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Polyphasic taxonomy of rhizobia associated with legumes occurring in South Africa

by

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Polyphasic taxonomy of rhizobia associated with legumes occurring in South Africa

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DEPARTMENT: Microbiology and Plant pathology

DEGREE: PhD (Microbiology)

SUMMARY

The advantageous association between rhizobia and leguminous plants has motivated numerous studies into the diversity and identity of the associated bacterial symbionts. This, as well as developments in molecular microbiology, has led to major revisions of rhizobial taxonomy. Previous investigations of the rhizobia, associated with various leguminous plants from South Africa, concluded that most of the indigenous strains were related to the genus *Bradyrhizobium* (Dagutat, 1995; Kruger, 1998). The other rhizobial genera represented, albeit to a lesser extent, were *Rhizobium, Sinorhizobium* and *Mesorhizobium*. The major shortcoming of these investigations was the lack of sufficient genotypic characterisation. Since the completion of these initial investigations, a new rhizobial genus and several new species have been described. These developments and the additional isolation of rhizobia, from previously uninvestigated legumes, necessitated a more detailed analysis of the indigenous rhizobia. The aim of this study was therefore to study the diversity of the indigenous strains, focusing particularly on genotypic traits.

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A selection of indigenous rhizobia was characterised by partial 16S rDNA sequencing, restriction fragment length polymorphism (RFLP) of the 16S-23S intergenic spacer (IGS) region, partial *nifH* sequencing and *nodC* RFLP. Based on 16S rDNA sequencing, most of the isolates could be assigned to a specific genus, most being related to the genus *Bradyrhizobium*. A group of isolates was also related to the genus *Methylobacterium*. The IGS-RFLP analyses were sufficiently discriminatory to indicate additional variation among isolates which showed little or no 16S rDNA sequence variation. The *nifH* phylogenetic groupings correlated well with those obtained by 16S rDNA sequencing. However, *nodC* RFLP indicated that the indigenous rhizobia carry diverse range *nodC* genotypes, with only a few showing host-specific associations. In the absence of sequence data of these *nodC* genotypes, their origin and correspondence to known *nodC* genes of other rhizobial genera, remain uncertain. Considering the results obtained here and the phenotypic characteristics determined previously, several novel *Bradyrhizobium* and *Mesorhizobium* strains were identified, however, their specific status should be validated by DNA homology studies.

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Polifasiese taksonomie van die rhizobia geasossieerd met inheemse peuplante in Suid Afrika

deur

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OPSOMMING

Op grond van die voordelige assosiasie tussen rhizobiums en peulplante, is talle studies na die diversiteit en identiteit van die endosimbionte geloods. Om hierdie rede, asook agv ontwikkelinge in molekulêre mikrobiologie, het daar grootskaalse hersienings van die taksonomie van rhizobiums plaasgevind. Vorige ondersoeke na rhizobiums, geassosieerd met verskeie peulplantspesies van Suid-Afrika, het aangetoon dat die meeste isolate aan die genus *Bradyrhizobium* verwant is (Dagutat, 1995; Kruger, 1998). Die ander rhizobium genusse, *Rhizobium*, *Sinorhizobium* en *Mesorhizobium* was wel teenwoordig, maar in mindere mate. Die grootste tekortkoming van hierdie studies was die gebrek aan voldoende genotipiese karakterisering. Sedert die voltooiing van hierdie ondersoeke is daar 'n nuwe rhizobiese genus, asook verskeie nuwe spesies beskryf. Hierdie ontwikkelinge en die isolering van rhizobiums vanuit voorheen onbestudeerde gasheerspesies, het 'n meer gedetailleerde ondersoek na die inheemse rhizobiums genoodsaak. Gevolglik was die doel van hierdie studie om die diversiteit van die inheemse rhizobia te bepaal deur spesifiek op meer genotipiese eienskappe te fokus.

