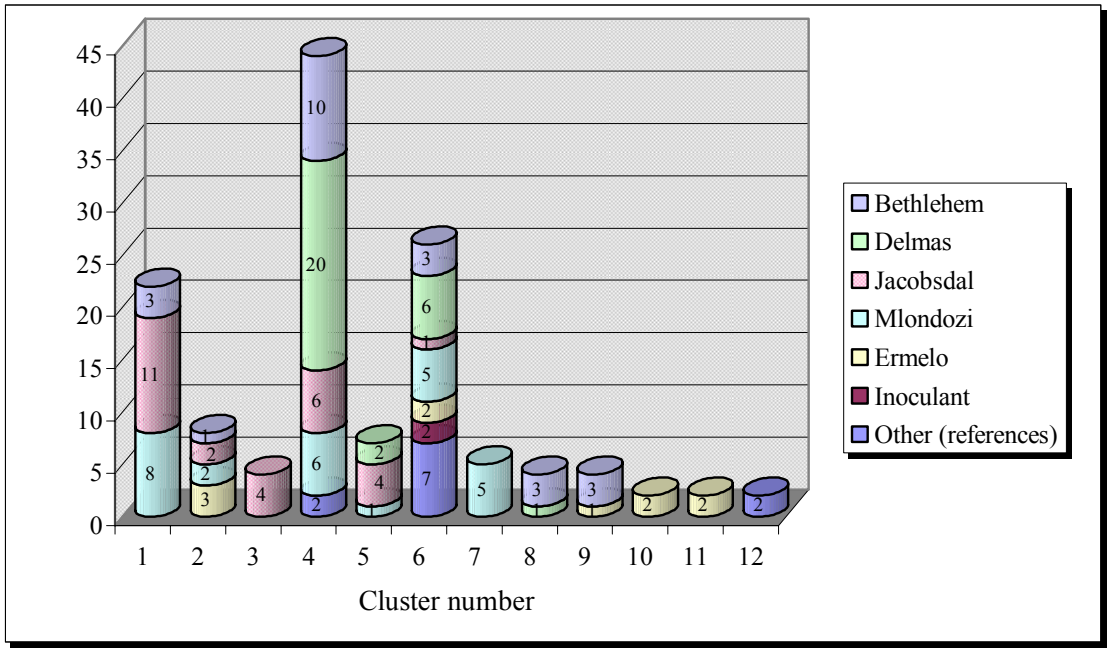


Figure 3.4 Distribution of isolates from the various geographical regions within different clusters of the SDS-PAGE tree. The colour code for each region is given in the figure legend.

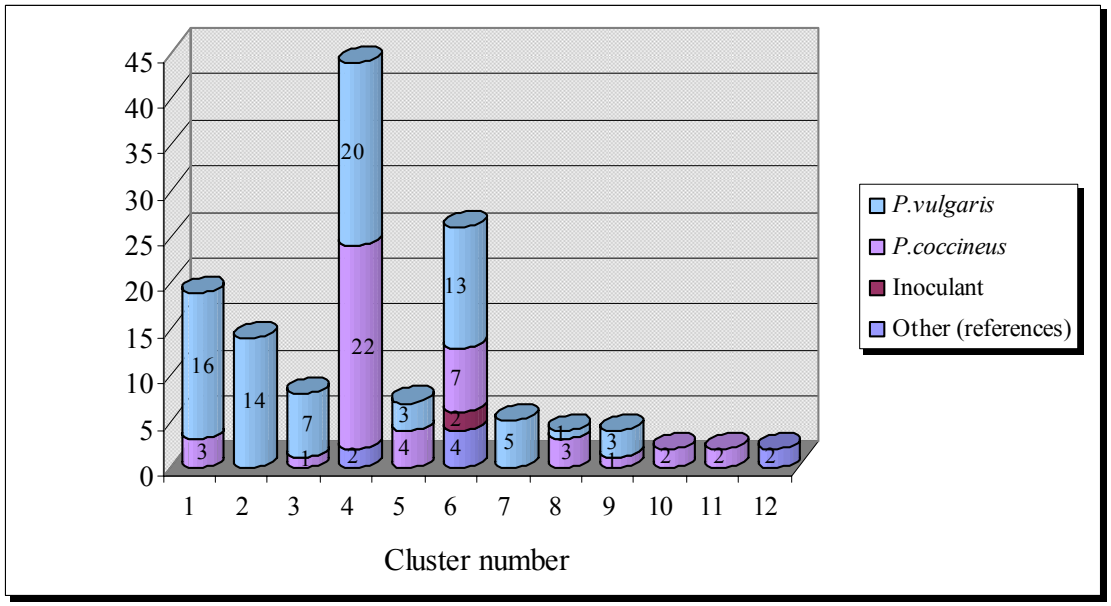


Figure 3.5 Distribution of isolates from the host plants within the different clusters of the SDS-PAGE tree. The colour code for each region is given in the figure legend. Note that the host plant for the *R. tropici* and *R. etli* reference strains is *P. vulgaris*.

3.3.3 16S rDNA amplification and partial 16S rDNA sequencing

The primers fD1 and rP2 (Weisburg *et al*., 1991) were able to amplify the 16S rRNA gene of the 32 selected isolates. The 16S rDNA amplification products were in the size range of approximately 1500 bp (results not shown).

Approximately 600 bp of the 3' end of the 16S rDNA gene was successfully sequenced for all of the selected isolates. These partial sequences were submitted to the GenBank database (www.ncbi.nlm.nih.gov/Genbank/). The results of the BLAST search and relevant accession numbers for the isolates used are in Table 3.7. A search of the NCBI's Nucleotide BLAST database, standard nucleotide-nucleotide BLAST/blastn, revealed that the majority of the *Phaseolus* isolates were found to have close homology to species within the genus *Rhizobium*, representing the species *R. tropici*, *R. etli* and *R. leguminosarum*. The isolate KB5 shows close homology to "*Rhizobium lusitanum*" (accession number AY738130) a new species not yet accepted as a valid species. A few isolates' BLAST results indicated that they were members of the genus *Rhizobium*, but they were not closely homologous to a specific species. The isolates PCE1, KB2 and KB8 were closely associated with "*Rhizobium* sp. Vif-1"; whereas other isolates showed close similarity to "Uncultured soil bacterium" (PCM6 and PCE7). There were also representatives of the genus *Burkholderia* in the β-subclass of the *Proteobacteria*; PCJ1 showed high homology to "*Burkholderia unamae*" (accession number AY391283) and PCE4 showed high homology to "*Burkholderia tropicalis*" (accession number AJ420332).

Table 3.7 The accession numbers allocated to the isolates of this study and results from NCBI♦ Nucleotide BLAST database search

Isolate	Accession number	BLAST search	% Similarity
PCD7	AY999303	*R. tropici* sub-group IIA	99%
PCD9	AY999304	*R. tropici* sub-group IIA	99%
PCM6	AY999305	"Uncultured soil bacterium"	99%
PCM9	AY999306	*R. tropici*	99%
PCJ1	AY999307	*Burkholderia unamae*	99%
PCJ4	AY999308	*R. leguminosarum*	99%
PCJ9	AY999309	*R. leguminosarum*	100%
PCB5	AY999310	*R. tropici* sub-group IIA	100%

Table 3.7 (continued)

Isolate	Accession number	BLAST search	% Similarity
PCE1	AY999311	"*Rhizobium* sp. Vif-1"	99%
PCE4	AY999312	*Burkholderia tropicalis*	99%
PCE6	AY999313	*R. leguminosarum*	99%
PCE7	AY999314	"Uncultured soil bacterium"	98%
KD1	AY999315	*R. tropici* sub-group IIA	100%
KD4	AY999316	*R. tropici* sub-group IIA	99%
KD7	AY999317	*R. tropici* sub-group IIA	100%
KD11	AY999318	*R. tropici* sub-group IIA	99%
KD13	AY999319	*R. tropici* sub-group IIA	99%
KD14	AY999320	*R. tropici* sub-group IIA	99%
KM2	AY999321	*Rhizobium etli*	99%
KM8	AY999322	*R. leguminosarum*	100%
KM10	AY999323	*R. tropici* sub-group IIA	100%
KM17	AY999324	*R. leguminosarum* bv. phaseoli	99%
KJ2	AY999325	*Rhizobium etli*	99%
KJ3	AY999326	*R. leguminosarum*	100%
KJ13	AY999327	*R. leguminosarum*	99%
KJ14	AY999328	*R. leguminosarum*	99%
KB1	AY999329	*R. tropici* sub-group IIA	99%
KB2	AY999330	*"Rhizobium* sp. Vif-1*"*	100%
KB5	AY999331	*"Rhizobium lusitanum"*	100%
KB8	AY999332	"*Rhizobium* sp. Vif-1"	99%
KB11	AY999333	*R. leguminosarum*	99%
KB13	AY999334	*Rhizobium etli*	98%

♦ National Centre for Biotechnology Nucleotide BLAST database [website address: www.ncbi.nlm.nih.gov/Genbank/]

3.3.4 Phylogenetic analysis of the 16S rRNA gene

A phylogenetic tree (Figure 3.6) was constructed by the Neighbour-joining algorithm (Saitou and Nei, 1987) in PAUP* 4.0 (Swofford, 2000). The rhizobial genera formed distinct clusters; namely the *Sinorhizobium*, *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* clusters, with species of the β-*Proteobacteria* genus *Burkholderia* belonging to an additional lineage. A substantial phylogenetic distinction between members of the α- and β-*Proteobacteria* was evident from the tree. Within the α-*Proteobacteria* there was a significant phylogenetic separation between *Azorhizobium* and *Bradyrhizobium* and the other rhizobial genera. The relatively close phylogenetic relationship between the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* was evident. The comparative 16S rDNA sequence analysis reflected the polyphyletic nature of the rhizobia.

The isolates PCJ1 and PCE4 formed a formed a well-resolved cluster with the *Burkholderia* reference strains. The branch leading to these strains was supported with a bootstrap value of 100%. These species are the novel rhizobia found in the ß-*Proteobacteria*.

Almost half of the isolates clustered on the *Rhizobium tropici* branch (KD7, KB1, KD11, KM10, PCD7, KD4, PCD9, KD1, KD13, KD14, PCB5, PCM9 and KB5), which formed a well-resolved cluster supported by a bootstrap value of 100%. Several isolates were found on the *R. leguminosarum-R.etli* branch. This included the isolates KB2, KB8 and PCE1 that showed high homology to "*Rhizobium* sp. Vif-1" with the BLAST search. This branch had a relatively low bootstrap value of 60%.