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'n Geselekteerde groep inheemse rhizobiums is dmv gedeeltelike 16S rDNS volgorde-bepaling, restriksiefragmentlengte-polimorfismes (RFLP) van die 16S-23S intergeniese skeier (IGS) gebied, gedeeltelike *nifH* volgorde-bepaling en *nodC*-RFLP gekarakteriseer. Op grond van 16S rDNA volgorde-bepaling, kon meeste van die isolate aan 'n rhizobiese genus toegewys word. Meeste was egter aan die *Bradyrhizobium* genus verwant. 'n Groep isolate was ook aan die genus *Methylobacterium* verwant. Die IGS-RFLP analise het ook 'n goeie onderskeidingsvermoë getoon en was in staat om addisionele verskille binne isolate, met geen of baie min 16S rDNS volgordevariasie, aan te toon. Die filogenetiese groeperings, op grond van *nifH*- en 16S rRNS-geenvolgordes, het goed ooreengestem. Daarenteen het die *nodC*-RFLP aangetoon dat die *nodC*-genotipe van die inheemse isolate baie divers is. Slegs by 'n paar isolate is 'n sterk ooreenkoms tussen *nodC* genotipe en gasheerspesie aangetoon. Aangesien DNS volgorde van hierdie genotipes onbekend is, is dit moeilik om hul oorsprong en ooreenkoms met *nodC*-genotipes van ander rhizobiese genera te bepaal. Deur die genotipiese resultate van hierdie studie en fenotipiese eienskappe, soos voorheen bepaal, gesamentlik te oorweeg is verskeie nuwe *Bradyrhzobium* en *Mesorhizobium* rasse geïdentifiseer. Die spesies-status van hierdie rasse moet nog deur DNA-homologie analises bevestig word.

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ARC	Agricultural Research Council
ATP	adenosine-5'-triphosphate
bp	base pair
°C	degrees Celsius
ClustalX	cluster analysis version X
dNTP	deoxyribonucleoside-5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminotetra-acetic acid
ERIC	enterobacterial repetitive intergenic consensus
Ha	hectar
lsu	large subunit
LMG	Laboratorium voor Microbiologie Gent Culture Collection, Gent, Belgium
M	Molar or Mega (10^6)
mg	milligram
ml	millilitre
MLEE	multilocus enzyme electrophoresis
mm	millimeter
mM	millimolar
mS	milli Siemens
m/v	mass per volume
NCBI	National Center for Biotechnology Information
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
r	Pearson product moment correlation coefficient
RAPD	Random amplified polymorphic DNA
REP	repetitive extragenic palindromic

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RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
ssu	small subunit
^{T}	type strain
T	Terra (10^{12})
U	units
UPGMA	unweighted pair group method of arithmetic averages
µg	microgram
µl	microlitre
V	volts
v/v	volume per volume

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CHAPTER 1

Introduction

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Our knowledge of the beneficial association of rhizobia with leguminous plants in utilising atmospheric nitrogen (biological nitrogen fixation) is very recent when compared to the very long period that leguminous crops have been cultured and valued for food and soil enrichment (Lim *et al.*, 1986). Soil nitrogen, originating from decomposing plant residues and microorganisms, is normally deficient for intensive crop production. This is the compelling reason to increase our understanding of biological nitrogen fixation as brought about by organisms such as rhizobia. Research has shown that the biological nitrogen fixation process is the most efficient way to supply the large amounts of nitrogen needed by legumes to produce high-yielding crops with high protein content. As an example, most grain legumes can obtain between 50% and 80% of their total nitrogen requirements from biological nitrogen fixation, while some like fababean will fix up to 90% (Peoples *et al.*, 1995).

The projected doubling of the world's population over the next fifty years is expected to increase pressure for more effective food production and the need for fixed nitrogen. Supplying this demand by industrial sources will increase the required nitrogen. However, the expanded use of the biological nitrogen fixation could reduce and replace the need for industrially produced fertiliser nitrogen. In South Africa this demand for available proteinaceous food is particularly current and can be met by establishing nodulated legumes, which are able to effectively fix nitrogen, on poor agricultural soils. Effective nitrogen fixation has been effectively achieved by inoculation of legume seeds with appropriate rhizobial inoculants before planting (Jansen van Rensburg *et al.*, 1969 & 1983). According to J.B. Skeen (Fertilizer Society of South Africa, president's report, 1996) the effective introduction of suitable legumes on 75% of natural veld could lead to the enrichment of soil with an estimated 400 000 tons of nitrogen, corresponding closely to the amount sold annually in the country. The need to select strains that are more effective and competitive for application in southern African agriculture should therefore be evident.

In recent years the classification of legume root-nodulating bacteria has undergone major revisions and improvements (Jordan, 1984). This has been the result of the application of polyphasic taxonomy, a term coined by Colwell (1970), and is used for the delineation of taxa at all levels (Murray, 1990). Polyphasic taxonomy involves techniques, which have

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various discriminatory powers, to resolve the complex intra- and intergenic relationships of different bacterium species (de Lajudie *et al.*, 1994). The transient nature of rhizobial taxonomy is mainly due to the application of molecular techniques within a polyphasic approach. Consequently, major revisions of the taxonomic outline of the root nodulating bacteria have taken place as shown in the second edition of Bergey's Manual of Determinative Bacteriology (Garrity, 2001).

South Africa has approximately 1400 legume species, growing under diverse geographical and climatological conditions. It is therefore expected that this diversity will be reflected in their symbiotic partners. Systematic analyses of these symbionts from diverse environmental conditions will also increase the chances of finding inoculant strains suited for effective application in South African agriculture.

Previous studies (Dagutat, 1995; Kruger, 1998) into the taxonomy of the indigenous South African rhizobia focussed on isolates from a range of leguminous plants. However, this research has been limited to the analysis of growth rate characteristics, colony morphology, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of total cellular proteins, substrate utilisation patterns and 16S rDNA-RFLP. According to Graham *et al.* (1991) both phylogenetic and phenotypic (symbiotic, cultural, morphological, and physiological) traits of a relatively large number of strains should be considered when proposing new addition to the taxonomy of the rhizobia. Clearly not all of these criteria were met and a more detailed analysis of the indigenous rhizobia was needed. The aim of this study was therefore to further investigate the diversity of the indigenous rhizobia by focussing mainly on molecular genetic techniques and morphological traits (on a limited number of new additions to the existing culture collections).

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CHAPTER 2

Aspects of Biological Nitrogen Fixation

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1. Introduction

The earth's population is expected to reach 8.3 billion by 2025, and to maintain the current level nutritional intake, crop production will have to increase dramatically. Seen against the backdrop of deteriorating environmental conditions, which is in part due to the injudicious use of fertiliser, this will be an almost impossible task.

The effective management of nitrogen (N_2) in the environment is an essential element of sustainable agriculture. It usually involves some use of biologically fixed nitrogen, which is less susceptible to volatilisation, denitrification and leaching (Graham & Vance, 2000b). It is estimated that approximately 80% of biologically fixed nitrogen involves the symbiosis between rhizobia and leguminous plants (Vance, 1998). Worldwide, legumes are grown on approximately 250 Mha and fix close to 90 Tg of N_2 per year (Fink *et al.,* 1999). Other organisms are also increasingly recognised as major contributors to overall nitrogen fixation. These include actinorhizal symbiosis and associative relationships, including sugarcane and *Acetobacter.* However, most of the investigations of such N_2-fixing organisms reflect a molecular rather than a field orientation (Graham & Vance, 2000b).

Scientific discoveries over the last three decades have led to a better understanding of the processes involved in nodulation and nitrogen fixation. Despite these developments, little impact has been made on the field level. This is clearly illustrated by the total absence or lack of good quality inoculants in many parts of the world (Graham & Vance, 2000a).

The capacity of soil to supply nitrogen declines rapidly as soon as agricultural activities starts. This represents a major problem to farmers since nitrogen should then be supplemented from other sources. Biological nitrogen fixation presents an environmentally sound alternative if farmers are going to meet the increased demand of food production.

Despite the obvious advantages of biological nitrogen fixation, a number of factors influences agricultural dependence. These include the variation in farmers' acceptance of inoculation technologies, the availability of cheap nitrogen fertiliser and the availability of land for lower-yielding crops, etc. Where there is wider acceptance for this approach, factors impacting on the symbiosis will need to be examined. These include breeding and selection

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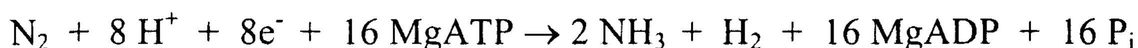
programmes aimed at alleviating nutrient deficiencies, pH and drought tolerances, etc. (Graham & Vance, 2000b).