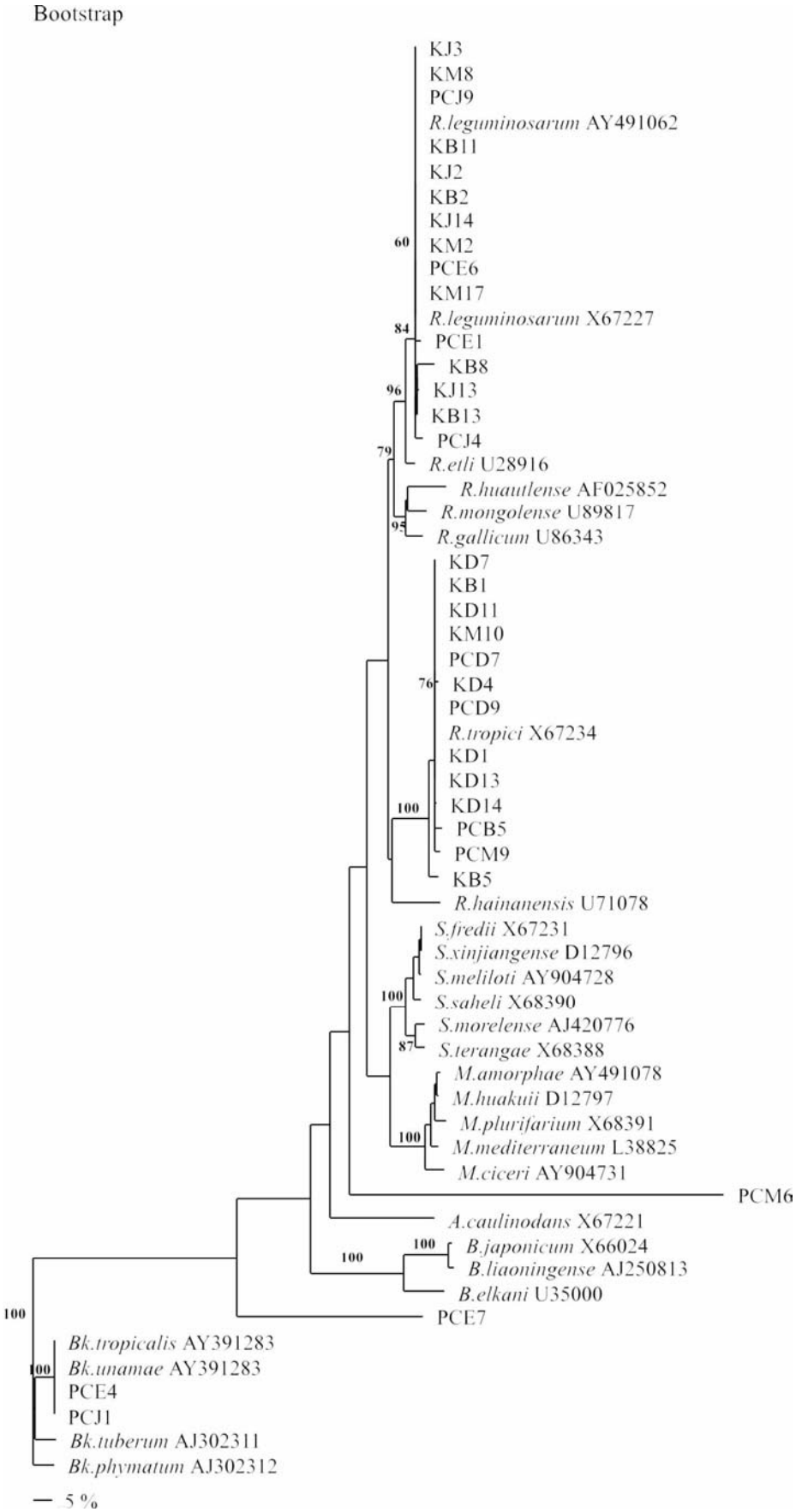

Bootstrap
KJ3
KM8
PCJ9
R.leguminosarum AY491062
KB11
KJ2
KB2
KJ14
KM2
PCE6
KM17
R.leguminosarum X67227
PCE1
KB8
KJ13
KB13
PCJ4
R.etli U28916
R.huautlense AF025852
R.mongolense U89817
R.gallicum U86343
KD7
KB1
KD11
KM10
PCD7
KD4
PCD9
R.tropici X67234
KD1
KD13
KD14
PCB5
PCM9
KB5
R.hainanensis U71078
S.fredii X67231
S.xinjiangense D12796
S.meliloti AY904728
S.saheli X68390
S.morelense AJ420776
S.terangae X68388
M.amorphae AY491078
M.huakuii D12797
M.plurifarium X68391
M.mediterraneum L38825
M.ciceri AY904731
PCM6
A.caulinodans X67221
B.japonicum X66024
B.liaoningense AJ250813
B.elkani U35000
PCE7
Bk.tropicalis AY391283
Bk.unamae AY391283
PCE4
PCJ1
Bk.tuberum AJ302311
Bk.phymatum AJ302312
5 %

Figure 3.6 (Previous page) Neighbour-joining tree showing the phylogenetic relationships of *Phaseolus* nodulating bacteria in comparison to other rhizobial genera in the α-*Proteobacteria* and the genus *Burkholderia* from the β-*Proteobacteria*. Horizontal branch lengths reflect the phylogenetic distances, while vertical branch lengths are uninformative. The scale bar indicates 5 nucleotide substitutions and bootstrap probability values of some of the major branching points are shown (As percentages of 1 000 replicas). GenBank accession numbers of the reference strains are indicated.

A. Azorhizobium, *B. Bradyrhizobium*, *Bk. Burkholderia*, *M. Mesorhizobium*, *R. Rhizobium*, *S. Sinorhizobium*

3.4 DISCUSSION

The association between indigenous rhizobial isolates and *Phaseolus* spp. was determined in this study by isolating putative rhizobial strains from the bean species *P. coccineus* and *P. vulgaris* and determining the relations among these isolates through comparison of their whole-cell protein profiles. Furthermore, partial 16S rDNA sequencing was performed to further determine the identity of representative isolates.

The indigenous isolates associated with the *Phaseolus* species all produced an acid reaction on YMA plates containing bromothymol blue, indicating that they represented fast and medium growing strains and not slow-growing bradyrhizobia. This was confirmed by the fact that none of the isolates showed any significant similarity to the whole-cell protein profiles of either of the *Bradyrhizobium* reference strains, nor did any of the 32 isolates that were subjected to partial 16S rDNA sequencing show any similarity to *Bradyrhizobium* species.

3.4.1 Comparison of rhizosphere soil properties

Environmental conditions such as soil acidity, high soil nitrogen fertility, trace elements and soil temperatures affect the symbiotic process. The failure of legumes to nodulate under acid-soil conditions is common in soils with a pH less than 5.0 (Zahran, 1999). The rhizosphere soils from this study had slightly acidic pH levels, but they all had a pH greater than 5.0, ranging from 5.4 to 6.1. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic nitrogen fixation (Bordeleau and Prevost, 1994). The species *R. tropici*, which commonly nodulates *Phaseolus* spp., has been found to be predominant in acidic soils (Laguerre *et al*., 2001).

The capacity for nitrogen fixation by a nodulated legume is influenced by mineral nitrogen in the rhizosphere soil. Combined nitrogen, in the form of nitrate and ammonia, is one of the many environmental factors that limit the development and success of the rhizobia-legume symbiosis in nature. Relatively low levels of available nitrate or ammonia promote the process of nodulation, while higher concentrations depress nodulation (Eaglesham *et al*., 1983). Rhizobia found in the rhizosphere soils at Delmas and Ermelo therefore had a higher potential for nodulation, as they have lower concentrations of combined nitrogen than the Bethlehem and Jacobsdal soil samples. When initial levels of available soil nitrogen are low, a lack of

elemental nitrogen (N_2) can reduce yield, nodulation and nitrogen fixation (Eaglesham *et al*., 1983) as the amount of nitrogen fixed is primarily controlled by the amount of available soil nitrogen (Van Kessel and Hartley, 2000). Therefore rhizobia located in the Bethlehem rhizosphere soil, with the highest concentration of nitrogen, have the greatest potential for biological nitrogen fixation (BNF).

The requirement of certain essential elements, for example calcium and phosphorous, is increased under severe stress conditions (Zahran, 1999). Phosphorus appears essential for both nodulation and nitrogen fixation (Pereira and Bliss, 1989) and specific nodule activity are directly related to the phosphorous supply (Leung and Bottomley, 1987). The rhizosphere soils from Bethlehem and Jacobsdal with high concentrations of phosphorus have an advantage over the Ermelo rhizosphere soil that had a very low level. Pereira and Bliss (1989) found variability of nitrogen fixation under low phosphorous availability between strains of *P. vulgaris*.

The rhizosphere soil samples from each location had relatively high concentrations of calcium. This element plays an essential role in bacterial cell division, elongation, and membrane structure and function (Torimitsu, *et al*., 1985). Vassileva *et al*. (1997) found that a low concentration of calcium (0.13 mM) at pH 4.5 greatly affected nodule number, nitrogenase activity and nodule structure of the common bean, *Phaseolus vulgaris*. At low pH, the addition of calcium to the soil improves both growth and ion uptake by roots (Torimitsu *et al*., 1985).

A report by Sangakkara *et al*. (1996) indicated that potassium could alleviate the effects of water shortage on symbiotic nitrogen fixation of *Vicia faba* and *P. vulgaris*. This is important in South Africa where there is a high occurrence of drought. While Delmas and Jacobsdal rhizosphere soils had high concentrations of potassium, the Ermelo rhizosphere soil had a very low concentration.

The most probable number (MPN) plant infection count was used for estimating viable cells of rhizobia. The rhizosphere soil at Delmas had a very high concentration of rhizobia, which would hinder any inoculation attempts in this region due to competition. The Ermelo rhizosphere soil, which had very few rhizobia, would not have a problem with competition.

These properties suggest that the Bethlehem and Jacobsdal rhizosphere soils would allow for optimal nodulation by rhizobia. However, considerations for suitable inoculants must take a rhizobial strain's ability to survive and nodulate under conditions that are unfavourable into account.

3.4.2 SDS-PAGE of whole-cell proteins

In this study, the isolates associated with the *Phaseolus* species clustered predominantly with *Rhizobium* reference strains. There was little similarity to the *Mesorhizobium* or *Sinorhizobium* reference strains and none of the isolates clustered significantly with the *Bradyrhizobium* or *Azorhizobium* reference strains, indicating that none of the isolates of this study represent these genera. This was not completely unforeseen as *Phaseolus* species are selective of the microsymbiont with which they form a symbiosis; preferring species from the genus *Rhizobium*. The absence of indigenous strains similar to *Azorhizobium* has been indicated in previous South African studies (Dagutat, 1995; Kruger, 1998; Joubert, 2002). Sub-cluster 6a within Section I contained three isolates that grouped with relatively high homology to the *Burkholderia* reference strains, indicating that they may be representatives of the genus *Burkholderia*. This was confirmed by sequencing analyses of the 16S rRNA gene, which grouped the isolates PCJ1 and PCE4 within the *Burkholderia* cluster of the phylogram (Figure 3.6). These results were unexpected as *Phaseolus* species were previously thought to only associate with *Rhizobium* species (Aguilar *et al*., 2001) and *Burkholderia* species were not previously known to nodulate *Phaseolus* plants. The *Burkholderia* cluster, containing members of the ß-*Proteobacteria*, was intermingled with reference strains from the α-Proteobacteria (*Rhizobium*) in the SDS-PAGE dendrogram. Kersters (1985) found that comparative protein electrophoresis is of little value when comparing distantly related bacteria, which may explain why bacteria from the β-*Proteobacteria* are intermingled with α–*Proteobacteria*. The relationships within this sub-cluster will have to be resolved using additional techniques.

None of the isolates of this study clustered with *Bradyrhizobium* reference strains, produced an alkaline reaction or showed homology to *Bradyrhizobia* based on 16S rDNA. It is therefore evident that none of the isolates used in this study were members of the genus *Bradyrhizobium*.

The isolate PCM6 was loosely associated with the *Mesorhizobium* and *Sinorhizobium* reference strains, yet it is not clear whether it is a member of either of these genera. Clustering of isolates may be ambiguous if there is not sufficient representation of different species of a particular genus. Including isolates similar to PCM6 or additional members of these genera may aid in resolving this cluster. Further analysis of this isolate is required.

Several isolates shared 94% similarity or more and they appear to represent the same strain of the same species. These include the isolates KM5 and KM12; PCM2 and KB3; KD5 and KD7; PCJ4 and PCJ9; PCD5 and KD21; PCJ5 and PCJ10; the two *R. etli* reference strains (LMG17827 and USDA 9032); KM1 and KM10; KD13 and KD16; the two inoculant strains, UD2 and UMR1899; and the three strains from Bethlehem, KB5, KB18 and KB20.

SDS-PAGE is a reliable method for comparison and grouping of large numbers of closely related isolates (Priest and Austin, 1993; Vauterin *et al*., 1993). Based on their protein profiles alone, many of the isolates are clearly differentiated and cannot be assigned to any of the previously recognized species. The identity of these isolates should be clarified by further genotypic studies.