2 Nitrogenase and Biological Nitrogen Fixation.

Elemental nitrogen is abundant in the earth's atmosphere and is essentially inert at room temperatures in the absence of a suitable catalyst. The reduction of N_2 to form ammonia requires a large amount of activation energy, as has been indicated in the industrial fixation of nitrogen to ammonia by the Haber-Bosch process. The abundance of N_2, contrasted with the difficulty of chemically utilising this source, creates a paradox, which nature has ingeniously solved through the process of biological nitrogen fixation. A relatively small number of microorganisms, termed diazotrophs, are capable of carrying out this process, contributing annually approximately 60% of the earth's fixed nitrogen (Kim & Rees, 1994).

Biological nitrogen fixation is catalysed by the nitrogenase enzyme system which consists of two metalloproteins: the iron (Fe-) protein and the molybdenum-iron (MoFe-) protein. These enzymes mediate an ATP dependent reduction of dinitrogen to ammonia. Under optimal conditions the overall stoichiometry of dinitrogen reduction has been established as (Kim & Rees, 1994):



$$N_2 + 8\,H^+ + 8e^- + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16\,P_i$$

3 Phylogenetic perspectives on nodulation

Nitrogen-fixing symbiosis is best known from its associations between leguminous plants and a diverse group of gram-negative soil bacteria, collectively called rhizobia. However, this nitrogen-fixing symbiosis is also known in several other plant groups. *Parasponia* (Ulmaceae family) is also nodulated by rhizobia, while cyanobacteria such as *Anabaena* and *Nostoc*, form symbiosis with plants such as the water fern *Azolla*, cycads and flowering plant *Gunnera* (Gunneraceae). The Gram-positive actinomycete *Frankia* nodulates nearly 200 species belonging to eight different families and it is considered that actinorhizal symbiosis fixes as much nitrogen as the legume-rhizobia symbiosis (Doyle, 1998).

Not all genera of legumes are nodulated. Most members of the subfamily Papilionoideae (including the typical beans) and Mimosoideae (acacias and mimosas) appear to be capable

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of nodulation. By contrast, only few members of the third subfamily, Caesalpinioideae are known to nodulate (de Faria *et al.*, 1989). The observation that rhizobia can infect the roots of non-nodulated legumes and that such plants show nitrogenase activity, led Bryan *et al.* (1996) to speculate that all legumes are capable of rhizobial symbiosis though not all are able to form nodules. This further compounds the issue of the origin of nodulation. This phenomenon of nodulation could have arisen once or have had multiple independent origins. The multiple origin theory of nodulation is supported by the following:

1. Based on chloroplast gene phylogeny, the subfamily Caesalpinioideae is composed of diverse elements including earliest diverging lineages of the Leguminosae (Doyle *et al.*, 1997), it is therefore suggested that nodulation occurred once in each of these subfamilies.
2. The actinorhizal nodules from different families are structurally diverse as would be expected in the case of multiple origins of nodulation (Doyle, 1998)

On the other hand, an assessment of the major nodule types in the Leguminosae revealed that the unbranched indeterminate "caesalpinioid" nodule type is scattered throughout the family (Doyle *et al.*, 1997). This is consistent with a single origin of nodulation within the family, with the caesalpinioid nodule type being the common ancestor.

It is clear that many arguments can be raised on whether nodulation arose once or more times. The other related key issue is: how did they evolve? To examine this issue, Gualtiery & Bisseling (2000) investigated the nodule-specific proteins, called nodulins. These proteins were expressed in other parts of the plant, suggesting that these may have been recruited from other developmental processes. Determining the ancestral expression patterns of these nodulins could perhaps give better insight into the evolution of nodulation. The hypothesis of an independent origin of nodulation would therefore mean an independent recruitment of nodulin genes. Different legumes would therefore have different copies of the nodulin genes. In contrast, the common ancestor species possessing homologous nodules would have recruited the same type of nodulin genes. These nodulin genes, as well as transcription factors, remain to be investigated in greater detail and could so provide insight into the origin of nodulation.

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4 The *Rhizobium* legume symbiosis

Most higher plants have the ability to form arbuscular endomycorrhiza (AM), a symbiotic association between the plant root and fungi of the order *Glomales*. These fungi grow toward the inner cortical cells of the root, where they differentiate into the highly branched structures, the arbuscules. Additionally, the fungus also forms hyphae outside the plant, which facilitate the uptake of nutrients. The *Rhizobium*-plant symbiosis is more complex and more specific, interacting symbiotically with only a few plant species. This interaction results in the formation of a new organ, the root nodule in which the bacteria are hosted in an ideal environment where they can reduce atmospheric nitrogen (Albrecht *et* al., 1999).

The process of rhizobium-legume infection consists of successive, discrete recognition events involving interactive, complementary plant and bacterial functions. Rhizobia in the rhizosphere are chemoattracted to the plant roots by compound such as amino acids, sugars organic acids and plant secondary metabolites. This is followed by bacterial attachment to the root hairs, which is considered a complex process involving multiple mechanisms which include plant lectins and bacterial fimbriae (Roth & Stacey, 1991). A study by van Workum *et al.* (1998) showed that exopolysaccharides of rhizobium accelerates root hair curling and infection to such an extent that rhizobial root penetration precedes a plant defense response. Root nodule formation involves the redirection of the development of fully differentiated plant cells and the responsible bacterial signals are the so-called nodulation (Nod) factors. The common basic Nod-factor structure (Fig. 2.1) of different rhizobia is: β-1,4-linked *N*-acyl-D-glucosamine backbone of almost four or five units, containing a fatty acid at the non-reducing terminal sugar (Carlson *et al.*, 1994).

The Nod-factor secreting rhizobia induce morphological changes of the root hairs, commonly referred to as the "shepherd's crook'-like curling of the root hairs. Although a curled root hair is not essential for infection , it is thought to facilitate infection. The microenvironment within such curls are used by the rhizobia to establish an infection site, where they locally degrade the plant cell wall and enter the root hair via invagination of the plasma membrane (Albrecht *et al.*, 1999). New cell wall material is deposited to form the infection thread. The infection thread continues to grow beyond the root hair cell and penetrates the cortex of the root (Dixon & Wheeler, 1986).

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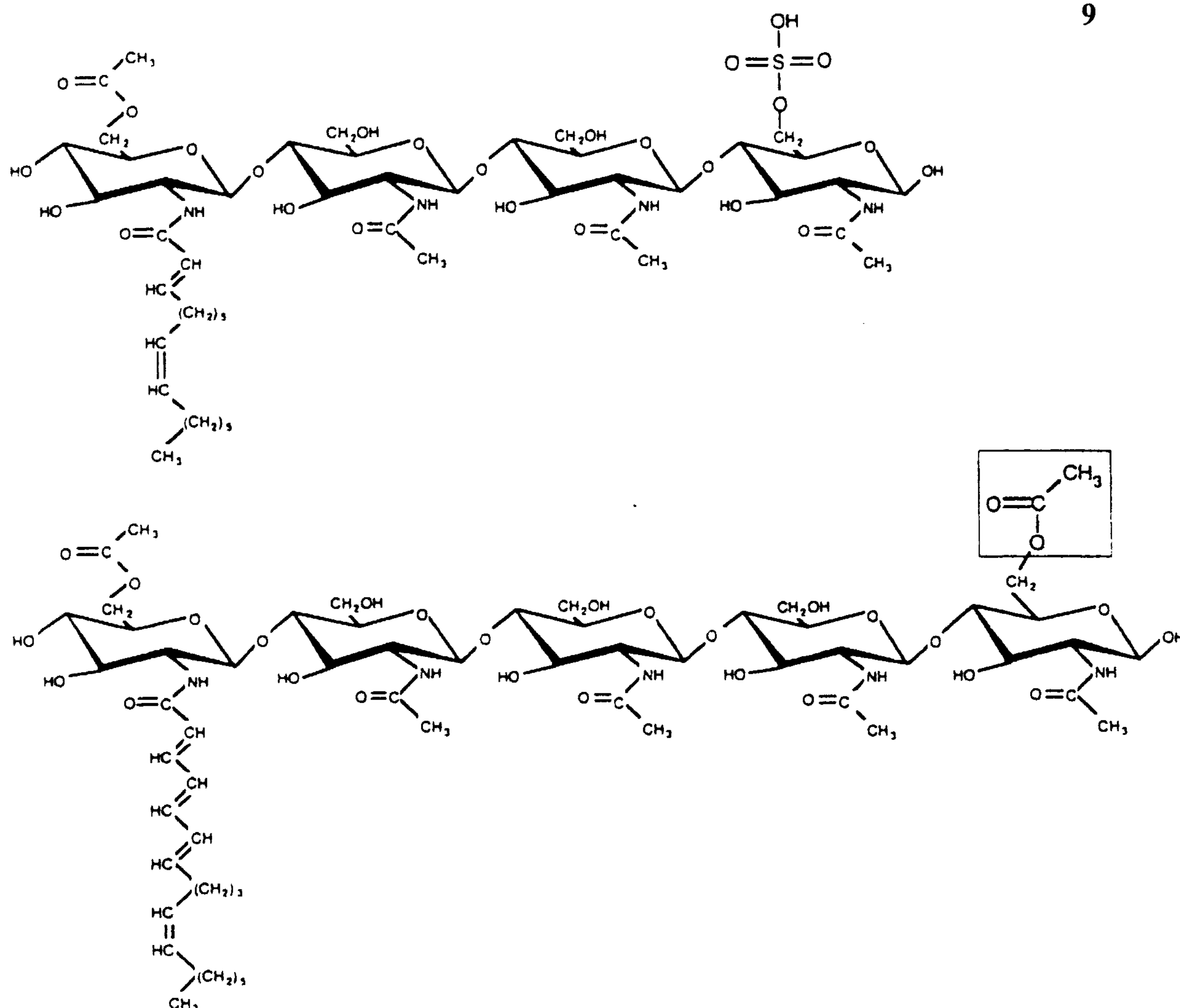