3.4.3 The clustering of isolates in relation to host plant species and geographic location

The bar graphs (Figure 3.4 and 3.5) that indicate the distribution of isolates allow visual understanding and easy interpretation of the dendrogram. When comparing the clusters it became apparent that a degree of specificity exists between both the host plant and location of origin. The geographic location played a role in the protein electrophoretic grouping of the isolates in several clusters (3, 7, 8, 9, 10 and 11) and sub-clusters (4b and 4d).

The host plant of origin also appears to have had an effect on the clustering; however, the isolates were only obtained from two different species of host bean plants, so the sample size was small. Additionally, there were nearly twice as many isolates from *P. vulgaris* than from *P. coccineus* plants, which must be taken into consideration. Isolates grouping according to host plant are found in clusters 3, 7, 8, 9, 10 and 11 and sub-clusters 1b, 1d, 2a, 3, 4b, 4d and 6c. Various clusters grouped according to both geographic locations as well as host specificity, including clusters 3, 7, 8, 9, 10 and 11 and sub-clusters 4b and 4d.

SDS-PAGE is useful for the initial grouping of large numbers of highly related strains into clusters having potential species status. Protein electrophoresis is more rapid than DNA re-association and has the advantage that clusters are formed from complete similarity matrices. This data would be enhanced by the addition of genotypic data to further differentiate these isolates and to get clarity on the status of clusters above the species level. Some of these isolates may represent new species within the rhizobial group or may belong to taxa outside this group.

3.4.4 Phylogenetic analysis

Under standardized conditions a degree of variation may exist for SDS-PAGE analyses (Vauterin *et al*., 1993) and techniques such as 16S rDNA sequencing are not liable to such variation, therefore partial 16S rDNA sequencing may enhance the results obtained from SDS-PAGE analysis, however, partial 16S rDNA sequences can be used to identify and assign organisms to phylogenetic groups but not to draw phylogenetic conclusions (Ludwig *et al*., 1998).

The phylogenetic tree was composed of two distinct lineages representing members of the α- and β-subclasses within the *Proteobacteria*. Within the α-subclass of the *Proteobacteria*, the genus *Bradyrhizobium* was clearly differentiated from the faster-growing rhizobial genera, including representatives of the *Rhizobium*, *Sinorhizobium* and the *Mesorhizobium*. The β-subclass of the *Proteobacteria* was represented by reference strains from the genus *Burkholderia*.

The partial 16S rDNA sequences were predominantly homologous to species within the *Rhizobium* genus. The 16S rRNA gene is highly conserved and limits the discriminatory power of 16S rDNA sequencing, restricting the resolution when comparing highly related organisms (Vandamme *et al*., 1996), therefore sequencing of the 16S rRNA gene is unable to resolve the position of closely related strains of a species (Leblond-Bourget *et al*., 1996; Vandamme *et al*., 1996). The *Rhizobium* species represented by the isolates of this study (*R. tropici*, *R. leguminosarum* bv. *phaseoli* and *R. etli*) are commonly associated with *Phaseolus* species. The higher occurrence of isolates with similarity to *R. tropici* than to *R. etli* may be explained by the fact that the isolates were all obtained from slightly acidic soils, and *R. tropici* has been found to be predominant in acidic soils (Laguerre *et al*., 2001). Rhizobia

nodulating common bean in South African soils were reported to be taxonomically related to *R. tropici* (Dagutat and Steyn, 1995).

According to Aguilar *et al*. (2001), *P. vulgaris* is highly discriminating with regard to the microsymbiont it associates with in nature. This study seems to support their findings, as the majority of isolates from *Phaseolus* spp. in this study represent *Rhizobium* species. Diouf and colleagues (2000) found indications that the bean rhizobia population in Africa has a restricted genetic diversity due to the fact that beans are an introduced crop in Africa. However, the isolates PCJ1 and PCE4 show high homology to *Burkholderia* species members of the β-*Proteobacteria*. *Burkholderia* species are not known to nodulate *Phaseolus* plants, but Gillis *et al*. (1995) isolated the nitrogen-fixing species *Burkholderia vietnamensis* from acidic soil. Therefore, while unexpected, the presence of these isolates is not altogether inexplicable.

The *Mesorhizobium* and *Sinorhizobium* reference strains each formed two distinct clusters, both supported with high bootstrap values. As was expected, none of the isolates from this study were found on these branches. The *Mesorhizobium* cluster was supported by a bootstrap value of 100, as was the *Sinorhizobium* cluster. There was very little distinction between the species *Sinorhizobium fredii* and *S. xinjiangense* in the *Sinorhizobium* cluster, as has been noted previously by Tan *et al*. (2001), Wei *et al*. (2002) and Yao *et al*. (2002), which raised questions about the taxonomic validity of *S. xinjiangense*.

Neither of the isolates PCM6 and PCE7 appear to be related to any of the known species of rhizobia, as they showed no significant phylogenetic relationship with any of the other rhizobia in the compiled phylogram and the BLAST search of their sequences showed high similarity to “Uncultured soil bacterium”. The isolate PCM6 is clearly divergent from the other isolates on the phylogram.

The 16S rDNA sequences of the *Rhizobium* genus display high similarity values, which makes it difficult to resolve the close relationships within the genus. The use of DNA-DNA hybridisation would help to clarify the position of the isolates from *Phaseolus* spp., as this method is still an important criterion for species delineation (Stackebrandt and Goebel, 1994).

3.5 CONCLUSION

SDS-PAGE was shown to be a useful method for the grouping of large numbers of highly related strains into clusters having potential species status. The clustering of isolates appears to have been affected by geographic location and host plant. Most of the isolates investigated belonged to the fast-growing *Rhizobium* genus, with some showing affinity to the ß-*Proteobacteria* genus *Burkholderia*. The comparison of the SDS-PAGE and 16S rDNA sequencing data confirmed that bacterial discrimination using SDS-PAGE is possible at and below the species level, but is not useful at the genus level and higher. This was evident from the occurrence of clusters containing potential *Burkholderia* strains amongst clusters containing *Rhizobium* strains. The isolates underwent further genotypic analysis to clarify their taxonomic position.

CHAPTER 4

GENOMIC DNA FINGERPRINTING OF PUTATIVE RHIZOBIA USING rep-PCR AMPLIFICATION

4.1 INTRODUCTION

Symbiotic, nitrogen-fixing bacteria interact with legumes in a readily identifiable manner by producing root nodules. In mature nodules, nitrogen fixation and ammonia assimilation occur (Caetano-Anollés, 1997). The taxonomy of the root-nodulating bacteria has changed considerably in the last few decades as new techniques are engaged and a greater variety of legumes are studied. It has recently become clear that the ability to nodulate and fix nitrogen is not restricted to the α-*Proteobacteria*, but that several species in the β-*Proteobacteria* (in the genera *Burkholderia* and *Ralstonia*) acquired the ability as well (Chen *et al*., 2001; Moulin *et al*., 2001; Vandamme *et al*., 2002). The first systematic investigation into the identity and taxonomy of root nodule bacteria associated with legumes in South Africa was carried out by Dagutat (1995).

Common bean (*Phaseolus vulgaris*) is one of the most important legumes grown for human consumption yet it is considered to be a poor fixer of N_2 compared to other legumes such as soybean and lupin (Hardarson, 1993). Nitrogen fixation by *P. vulgaris* should not be overlooked, as research by Hardarson (1993) showed that on average 40% of bean nitrogen could be derived from the atmosphere. A single wide-spectrum *Rhizobium* strain, UD2, is currently used to inoculate species of *Phaseolus* planted in South Africa. Inoculation experiments with UD2 gave erratic results (Strijdom 1998) and N_2-fixing effectiveness of this strain is poor compared to that of other inoculant strains (I.J. Law, personal communication). An alternative strain, UMR1899 was found to be effective on species of bean, but is less effective on common bean than strain UD2. Previous studies on rhizobia associated with leguminous plants in South Africa have helped to improve our knowledge of South African root-nodulating bacteria (Dagutat, 1995; Kruger, 1998; Le Roux, 2002; Jaftha, 2002; Kock, 2004), but none of these studies focused on *Phaseolus* spp. and there is a need for better inoculant strains for beans in South Africa (I.J. Law, personal

communication). The survey of rhizobial populations in selected rhizosphere soils of bean production areas will yield information concerning the genetic composition of bean rhizobia in South African soils and ultimately contribute to the identification of a suitable indigenous inoculant for use in bean production in South Africa.

The widely planted common bean (*P. vulgaris*) and the red kidney bean (*P. coccineus*; from the Latin *coccineus*, meaning scarlet) were used in this study. These beans are not indigenous to South Africa; they were introduced to Africa from South America. Symbionts of bean are genetically heterogeneous and effective populations of rhizobia exist in bean production areas, therefore it is highly probable that indigenous rhizobia can nodulate beans (Strijdom, 1998).

Repetitive sequence-based PCR is a powerful tool, as it allows the fingerprinting of individual genera, species and strains. This technique has been used repeatedly for the study of rhizobia. Combinations of BOX-, ERIC- (Enterobacterial Repetitive Intergenic Consensus) and REP-PCR (Repetitive Extragenic Consensus) patterns have been used to type *R. meliloti* (de Bruijn, 1992) and to determine the genetic variability within rhizobial isolates (Laguerre *et al*., 1997). The method was used to study *Bradyrhizobium* isolates (Vinuesa *et al*., 1998; Sikora *et al*., 2002) and to determine the diversity of *R. etli* strains which nodulate *P. vulgaris* in Argentina (Aguilar *et al*., 2001).

The aim of this study was to investigate the diversity of root nodule-associated bacteria of two *Phaseolus* species in South Africa. The root nodules of *Phaseolus* plants were collected from various bean-growing regions in South Africa and the bacterial strains were isolated from these root nodules. These isolates were previously characterised using SDS-PAGE of whole-cell proteins (Chapter 3). In this study, rep-PCR was used to differentiate between the strains from these plants. The isolates were compared to known rhizobia genera to obtain a classification of strains. Type and/or reference strains of the genus *Rhizobium* and the ß-*Proteobacteria* genus *Burkholderia* were included in the rep-PCR analysis, as well as the two current inoculants for *Phaseolus* species in South Africa, UD2 and UMR1899.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains used and maintenance

One hundred and eighteen putative rhizobial strains obtained from the rhizobial collection of the ARC-Plant Protection Research Institute (Private Bag X134, Pretoria 0001, South Africa) courtesy of Dr Ian Law were previously obtained from the root nodules of two *Phaseolus* species. These strains were used in the SDS-PAGE analyses and are listed in Table 3.1 in Section 3.2.1. The inoculant strains UD2 and UMR 1899, also obtained from the ARC-Plant Protection Research Institute were included in this study. Reference cultures from the *Rhizobium* genus were obtained from the bacterial culture collections of the Laboratorium voor Microbiologie (LMG), Belgium and the United States Department of Agriculture-ARS (USDA) *Rhizobium* Culture Collection, Maryland, USA and from Laboratoire des Symbioses Tropicales et Méditerranéennes (STM), Montpellier, France. A *Burkholderia* reference strain was donated by Dr M.M. Kock, University of Pretoria (Kock, 2004) [Table 4.1].

The purity of cultures was verified by repeated streaking of single colonies on YMA plates supplemented with Congo red medium, as well as by microscopic examination of Gram-stained cells and phase contrast microscopy of wet preparations. Purified cultures were maintained on YMA slants at 4°C. For long-term storage, cultures were grown in yeast extract broth (YMB) for 5 – 7 days at 28°C with rigorous shaking. The resultant suspensions were mixed 1:1 with 50% (v/v) glycerol in sterile cryotubes and stored at both –20° and –70°C.

Table 4.1 List of rhizobial reference strains analysed for the rep-PCR study.

Reference strain	Strain number	Host plant
Rhizobium etli bv. phaseoli	LMG 17827^{T}	*Phaseolus vulgaris*
Rhizobium hainanense	USDA 3588^{T}	*Desmodium sinuatum*
Rhizobium huautlense	USDA 4900^{T}	*Sesbania herbacea*
R. leguminosarum bv. trifolii	LMG 8820^{T}	*Trifolium repens*
Rhizobium mongolense	LMG 19141^{T}	*Medicago ruthenica*
Rhizobium tropici	USDA 9030^{T}	*Phaseolus vulgaris*
Burkholderia phymatum	STM 815^{T} ✧	*Aspalathus* spp.
Burkholderia tuberum	UCT36*	*Cyclopia galioides*

^{T} Type strain

LMG Laboratorium voor Microbiologie Gent Culture Collection

USDA United States Department of Agriculture-ARS National Rhizobium Culture Collection

STM Laboratoire des Symbioses Tropicales et Méditerranéennes

NS Not stated

✧ The type strain for *Burkholderia phymatum* was donated by C. Boivin-Masson, 34398 Montpellier Cedex 5, France.

* The strain for *Burkholderia tuberum* was a gift from Dr M. M. Kock, University of Pretoria

4.2.2 Extraction of genomic DNA

A method for proteinase-K (Roche Molecular Biochemicals) treated cells as described by Lemanceau *et al*. (1995) and modified by Laguerre *et al*. (1997) as previously described in Section 3.2.4 (ii) was used. Isolates were incubated on tryptone yeast extract medium (TY) agar slants at 28°C for 5 days. TY reduces slime formation by the rhizobia. The cells were harvested by adding sterile double distilled water and gently shaking until the cells were suspended. Cell suspensions were transferred to sterile plastic tubes and vortexed to ensure a uniform suspension. The optical density (OD) of cell suspensions was measured at 620 nm using the sterile water as a blank. The number of cells needed for genomic extraction was calculated using the formula

V (µl) = 0.2/ OD_{620} x 1000. This results in a DNA concentration of approximately 50 µg/ml. The appropriate volume of cells was transferred to a clean Eppendorf tube and centrifuged at 12 000rpm (x g) for 5 minutes. The supernatants were discarded and the pellet blotted dry. The cell pellet was resuspended in 100 µl of ultra-high quality (UHQ) water, 100 µl Tris-HCl (10 mM, pH 8.2) and 10 µl proteinase K (15.6 mg/ml) (Roche Molecular Biochemicals) and incubated overnight at 55°C. The proteinase K was inactivated by heating the suspension for 10 minutes at 96°C. The cell lysates were stored at -20°C.

4.2.3 rep-PCR amplification

Each PCR amplification was carried out in 50 µl reaction volumes containing 5 µl of the cell lysate, 50 pmol of each primer, 250 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Supertherm Taq DNA polymerase (SouthernCross Biotechnology). The primers used for BOX, ERIC and REP PCR respectively are listed in Table 4.2. Amplifications for all three types of PCR reaction were carried out in an Eppendorf Mastercycler gradient thermocycler using the following general thermal profile for all three types of PCR reactions:
An initial denaturing step at 95°C for 7 minutes; followed by 35 cycles of denaturation at 94°C for 1 minute, annealing for 1 minute at a different temperature for each different type of PCR, extension at 65°C for 8 minutes and a final extension at 65°C for 16 minutes. The annealing temperatures are listed below with variations to this profile.

BOX PCR amplification: The thermal profile as described by Versalovic *et al*. (1994) was used, with an annealing temperature of 53°C. The final extension was 15 minutes.

ERIC PCR amplification: The thermal profile as described by de Bruijn (1992) was used, using 35 cycles of denaturation instead of 30. The annealing temperature used was 52°C.

REP PCR amplification: The thermal profile as described by de Bruijn (1992) was used, using 35 cycles of denaturation instead of 30. The annealing temperature used was 44°C instead of 40°C.

Table 4.2 Primers used in this study

Primer❖	Sequence (5′ to 3′)	Target region	Reference
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C	ERIC region	Versalovic *et al.*, 1991
ERIC2	AAG TAA GTG ACT GGG GTG AGC G	ERIC region	Versalovic *et al.*, 1991
REP1R-I	III ICG ICG ICA TCI GGC*	REP region	Versalovic *et al.*, 1991
REP2-I	ICG ICT TAT CIG GCC TAC*	REP region	Versalovic *et al.*, 1991
BOXA1R	CTA CGG CAA GGC GAC GCT GAC G	BOX region	Versalovic *et al.,* 1994
NodA1	TGC RGT GGA ARN TRN NCT GGG AAA*	*nodA* gene	Haukka *et al*., 1998
NodA2	GGN CCG TCR TCR AAW GTC ARG TA*	*nodA* gene	Haukka *et al*., 1998

❖ All primers were synthesized by Roche Molecular diagnostics, Mannheim, Germany

* I = Inosine, B= G/C/T, K= G/T, N= A/G/C/T, R= A/G, S= G/C, W= A/T

(i) Analyses of the rep-PCR DNA fingerprints

Aliquots of the PCR products (10 µl) were examined by horizontal gel electrophoresis using 1.5% (m/v) agarose gels (Promega) in a 1 x TAE buffer (40 mM Tris-HCl, 20 mM NaOAc and 1 mM EDTA pH 8.5) stained with ethidium bromide (10 mg/ml). PCR products were visualised by UV fluorescence. The standard marker 1 Kb PLUS DNA Ladder (GibcoBRL®) was included on each gel (four lanes per gel of 22).

Images of the gels were captured in a format compatible with the GelCompar II computer programme (Applied Maths, Kortrijk, Belgium). Analysis was performed using the GelCompar II computer programme. The standard marker 1 Kb PLUS DNA Ladder (GibcoBRL®) was used as the standard to normalise the gels by aligning the four lanes within each gel.

The dice correlation coefficient (Nei and Li, 1979) was used to calculate a distance matrix and the unweighted pair group method with arithmetic mean (UPGMA) was used to construct

individual dendrograms for each rep-PCR type. The final dendrogram was constructed by combining the BOX, ERIC and REP data sets, as described by Vinuesa *et al*. (1998). The similarity between organisms was calculated as the average from the three experiment types, and the unweighted pair group method of arithmetic averages was used to cluster strains.
To determine reproducibility, rep-PCR amplifications and electrophoresis of the same samples were repeated independently.

(ii) Comparison of SDS-PAGE data and combined rep-PCR data

The clustering of isolates was compared between the dendrograms derived from SDS-PAGE and combined rep-PCR. The attributes of these two dendrograms are discussed in section 4.4.3.

4.2.4 Amplification and sequencing of the *nodA* gene

Amplification of the *nodA* was employed to determine whether isolates from Section III of the rep-PCR dendrogram (Figure 4.1, section 4.3.2) were nodulators or contaminants. The partial *nodA* gene of selected isolates was amplified with the primers NodA1 and NodA2 (Table 4.2). The PCR mixture contained: 5 µl of the cell lysate, 50 pmol of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 U Gold Taq DNA polymerase (Southern Cross Biotechnology) in a 50 µl reaction volume. The PCR reactions were done on an Eppendorf thermocycler using the following thermal profile: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (50°C for 45 seconds) and extension (72°C for 1 minute). This was followed by a final extension step at 72°C for 5 minutes.

The concentration, purity and size of the products were evaluated by running an aliquot (5 µl) of each reaction on 0.9% (m/v) horizontal agarose gels (Promega) (results not shown). The standard marker molecular marker VI (Roche Molecular Biochemicals) was included on each gel. Where additional products of amplification were observed, the desired band was excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) as described by the manufacturer.

The purified *nodA* products were sequenced with the forward primer NodA1 (Haukka *et al*., 1998). Sequencing was carried out in 10 µl reaction volumes: Approximately 50 ng purified 16S rDNA template, 2 µl BigDye^{TM} Terminator RR Mix (PE Applied Biosystems), 10 pmol of the primer. The sequencing reaction was carried out in an Eppendorf Mastercycler gradient thermocycler with an initial denaturation at 96°C for 5 seconds followed by 25 cycles of denaturation (96°C for 10 seconds), annealing (50°C for 5 seconds) and extension (60°C for 4 minutes). Sequencing samples were submitted to the sequencing facility of the University of Pretoria for electrophoresis on the Perkin-Elmer ABI 377 Automated Sequencer.

4.3 RESULTS

4.3.1 rep-PCR amplification

In this study, repetitive sequence-based PCR (BOX, ERIC and REP) was used for the characterisation of a large group of isolates by including reference strains of various species of the genus *Rhizobium* as well as a representative from the genus *Burkholderia*. The banding patterns of the BOX-, ERIC- and REP-PCR for each isolate are indicated in Figure 4.1. Partial *nodA* sequences were determined for the isolates in section III of the combined rep-PCR dendrogram.

The ERIC-PCR amplifications were originally carried out using the primer set ERIC-1R and ERIC2, but difficulties were encountered. Sequential PCR reactions gave erratic results. On repeating experiments, many isolates were not amplified or lacked specific bands resulting in differing patterns. Versalovic *et al*. (1991) and Niemann *et al*. (1997) had also encountered similar problems using this primer pair. They found that the ERIC2 primer generated most of the PCR products in ERIC-PCR and that the ERIC1 primer hampered amplification therefore Niemann *et al*. (1999) removed the ERIC1 primer from the reaction. Their approach was used in this study by omitting the ERIC1 primer to simplify the PCR reaction.

The PCR amplification using the BOX primer generated the highest number of bands, containing 12 to 20 bands per strain, with an average of 15 bands per strain. The size of the amplified

products ranged from 200bp to 5 000bp (estimated using the DNA molecular weight marker as a standard), but the majority of these fragments were found in the range of 500bp to approximately 3 000bp.

In amplification reactions with the ERIC primer, the number of DNA fragments ranged from 8 – 16 per strain, averaging 10 bands per strain. The size of the amplified products ranged from 200bp to approximately 4 500bp. The majority of these fragments were detected in the range of 200 – 2 000bp.

The REP primers generated a larger number of bands than the ERIC primer, but less than the BOX primer. These fragments were 100bp – 5 000bp in size, and were more dispersed than either BOX- or ERIC -PCR fragments, with most isolates having a range of small to large DNA fragments. REP fragments of both 400bp and 1 000bp were commonly found in most of the isolates. There were 8 – 18 bands per isolate, averaging 12 bands per strain.

The bands found to be evident in a large number of isolates and easily distinguished and intense were considered “major” bands, while those that were less intense and less common were considered as “minor” bands. This was a visual observation to aid in the discussion and was not a consideration in the analysis of the banding patterns. Although it was often found that “minor” bands were difficult to visualise or “major” bands were found to be less intense on repeating the experiment, constant reaction conditions yielded good reproducibility and the characteristic bands were still evident. The reproducibility was found to be in the range of 88% to 90% with BOX-PCR fingerprinting found to have the highest reproducibility.

4.3.2 Analyses of the rep-PCR DNA fingerprints

Three individual dendrograms were constructed from the profiles of each primer set (results not shown). There was generally good correlation between clusters in these dendrograms, but some strains showed no association between experiment types. To maximise strain discrimination, a dendrogram was generated from combined BOX-, ERIC- and REP-PCR data sets.

The isolates and reference strains grouped into three major sections (arbitrarily defined) that could be further divided into 16 clusters and three loosely associated strains (Figure 4.1).

Section I contained 71 isolates, the two inoculant strains and the six *Rhizobium* reference strains. Section II comprised 34 isolates and no reference strains and section III had 13 isolates and the reference strain, *Burkholderia phymatum* (STM 816) and the *Burkholderia tuberum* isolate (UCT36).