Figure 2.1. The major Nod factors produced by *Sinorhizobium meliloti* (top) and *Rhizobium leguminosarum* bv. *vicae* (bottom). The major differences concern the specific decoration at the reducing terminal sugar unit and the structure of the acyl chain. The *S. meliloti* Nod factor contains four glucosamine units, an acyl chain of 16C-atoms, an acetyl group at the non-reducing end and a sulphate group at the reducing terminal sugar residue. *R. leguminsarum* : the glucosamine backbone is four or five units carrying an acyl chain of 18 C-atoms (Carlson *et al.*, 1994)

Clusters of cortical cells, as determined by the host species, are mitotically activated by Nod-factors to form a nodule primordium. The infection thread grows, reaches the primordium and the bacteria are released. The bacteria enter the cytoplasm via an invagination of the host-cell membrane, forming a so-called symbiosome. Within the host cytoplasm the bacteria remain surrounded by a host membrane (also termed peribacteriod membrane) and is never in direct contact with the host cytoplasm. Upon infection, the nodule primordia form a meristem as well as different tissues that form a nodule. Bacteria within the symbiosome are referred to as bacteroids due to the series of biochemical and cellular changes they have undergone (Albrecht *et al.*, 1999; Roth & Stacey, 1991).

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A differentiation is made between effective and ineffective nodules. In the former nitrogen is fixed effectively to the benefit of the host plant, while in the latter no nitrogen is fixed or the level of fixation is so low that it is of no benefit to the plant. This ineffectivity might be ascribed to the inability of the bacteria to emerge from the infection thread or the lack of production of nodule tissue. Senescence of nodules is also a well-known occurrence, typified by a total disintegration of nodules and is essentially controlled by the plant (Roth &Stacey, 1991).

5 Molecular basis for nodulation

In rhizobia the nodulation genes are organised in several operons, located either on the chromosome or on a large symbiotic (Sym) plasmid. The nodulation genes may be classified into three groups: the regulatory *nodD* gene, the so-called "common" *nod*-genes and host specific nodulation genes (Albrecht *et al.*, 1999).

These *nod* operons are preceded by a conserved *cis*-regulatory element, the *nod* box. The regulatory NodD–protein, induced by plant flavonoids, binds to the *nod* box and activates the transcription of the *nod* genes. The interaction of NodD-protein with specific plant flavonoids of the host plant represents the first level host-specific interaction (Schultze & Kondorosi, 1998). Some rhizobial species appear to have more than one *nodD* gene e.g. *Bradyrhizobium* species, *Sinorhizobium meliloti* and *Sinorhizobium fredii*. In contrast, *R. leguminosarum* bv. *vicae* contains only a single *nodD*-gene. These copies are generally highly homologous producing NodD proteins capable of interacting with a range of different inducer molecules (Roth & Stacey, 1991).