Section I

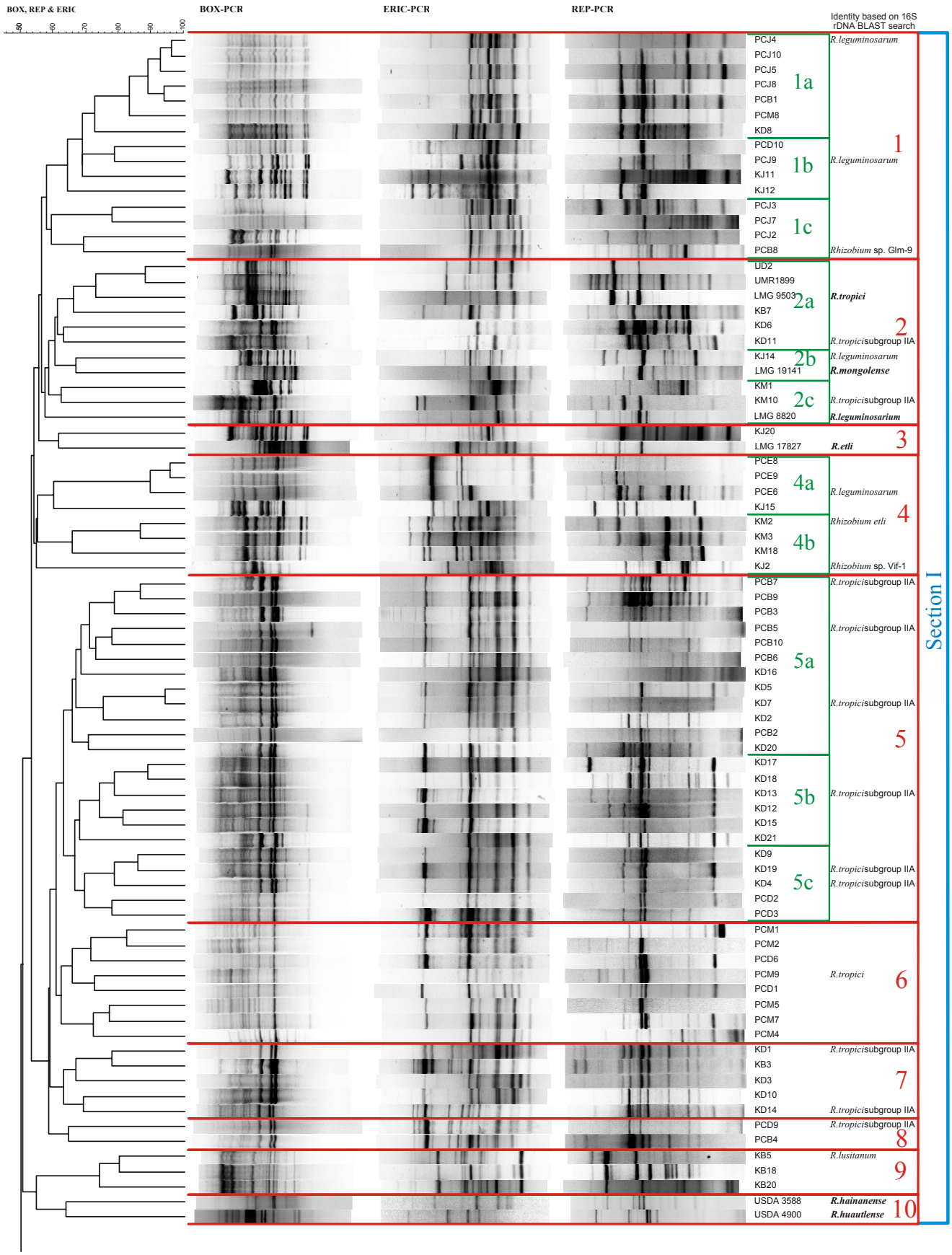
This section was the largest, comprising ten clusters (1 – 10) as well as all six of the *Rhizobium* reference strains and the two inoculant strains. There was a total of 71 isolates in this section, which contained representatives from all five localities.

Cluster 1

This cluster contained 15 isolates, grouping at an overall similarity of 57.8% and could be further divided into two subgroups. The isolates of cluster 1 had characteristic bands for all three fingerprint types. The BOX pattern had common bands of 400, ~ 750 and 1 000bp.

Sub-cluster 1a: Consisted of eleven isolates, which joined at 64.0%. The isolates PCJ4, PCJ10, PCJ5, PCJ8 and PCB1 all shared significant homology (89.0%). The BOX fingerprinting patterns for these five isolates were nearly identical. Differences were found in the ERIC and REP patterns of these isolates, which had characteristic bands, but had slight variation in the minor bands.

Sub-cluster 1b: Contained four isolates from *P. coccineus* that grouped together at 59.2%. Three of these isolates were obtained from Jacobsdal. PCJ3 and PCJ7 were the most related, with matching bands for BOX and similar bands for ERIC. While the REP patterns for PCJ3 and PCJ7 looked different, they contained many similar bands of different intensities.


BOX, REP & ERIC
50
60
70
80
90
100
BOX-PCR
ERIC-PCR
REP-PCR
Identity based on 16S rDNA BLAST search
PCJ4
PCJ10
PCJ5
PCJ8
PCB1
PCM8
KD8
1a
R.leguminosarum
PCD10
PCJ9
KJ11
KJ12
1b
R.leguminosarum
1
PCJ3
PCJ7
PCJ2
PCB8
1c
Rhizobium sp. Glm-9
UD2
UMR1899
LMG 9503
KB7
KD6
2a
R.tropici
KD11
R.tropici subgroup IIA
2
KJ14
2b
R.leguminosarum
LMG 19141
R.mongolense
KM1
KM10
2c
R.tropici subgroup IIA
LMG 8820
R.leguminosarium
KJ20
LMG 17827
R.etli
3
PCE8
PCE9
PCE6
KJ15
4a
R.leguminosarum
KM2
Rhizobium etli
4
KM3
KM18
4b
KJ2
Rhizobium sp. Vif-1
PCB7
PCB9
PCB3
PCB5
PCB10
PCB6
KD16
KD5
KD7
KD2
PCB2
KD20
5a
R.tropici subgroup IIA
R.tropici subgroup IIA
R.tropici subgroup IIA
5
KD17
KD18
KD13
KD12
KD15
KD21
5b
R.tropici subgroup IIA
KD9
KD19
KD4
PCD2
PCD3
5c
R.tropici subgroup IIA
R.tropici subgroup IIA
PCM1
PCM2
PCD6
PCM9
PCD1
PCM5
PCM7
PCM4
R.tropici
6
KD1
KB3
KD3
KD10
KD14
R.tropici subgroup IIA
R.tropici subgroup IIA
7
PCD9
PCB4
R.tropici subgroup IIA
8
KB5
KB18
KB20
R.lusitanum
9
USDA 3588
USDA 4900
R.hainanense
R.huautlense
10
Section I

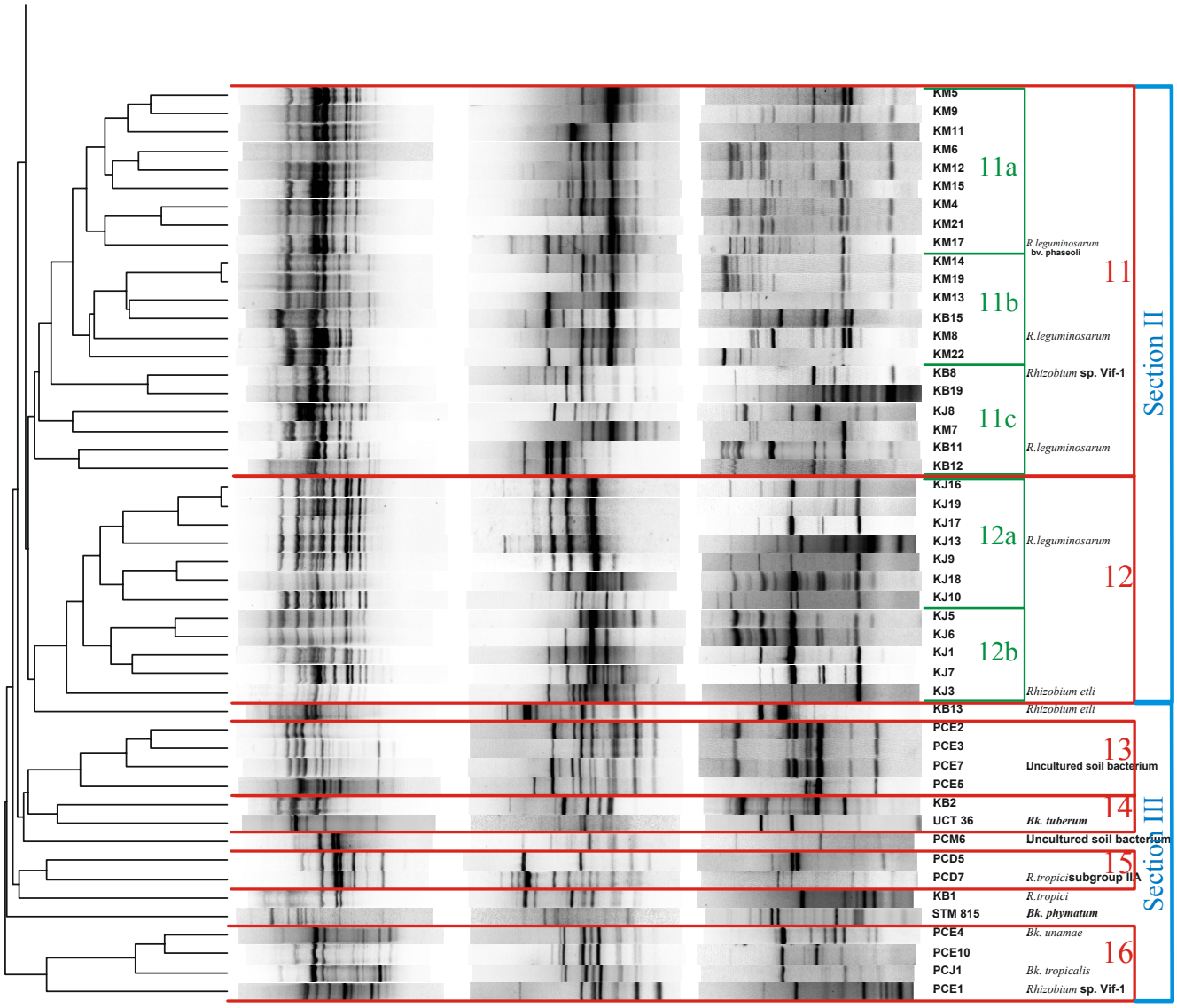


Figure 4.1 Detailed UPGMA dendrogram constructed from the combined rep-PCR DNA fingerprints of isolates and reference strains of the genera *Burkholderia* and *Rhizobium*. The levels of similarity are expressed as percentages on the x-axis. The normalized banding patterns representing BOX-, ERIC- and REP-PCR are found adjacent to each branch. The reference strains are indicated in bold. *Bk. Burkholderia*, *R. Rhizobium*

Cluster 2

Cluster 2 consisted of six isolates, three reference strains and two inoculant strains. The cluster was divided into three, with each sub-cluster containing one reference.

Sub-cluster 2a: The two inoculant strains, UD2 and UMR1899 show significant similarity to each other and the reference strain *R. tropici* (LMG 9503). These three strains all had near-identical patterns (BOX) or similar patterns (ERIC and REP). The isolates KB7, KD6 and KD11 grouped with these references at 61.2% similarity. They shared similar bands with the reference strains, particularly the ERIC and REP patterns, but they did not share similar BOX profiles.

Sub-cluster 2b: Contained the isolate KJ14 joined to the reference strain *R. mongolense* (LMG 19141) by 66.7% similarity. The ERIC profiles resembled each other, while the BOX and REP profiles differed, but had matching bands.

Sub-cluster 2c: This group contained two strains, with the reference strain *R. leguminosarum* bv. trifolii (LMG 8820) joining the rest of cluster 2 at 57.4% similarity. The isolates had very similar BOX and ERIC DNA profiles.

Cluster 3

There were only two representatives in this cluster; KJ20 joined the reference strain *R. etli* (LMG 17827) with 61.6% similarity. They had dissimilar patterns, but they had many bands in common.

Cluster 4

Cluster 4 had eight strains, which were divided to form two groups. Both sub-clusters were comprised of four isolates, with three closely related strains and one joining at a lower similarity. The sub-clusters were easily distinguished by their DNA profiles.

Sub-cluster 4a: The three isolates in this group originating from *P. coccineus* and obtained from Ermelo share significant homology, with a similarity value of 89.4%. These isolates shared very similar profiles and thus most likely have similar genotypes. The isolate PCE6 had many

additional bands in the REP pattern, but shared the same bands that the other two strains had. The single isolate KJ15 joined the sub-cluster at a relatively low similarity (59.9%). The position of KJ15 within this sub-cluster is due to the fact that it shares a few characteristic bands with all three experiment types; however no correspondence was noted between profiles for the ERIC and REP experiments.

Sub-cluster 4b: The three strains that were isolated from *P. vulgaris* plants in the Mlondozi area grouped at a similarity of 65.8%. These three isolates appeared to have the same BOX profiles. The ERIC and REP profiles were very similar, but not all of the bands were evident for all three isolates. The isolate KJ2 joined the rest of the cluster at 54.8% similarity. It shared related bands, but the profiles were dissimilar.

Cluster 5

This was the largest cluster. It was composed of 23 isolates grouping at 63.0%. The isolates had characteristic bands: most of the BOX profiles had two bands in the region of 650 bp (some isolates only contained one band) and another at 1kb; ERIC had bands of 300, 400 and 650 bp long, while REP had bands of approximately 300 and 400 bp as well as 1 kb in common. The cluster was separated into three smaller groups. The strains in this cluster were obtained from either Bethlehem or Delmas.

Sub-cluster 5a: Half of the isolates in cluster 5 are found within this group. The 12 isolates clustered at 65.6% similarity, with KD5 and KD7 showing the closest relationship (94.1% similarity) due to their matching profiles.

Sub-cluster 5b: All six isolates in this group were isolates from *P. vulgaris*, from Delmas. The patterns of these isolates resembled each other, with some isolates having additional bands for the REP experiments.

Sub-cluster 5c: This group had five isolates clustering at 69.4%. All five isolates were obtained from Delmas. They also had similar profiles.

Cluster 6

Cluster 6 had eight isolates originating from *P. coccineus* from either Mlondozi or Delmas. These isolates shared characteristic banding patterns, similar to those in cluster 5. BOX had bands of 300, 650 and 1 000bp; ERIC had bands of 200, 300, 450 and 650 bp in common and REP had common bands of 250, 450 and bands up to 1 000 bp in length. This cluster was divided into two smaller groups based on their DNA profiles, sharing 62.1% homology.

Sub-cluster 6a: Contained five strains that had similar DNA profiles. All of these isolates had characteristic bands, but for some of the isolates, they were less intense. Many of the isolates had additional minor bands.

Sub-cluster 6b: These three isolates were all obtained from Mlondozi. They were separate from the rest of the cluster based on their ERIC and REP patterns.

Cluster 7

All five samples were isolated from *P. vulgaris*. All of their BOX and REP profiles were similar, while the ERIC patterns were more divergent. These isolates grouped at 63.0%.

Cluster 8

This cluster contained only two isolates. They shared near-identical BOX patterns, but they did not have the same REP and ERIC profiles and only shared a few similar bands. This is reflected in their similarity (64.6%).

Cluster 9

There were only three isolates in this cluster. KB5, KB18 and KB20 were all obtained from *P. vulgaris* in the Bethlehem area. They shared a common BOX pattern and similar, yet distinct patterns for ERIC and REP. They had a similarity of 80.0%.

Cluster 10

The two reference strains *R. hainanense* (USDA 3588) and *R. huautlense* (USDA 4900) formed cluster 10, with a similarity value of 63.7%.

Section II

Section II contained 34 isolates, in two clusters (11 and 12) with one loosely associated isolate. The majority of the isolates were from Mlondozi or Jacobsdal.

Cluster 11

The 21 isolates all originated from *P. vulgaris* plants. Most of these were obtained from Mlondozi (15), but five were collected from Bethlehem and one from Jacobsdal. This cluster had characteristic bands, both ERIC and REP fingerprints had a band of 500bp (many of the isolates had a double band); the BOX fingerprints had bands at 850 and 1 000bp. The isolates shared 52.2% similarity and were divided into three sub groups.

Sub-cluster 11a: Contained nine isolates all originating from Mlondozi. The BOX profiles for the isolates in this sub-cluster all resembled one another, in presence, position and intensity of bands.

Sub-cluster 11b: This group contained six isolates with 65.9% similarity. KM14 and KM19 shared the highest homology with a value of 98.5%. These PCR profiles appear identical, except for the absence of a single band for each type.

Sub-cluster 11c: The six isolates in this sub-cluster formed three sets of pairs that were loosely associated with the rest of cluster 11.

Cluster 12

All 12 isolates in this cluster were obtained from the root nodules of *P. vulgaris* plants, found in Jacobsdal. They two subgroups joined at an overall similarity of 60.3%. All of the isolates had characteristic bands. BOX had many while ERIC had bands of 500bp, 850 and 1 000bp, while REP had bands of 400bp and approximately 1 000bp.

Sub-cluster 12a: Contained seven isolates that clustered at 68.6% relatedness. The isolates KJ16 and KJ19 showed the highest relatedness (98.5%), with near identical profiles for the experiment types. The REP patterns of this cluster had additional minor bands.

Sub-cluster 12b: Four of the five isolates clustered at 71.3%. These isolates appeared to have identical BOX and REP profiles. The isolate KJ3 joined these isolates and those of sub-cluster 12a at 60.3% similarity. It had differing patterns, but shared many bands with the rest of this cluster.

Section III

This section contained four clusters (13 – 16) and three loosely associated strains, one of which was the reference strain *B. phymatum* STM 815. There were 13 isolates in this section, seven of which originated from *P. coccineus* plants in the Ermelo region. These isolates did not show significant homology to the rest of the tree and were considered 'outlying isolates' as they show unexpected genus affiliations.

Cluster 13

The four *P. coccineus* isolates in this group originated from Ermelo and clustered with a similarity value of 63.7%. The profiles of the isolates were alike, particularly the REP patterns. The ERIC pattern showed variation in the intensity of bands and some of the bands in the BOX pattern were not properly aligned.

Cluster 14

Cluster 14 was made up of the isolate KB2 grouping with the *Burkholderia tuberum* reference isolate, UCT 36 with a relatively low value of 58.0%. There profiles were not similar, but they shared some minor bands.

Cluster 15

This grouping contained only two isolates, PCD5 and PCD7. They were clearly divergent from each other and the other members of this section. While they had similar BOX patterns, there was very little correspondence between the other patterns of these isolates, but they did have a few bands in common.

Cluster 16

The final cluster contained four isolates from *P. coccineus*, three of which were from Ermelo, with 55.5% similarity. The isolates PCE4 and PCE10 shared 84.5% similarity. The profiles within the ERIC results showed the greatest resemblance. These isolates were found to show high homology to *Burkholderia* strains.

The loosely associated strains

Branching points consisting of only one isolate having a similarity of 55.0% or less to any of the other branching points within the dendrogram were considered as loosely associated. The isolate KB13 was loosely associated with cluster 12, sharing 52.5% similarity with these isolates. It had a BOX profile similar to those in cluster 12, but the REP and ERIC profiles were vastly different. The single isolate PCM6 was loosely associated with the isolates in clusters 13 and 14. It shared a similarity of 49.6% with these clusters. The single isolate KB1 was loosely associated with cluster 15 at 49.8%. It had similar DNA bands for BOX, but varied vastly in the REP and ERIC profiles. The reference strain *B. phymatum* (STM 815) showed no significant homology to any of the isolates. It was joined to section II by 47.1%. This strain did not share characteristic bands with other isolates.

Two bar graphs that compare the clusters were created. These graphs reveal the distribution of isolates from the different geographic regions (Figure 4.2) and the distribution of isolates from the same host plant (Figure 4.3) throughout the dendrogram. Each bar represents one cluster and the number of isolates within each section is displayed.

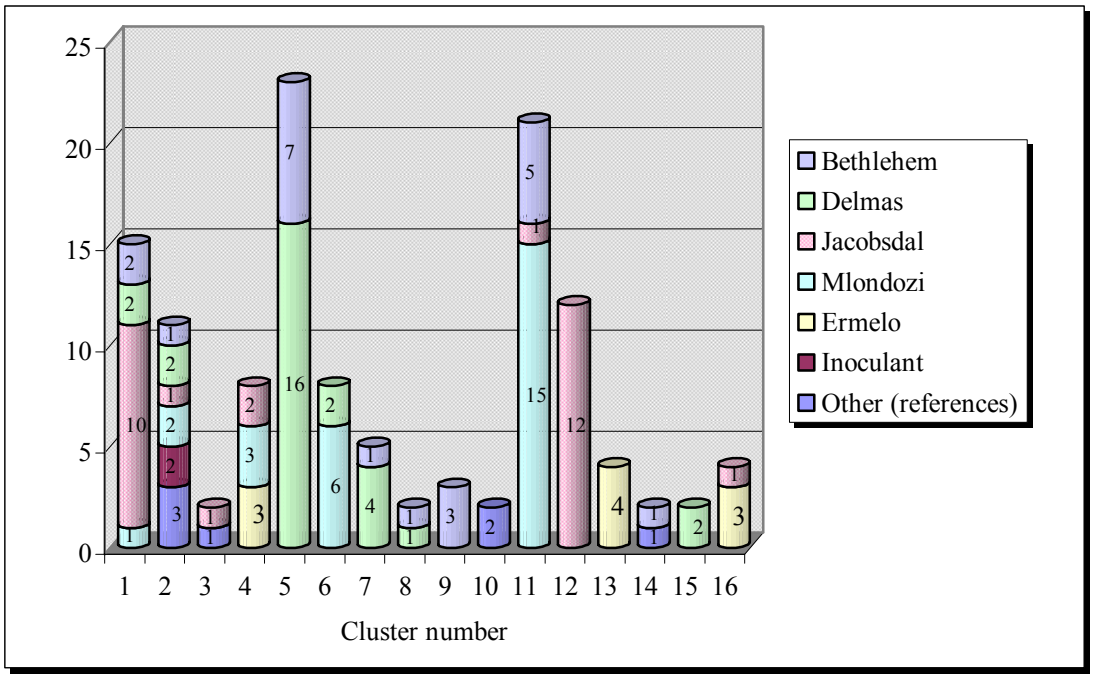


Figure 4.2 Distribution of isolates from various geographical regions within different clusters. The colour code for each region is indicated

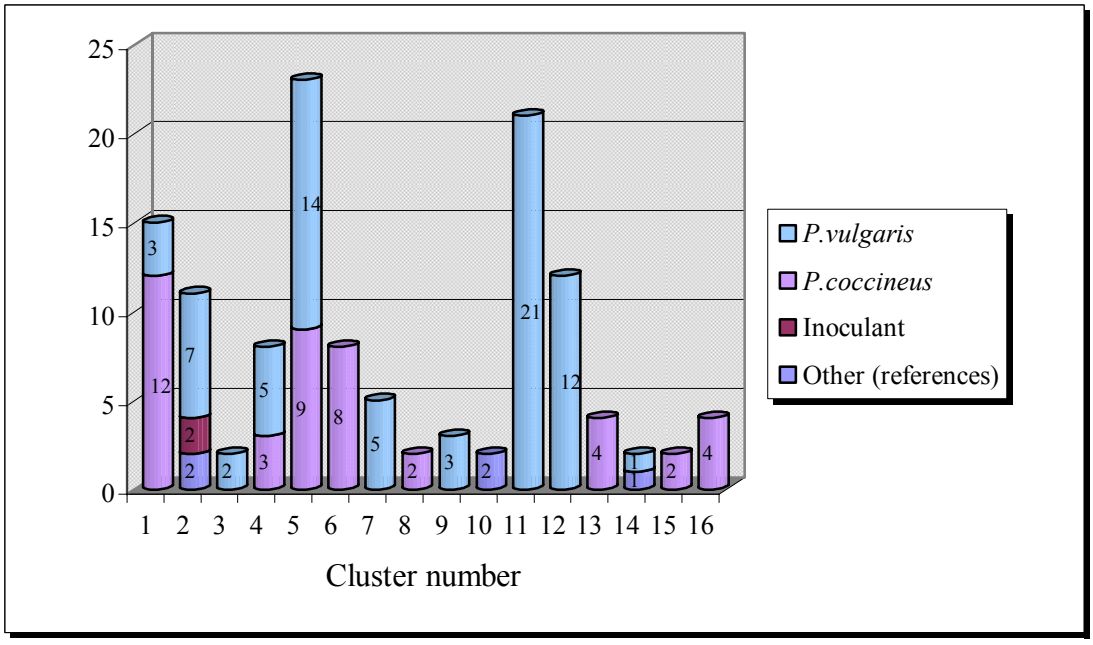


Figure 4.3 Distribution of the isolates from the host plants within different clusters.