In response to the release of the appropriate inducers by the host plant, the rhizobia synthesise and secrete a family of Nod-factors. The synthesis of the basic Nod-factor structure is catalysed by the bacterial NodA-, NodB- and NodC-proteins. NodC (*N* –acetyl glucosaminyl-transferase) catalyses the synthesis of the chitin oligomer and controls the length of the backbone, NodB deacetylates the terminal non-reducing glucosamine unit of the oligomer, which is substituted by an acyl chain by NodA (Albrecht *et al.*, 1999; Perret *et al.*, 2000). According to Carlson *et al.* (1994), several other species-specific nod-proteins modify the terminal sugar residue or determine the nature of the acyl chain. Such modifications determine biological activity and host specificity of these Nod factors. The products of the

other *nod* genes play subtle roles in nodulation, perhaps by permitting interaction with certain plants or by protecting the Nod factors from degradation (Perret *et al.*, 2000). An outline of the signal exchange in the *Rhizobium*-plant symbiosis is given in Fig. 2.2.

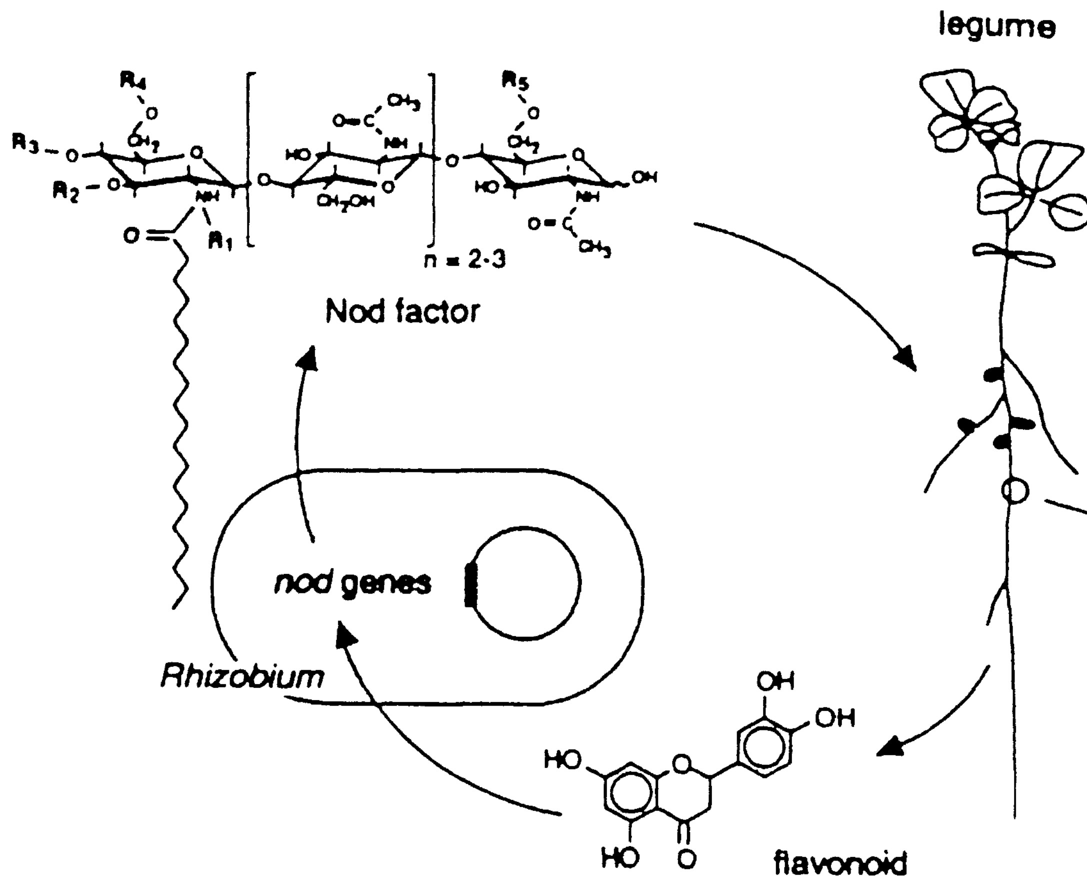

Figure 2.2. Signal exchange in the *Rhizobium*-legume symbiosis. Flavonoids induce the rhizobial *nod* genes. This leads to the production of nodule-inducing (Nod) factors, lipochitooligosaccharides (LCO), which are differently modified depending on the *Rhizobium* species (Schultze & Kondorosi, 1998).