The colour code for the host plant is indicated. Note that the host plant for the *R. tropici* and *R. etli* reference strains is *P. vulgaris*

4.3.3 Amplification and sequencing of the *nodA* gene

A 450 bp fragment of the partial *nodA* gene was amplified and sequenced for isolates PCM6, PCD5, PCD7, KB1, PCE4 and PCE10. Although amplification products were also observed for isolates KB2, PCJ1 and PCE1, these were too faint to allow further manipulation (e.g. sequencing). All four isolates from cluster 13 (PCE2, PCE3, PCE5 and PCE7) did not generate any amplification product of the *nodA* gene by the primers used.

Comparing the obtained sequence data to the NCBI's Nucleotide BLAST database, standard nucleotide-nucleotide BLAST/blastn, revealed that the majority of the *Phaseolus* isolates were found to be related to the *nodA* gene of *R. tropici*. The *Burkholderia* isolates showed homology to the *nodA* gene of other *Burkholderia* strains.

4.4 DISCUSSION

The diversity of rhizobial isolates associated with *Phaseolus* species occurring in South Africa was determined by the isolation of putative root nodule-bacteria from species of *Phaseolus* and fingerprinting their DNA by rep-PCR in conjunction with BOX, ERIC and REP primers. The indigenous rhizobia associated with *Phaseolus* represented fast and medium growing strains clustering with reference strains of the genus *Rhizobium*. None of the isolates clustered significantly with the reference strain *Burkholderia phymatum* (STM 815), but there were a few isolates within the same section.

4.4.1 Analyses of the rep-PCR product

The ERIC primer set generated the lowest number of bands, but several intense and discriminative bands in the ERIC data set were found to be useful and characteristic for the grouping of strains. Laguerre *et al*. (1996) found that DNA banding patterns of the same isolate produced by rep-PCR could differ depending on the supplier of primers, batches of primers, different *Taq* enzymes and for the type of thermal cycler used. Liu *et al*. (1995) found that general reproducibility with ERIC-PCR was good, but sometimes the major bands produced were

less intense and the minor bands were difficult to visualise. Differences in DNA template concentration may also affect the intensities of the bands produced in rep-PCR. Liu *et al*. (1995) found that ERIC-PCR is more vulnerable to alterations in DNA template concentration than it is to subtle changes in annealing temperature, primer concentration and Mg^{2+} concentration.
To avert these problems, the experiments were done under constant conditions; the same thermal cycler was used for all of the PCR reactions, enzymes and primers were obtained from the same manufacturer and the DNA was extracted by the proteinase K method (Laguerre *et al*., 1997), which yields a steady concentration of DNA.

When using only one primer for PCR amplification, as in the case of rep-PCR using the BOX and ERIC2 primer, a PCR fragment can only arise if two targeted regions (either the BOX or ERIC elements) are inversely orientated and situated at an appropriate distance from each other. As the BOX primer produced more bands than the others, with a narrower size range, the BOX element appears to be more evenly distributed throughout the genome, resulting in similar fragment sizes.

The individual dendrograms were found to be in general agreement, but different relationships between subgroups were better established when the results were combined. By combining the DNA fingerprints from all three rep-PCR reaction, a higher number of bands were used for cluster analyses. This enabled more reliable groupings of the strains as well as a higher degree of differentiation among the strains. The combination of the BOX-, ERIC and REP patterns maximizes strain discrimination, as the genome is more extensively covered than using these fingerprinting techniques alone (Vinuesa *et al*., 1998). The DNA fingerprinting profiles of some isolates that have been placed together may not resemble each other for one or two of the experiment types. This is due to the fact that the similarities for each profile affect how the isolates cluster. So, while an isolate may have a highly comparable pattern for one set of primers, it may be dissimilar for another. For example, the PCE1 pattern for ERIC is similar to those for the rest of the cluster, while the pattern differs for both BOX and REP, with bands evident in different size ranges and quantities. This usually results in the isolate being included in the cluster, but with lower similarity values. The isolate KJ15 was grouped in sub-cluster 4a as it shares significant bands with the rest of the isolates, even though it doesn't appear to share a DNA profile with any of them. Sikora *et al*. (2002) found that *B. japonicum* strains were more

efficiently distinguished by using the combined results from ERIC- and REP-PCR experiments. Many other researchers have used the combined data from BOX, ERIC and REP-PCR in the study of rhizobia: Aguilar *et al*. (2001), Laguerre *et al*. (1997) and de Bruijn (1992).

The reproducibility for the combined data was found to be in the range of 88% - 90%, which is consistent with other studies (Rademaker *et al*., 1998; Vinuesa *et al*., 1998). Typing and identification in DNA fingerprinting techniques can be standardised by defining windows of similarity. Rademaker *et al*. (2000) defined windows of similarity in their study of *Xanthomonas* using AFLP. In this study, comparable windows of similarity were defined. Patterns of 90 – 100% homology indicated identical strains, patterns with 70 – 90% homology were indicative of different strains from the same species, while 50 – 70% homology was an indication of isolates from different species of the same genus and less than 50% represented isolates from different genera. Based on these windows of similarity, similarity values of 90% or more shared between strains were considered as representing strains of the same genotype, for example PCJ4, PCJ5 and PCJ10 in cluster 1a and PCE6, PCE8 and PCE9 in cluster 2. Different rhizobial species were well separated below the similarity value of 70% in the analyses.

A small number of isolates correspond to the β-subclass *Proteobacteria* genus, *Burkholderia*. All of the isolates in section III, including the *Burkholderia* reference, were only distantly related to the rest of the isolates. These, and other isolates that were not related to any of the reference strains included, warrant further investigation and could merit the description of novel bacteria.

The four loosely associated strains (KB13, PCM6, KB1 and the reference strain *B. phymatum* [STM 815]) did not cluster significantly with any other isolates. Apart from KB13, all of these isolates are outlying isolates found in Section III. However, the isolates in cluster 13 and those in cluster 16, which are also ‘outlying isolates’, shared homology to each other. A larger sample size with more than two host species may resolve the branching in this portion of the dendrogram better.

When Rademaker *et al*. (2000) compared rep-PCR to DNA-DNA homology studies they found that there was a high correlation, suggesting that rep-PCR analyses reflect the genotypic and phylogenetic relationships of the organisms studied. However, to compare closely related species,

hybridization of total genomic DNA will provide a more reliable estimate of overall genetic similarity (Young, 1996).

4.4.2 The clustering of isolates in relation to host plant species and geographic location

The graphs in figures 4.2 and 4.3 exhibit the composition of each cluster and allow visual understanding of the dendrogram. When considering these graphs with the dendrogram, it becomes apparent that a degree of specificity exists for both host plant species and location. Many of the clusters are composed of isolates from either the same host plant species or the same geographic location. Other researchers investigating bacterial diversity using rep-PCR found that clustering correlated with environmental niche. In their study of rhizosphere *Burkholderia* spp., Richardson *et al*. (2002) found that there appeared to be some correlation between clustering and the geographic origin of the isolates and Latour *et al*., (1996) found that rep-PCR profiles of fluorescent pseudomonads appeared to correlate with soil type. Zhang *et al*. (1999) also found that strains from the same cultivar and location clustered together when using rep-PCR fingerprinting.

Several clusters or sub-clusters were comprised of isolates obtained from the same *Phaseolus* species. The isolates in the clusters 6, 7, 8, 9, 11, 12, 13 and 16, as well as sub-clusters 1c, 4b and 5b grouped according to the host plant of origin. Several of these clusters contained a large number of isolates; cluster 6 contained eight isolates from *P. coccineus,* cluster 11 had 21 isolates from *P. vulgaris* and cluster 12 contained 12 isolates, also from *P. vulgaris*. One must bear in mind that the sample size was small as the isolates were obtained from only two different species of host bean plants. Also, there were nearly twice as many isolates from *P. vulgaris* than from *P. coccineus* plants, so this was not necessarily an indication of the real situation of soil rhizobia in South Africa. Several of these clusters contain isolates that predominantly share more than 70% similarity, indicating that they are different strains of the same species (Clusters 5b, 9, 11a, 11b, 13 and 16), while the rest were different species within the same genus based on the windows of similarity.

The geographic location, and therefore the rhizosphere soil composition, also appears to have played a role in the grouping of strains. The isolates in clusters 6, 9, 12, 13 and 15 and those in

sub-clusters 5b, 5c, 11a and 11b originated from the same location. The isolates in cluster 5 were all isolated from either Bethlehem or Delmas. These locations shared similar soil compositions of sand, silt and clay as discussed in Section 3.4.1. The Bethlehem rhizosphere soil appears to have contained the greatest diversity of rhizobia, as the isolates from this location were distributed throughout the dendrogram in several clusters. The isolates from Delmas, Jacobsdal and Mlondozi were also found in several clusters in the dendrogram, but these locations show less diversity. Clusters were composed of a large number of isolates from the same location: ten isolates from Jacobsdal in cluster 1, sixteen Delmas isolates in cluster 5, fifteen isolates from Mlondozi in cluster 11 and twelve isolates from Jacobsdal in cluster 12. A high degree of homology was evident for the ten isolates obtained from Ermelo. These isolates grouped with other Ermelo isolates, almost exclusively. Three isolates clustered together with significant homology in sub-cluster 4a (PCE6, PCE8 and PCE9), but most of the Ermelo isolates were found in section III (PCE2, PCE3, PCE5 and PCE7 in cluster 13; PCE1, PCE4 and PCE10 in cluster 16). This may be because the Ermelo rhizosphere soil was the most divergent of the rhizosphere soils tested, having nutrient poor soil with the lowest concentrations of each of the essential elements tested: calcium, phosphorous, nitrogen, potassium, magnesium and sodium. These trace elements affect the symbiotic process.

Various isolates clustered according to both geographic location as well as host specificity. They included the sub-clusters 5b, 5c, 6b, 11a and clusters 7, 12 and 13. Many clusters were made up with the majority of the isolates originating from the same host and geographic location, but also contained a few other isolates, such as cluster 5 that contained a majority of *P. vulgaris* isolates from Delmas, but also included *P. coccineus* isolates from Bethlehem. This is clearly demonstrated in Figures 4.2 and 4.3.

Based on windows of similarity, values of 90% or more shared between strains were considered as representing the same strain and in all the instances where more than 90% similarity were obtained, the isolates had the same host plant species and the majority of isolates also had the same geographic location (except for PCJ8 and PCB1).

4.4.3 Correlation between SDS-PAGE and rep-PCR

Polyphasic taxonomy aims to integrate phenotypic, genotypic and phylogenetic information for the description of taxa at all levels. Combining the results from several methods is complex in terms of time, labour and technical characteristics. Phenotypic data does not correlate completely with genotypic data (Van Rossum *et al*., 1995) as was found in this study, in this instance the comparison of whole cell proteins with the distribution of repetitive elements. While there were discrepancies between the results, the combined rep-PCR analyses supported many of the results of the SDS-PAGE of whole cell proteins. Due to the large number of isolates in the data set, not all clusters of the two dendrograms were compared, but rather the significant similarities as well as noteworthy discrepancies between them.

Variations between the SDS-PAGE and rep-PCR analyses

Both SDS-PAGE analyses and combined rep-PCR analyses are able to resolve isolates infraspecies, down to strain level (Rademaker and de Bruijn, 1997). However, Willems *et al*. (2000) found that SDS-PAGE analysis of whole cell proteins corresponds poorly with genotypic data. The group of isolates in rep-PCR cluster 5 were distributed throughout the SDS-PAGE dendrogram. Isolates that showed a very high degree of similarity in the rep-PCR dendrogram were often separated in the SDS-PAGE dendrogram. The high similarity between the isolates PCJ8, PCJ4, PCJ5 and PCJ10 in the rep-PCR dendrogram are supported by the DNA fingerprint profiles, particularly the BOX and ERIC profiles, yet these results are not supported by the SDS-PAGE dendrogram, where the isolates PCJ4 and PCJ8 (sub-cluster 4c) were separated from the isolates PCJ5 and PCJ10 (cluster 5). The isolate PCE1 grouped with the isolates KB5, KB18 and KB20 in cluster 9 of the SDS-PAGE, yet in the rep-PCR tree it was found grouping with the *Burkholderia* cluster (cluster 16).

Similarities between the SDS-PAGE and rep-PCR analyses

Many of the isolates that clustered together for SDS-PAGE of whole cell proteins were also clustered together for the combined rep-PCR analyses. The isolates in SDS-PAGE cluster 5 are also found in the rep-PCR cluster 1. The SDS-PAGE sub-cluster 2a contained three isolates (PCE6, PCE8 and PCE9) that were found as a tight cluster in the combined rep-PCR sub-cluster

4a. These three isolates shared 75.8% similarity in SDS-PAGE and 89.4% similarity to each other in rep-PCR. The isolates KB5, KB18 and KB20 are clearly similar as they clustered together in the SDS-PAGE cluster 9 and in the rep-PCR cluster 9, both with high levels of similarity (94.5% and 80.0% respectively). The isolates PCE2, PCE3, PCE5 and PCE7 appear to belong to the same species. They form cluster 13 for the rep-PCR dendrogram and clusters 10 and 11 for SDS-PAGE. The isolates KM14 and KM19 are clearly similar; in the rep-PCR analysis (cluster 11) they share 97.6% similarity and in the SDS-PAGE analysis (cluster 7) they are 93.2% similar.

Pairs of isolates that shared high similarities to each other appear to represent the same strain of bacterium. Several pair-wise groupings of isolates were in agreement between the SDS-PAGE and combined rep-PCR dendrograms and it may be concluded that these isolates were members of the same strain. These include KJ16 and KJ19 from SDS-PAGE cluster 3 and rep-PCR cluster 12; KD4 and KD19 from SDS-PAGE cluster 4b and rep-PCR cluster 5c; KD5 and KD7 in SDS-PAGE cluster 4b and rep-PCR cluster 5a; KB8 and KB19 from SDS-PAGE cluster 4d and rep-PCR cluster 11c; the inoculant strains UD2 and UMR 1899 found in SDS-PAGE sub-cluster 6f and rep-PCR cluster 2 and the isolates KM14 and KM19 in SDS-PAGE cluster 7 and rep-PCR cluster 11b. The isolates PCJ5 and PCJ10 found in SDS-PAGE cluster 5 and rep-PCR cluster 1a share very high homology.

Studies correlating genetic and phenotypic data are essential for determining the taxonomic significance of results obtained from various techniques, but there is no standard approach to compare methods, particularly methods with different targets (genotypic data and phenotypic data). Van Rossum *et al*. (1995) highlighted the difficulties of a polyphasic approach. They studied *Bradyrhizobium* strains using a polyphasic approach involving several genetic and phenetic features, including SDS-PAGE protein fingerprints, 16S rDNA analyses and RAPD fingerprinting. They found that strains clustered together by one method were often scattered over various separate clusters when using a different method and they found several inconsistencies across the various methods.

4.4.4 Sequencing of the *nodA* gene

The isolates from Section III were targeted for analysis of the *nodA* gene because they are 'outlying'; they are only distantly related to the other isolates in the study and are not significantly related to any of the *Rhizobium* reference strains. In addition, several isolates appear to be related to *Burkholderia* strains and therefore require further investigation. Sequence analysis of the *nodA* gene was conducted on these isolates in order to determine whether they contain the *nodA* gene necessary for nodulation, which indicates that they are rhizobia that have the capacity to nodulate, or if the *nodA* gene is absent, indicating that they may be contaminants.

The successful sequencing of the isolates with strong DNA bands (PCM6, PCD5, PCD7, KB1, PCE4 and PCE10) confirms that these isolates contain the *nodA* gene and are therefore likely to be capable of nodulation. To confirm this, nodulation studies would be required The *nodA* has been found to be a good symbiotic marker, since *nodA* analysis reflects Nod-factor features and the gene is present in all rhizobia as a single copy (Haukka *et al*., 1998). To determine whether these isolates are capable of nitrogen fixation, inoculation studies will be needed and nitrogen assays done.

Several isolates amplified the *nodA* gene, but generated a very low concentration of DNA. Upon repeating the *nodA* amplifications of these isolates the faint band was always present. Therefore, the *nodA* gene of was likely to be present in the genome of these isolates, but was not optimally amplified. This may be because the primers were not optimal to amplify the product sufficiently. These isolates could not be sequenced, as there was not enough target DNA.

The four isolates from cluster 13 (PCE2, PCE3, PCE5 and PCE7) did not produce an amplification product with the NodA1/NodA2 primer pair therefore they do not appear to contain the *nodA* gene. In rhizobia, *nod* genes are organized in several operons, located on the chromosome or on large symbiotic plasmids. The symbiotic genes can be acquired and lost through horizontal gene transfer of these plasmids (Haukka *et al*., 1998). The isolates lacking the *nodA* gene may have lost their symbiotic plasmid containing the *nod* genes. This loss would result in the inability of the bacteria to elicit nodule formation of the plant (Van Rhijn and Vanderleyden, 1995). Alternatively, the primers used may be unsuitable to amplify the *nodA*

gene of these isolates and need to be optimised. The isolate PCE7 showed close homology to an "Uncultured soil bacterium" when the 16S rRNA gene was sequenced and all four of the isolates clustered together in both the rep-PCR (cluster 13) and SDS-PAGE (cluster 17) trees implying that they are closely related to each other, while showing little relation to any other isolates. The lack of *nodA* gene could also be explained if these isolates are not root-nodulating bacteria and are merely opportunistic endosymbionts or plant growth promoting rhizobacteria (PGPR) that do not contain the *nodA* gene. However, no conclusions should be drawn and further investigation of these isolates is required.

4.5 CONCLUSION

DNA fingerprinting using the combined profiles of BOX-, ERIC- and REP-PCR proved to be a rapid technique for the description of a large collection of closely related bacteria. Both geographic location and host plant appear to have played a role in the clustering of isolates. The combined rep-PCR results corroborated many of the results for SDS-PAGE of whole-cell protein; the majority of isolates represented strains from the genus *Rhizobium* with a small percentage belonging to the genus *Burkholderia*. Analysis of the *nodA* gene demonstrated that the majority of isolates tested contain the *nodA* gene, which is an indication of nodulating ability. A number of the root-nodulating bacteria of the *Phaseolus* spp. appear to constitute several novel nodulating genotypes and DNA-DNA hybridisation would have to be done to determine the possible species status of these isolates (Dupuy *et al*., 1994) as this method is an important criterion for species delineation (Stackebrandt and Goebel, 1994).

CHAPTER 5

CONCLUDING REMARKS

In this study the diversity of rhizobial isolates associated with two bean species (*P. coccineus* and *P. vulgaris*) occurring in South Africa was determined in order to contribute to finding a suitable inoculant for beans. SDS-PAGE of whole cell proteins was used for the grouping of a large number of isolates into clusters having potential species status. As studies correlating genetic and phenotypic data are crucial for the description of taxa at all levels, partial 16S rDNA sequencing was performed on selected isolates to determine their affiliations at the genus level and repetitive-sequence based DNA fingerprinting was performed on all of the isolates to discriminate at the sub-species level.

SDS-PAGE was found to be helpful in the initial clustering of the collection of root nodule bacteria obtained from the different bean rhizobia and it provided differentiation at intraspecies level. However, due to its susceptibility to changes in growth conditions and its inability to distinguish above species level, SDS-PAGE data should not be used alone, but should rather form part of a polyphasic approach including genotypic data.

The resolution power of 16S rDNA sequences is restricted when comparing highly related organisms. The partial 16S rDNA sequences helped to assign organisms to a specific genus. As was expected, the majority of isolates sequenced belonged to species in the genus *Rhizobium* (specifically *R. tropici*, *R. leguminosarum* and *R. etli*); however a few isolates showed affinity to the ß-*Proteobacteria* genus *Burkholderia*. A comparison of the SDS-PAGE and 16S rDNA sequencing data confirmed that bacterial discrimination using SDS-PAGE is not useful at the genus level and higher as clusters containing potential *Burkholderia* strains were found amongst clusters containing *Rhizobium* strains.

Repetitive-sequence-based PCR analysis was an efficient method for determining the diversity of rhizobia at the strain level. Strain discrimination was maximised by combining the fingerprint patterns from BOX-, ERIC- and REP-PCR due to the higher number of bands used for cluster

analyses. Many of the results for SDS-PAGE of whole-cell protein corroborated with the results for combined rep-PCR, but the phenotypic and genotypic data in this study did not correlate completely; in some instances there were discrepancies between these techniques. From the SDS-PAGE and combined rep-PCR data it was evident that both geographic location and host plant played a role in the clustering of isolates for both of these techniques and therefore that a degree of specificity exists for these isolates.

The study of the *nodA* gene was done to determine whether the selected isolates, showing unexpected genus affiliations, were capable of nodulation or were merely contaminants. For some of these isolates, the *nodA* gene was successfully amplified and these isolates may be ruled out as contaminants, as the presence of the *nodA* gene is an indication that nodulation is possible. However, the isolates where the *nodA* gene did not amplify are either contaminants that do not contain the gene or the primers may be unsuitable to amplify the *nodA* gene of these isolates.

Bean plants in South Africa appear to be nodulated predominantly by *Rhizobium* species, particularly *R. tropici*, *R. leguminosarum* and *R. etli* that are commonly associated with *Phaseolus* species. It was previously thought that *Phaseolus* spp. are only nodulated by species from the genus *Rhizobium*, but in this study they were found to be nodulated by *Burkholderia* species as well: a small percentage of the isolates studied appear to be *Burkholderia*, one of the novel root-nodulating genera in the β-*Proteobacteria* that have not previously been reported to nodulate *Phaseolus* spp.

Isolates not showing particular affinity towards any of the reference strains should be investigated to clarify their status. The isolates PCM6 and the closely related group including PCE2, PCE3, PCE5 and PCE7 may be members of a novel genus in the α-*Proteobacteria*. The taxonomic position and identity of the isolates that were clearly differentiated and could not be assigned to any of the previously recognised species should be further clarified by DNA homology studies.

The investigation of agriculturally important leguminous crops helped to gain more information on the legume-rhizobium symbiosis. The characterisation of root-nodulating rhizobia of legumes associated with a specific crop led to the description of novel rhizobia and seemingly unrelated

isolates. A polyphasic approach was used to incorporate different types of data (phenotypic, genotypic and phylogenetic), allowing more reliable resolution of the interrelationship among these bacteria. There appears to be great strain diversity amongst these isolates, which will assist in the search for a suitable inoculant strain for *Phaseolus* species in South Africa.

Future work should include inoculation studies, nodulation studies and nitrogen-fixation assays. These would help to determine whether the isolates are capable of nodulation and nitrogen-fixation, how effectively they can compete with indigenous rhizobia present in the soil and their nitrogen-fixation efficacy. These factors demonstrate an isolates' suitability as an inoculant and will contribute to the selection of future inoculant.

CHAPTER 6

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