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6 Conclusion

Developing countries are faced with the dilemma of increasing food production due to escalating population numbers. However, the problem is compounded by the low nutrient status of agricultural soils. The demand for chemical nitrogen fertiliser is therefore expected to increase with a consequent increased negative effect on the environment. The challenge is therefore to find an alternative to chemical nitrogen fertiliser. Biological nitrogen fixation is an approach to meet this goal. It is therefore important to have an understanding microbes involved in biological nitrogen fixation and the signal exchange between the symbiotic partners. In this way symbiotic interactions of agricultural significance may possibly be identified.

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CHAPTER 3

Approaches In Bacterial Systematics

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1. Introduction

Developments in molecular microbiology now underpin many exciting new methods which have not only been used for the classification and identification of bacteria, but have also provided insight into procaryote evolution. Bacterial systematics has really come of age as it is seen as a fundamental scientific discipline which addresses some of the basic questions facing humankind, such as the extent of microbial diversity and its role in sustainable agriculture (Goodfellow & O'Donnel, 1993).

The three interrelated areas of systematics include classification, nomenclature and identification. Van Berkum & Eardly (1998) describe these areas as follows:

- Classification: the arranging of organisms into taxonomic groups based on similarities.
- Nomenclature: the assignment of names to the taxonomic groups according to international rules.
- Identification: the process of determining whether a new isolate belongs to one of the established and named groups.

The once dull and boring subject area of systematics has developed into an exciting and rapidly developing discipline recognised by diverse members of the scientific community. These include epidemiologists, molecular ecologists and biologists, geochemists, agriculturists, etc. Since systematics is data dependent, many of the advances made within this field are due to the manner in which data is collected and analysed. In many instances multiple traits are analysed to establish the relationships between microorganisms. Consequently, current systematics requires an understanding of microbial chemistry, molecular biology, microbial physiology and data handling procedures (Goodfellow & O'Donnell, 1993).

The integration of multiple traits, termed polyphasic taxonomy by Colwell (1970), arose about 30 years ago. This approach aims to integrate phenotypic, genotypic and phylogenetic information for the delineation of taxa at all levels. Genotypic information is derived from the nucleic acids (DNA and RNA) while phenotypic information is derived from proteins and their function, chemotaxonomic markers, and other expressed features (Vandamme *et al.*,

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1996). Information from a variety of molecules may be used within a polyphasic approach. However, the technical complexity in terms of time and labour often determines the sequence of application since not all can be applied to large numbers of isolates. Additionally, it is also important to understand at which level these molecules carry information. An abridged presentation from Vandamme *et al.* (1996) of the level of taxonomic information from the different techniques is given in Fig. 3.1.

The classification of rhizobia based on plant infection tests has been abandoned since the genes coding for nodulation, host specificity and nitrogen fixation are sometimes located on transmissible symbiotic plasmids. The International Subcommittee for the Taxonomy of *Rhizobium* and *Agrobacterium* proposed minimal standards for the description of species of root- and stem-nodulating bacteria (Graham *et al.*, 1991). These include symbiotic performance with selected hosts, cultural and morphological characteristics, DNA-DNA relatedness, rRNA-DNA hybridisation and 16S rDNA analysis, DNA restriction fragment length polymorphisms, and multilocus enzyme electrophoresis. The aim of this section is therefore to discuss some of the major categories of taxonomic techniques required to study bacteria at different levels, their general concept and application in rhizobial taxonomy.

2 Phenotypic Methods

Phenotypic methods comprise all those not directed toward DNA or RNA, and as such also include chemotaxonomic techniques. The term "chemotaxonomy" refers to the application of analytical methods to collect information on various chemical constituents of the cell to classify bacteria. More detail on this approach, classical phenotypic analyses and numerical analyses will be discussed in subsequent sections.

2.1 Classical phenotypic analyses

Classical or traditional phenotypic traits, such as morphological, physiological and biochemical features, form the basis for the formal description of taxa, from species and subspecies up to genus and family level. Very often, highly standardised procedures are required to obtain reproducible results within and between laboratories when considering some of these phenotypic traits. There are also instances where the paucity of these phenotypic characters can cause problems in the description or differentiation of taxa. For

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such bacteria, alternative chemotaxonomic or genotypic methods are often required to reliably identify strains (Vandamme *et al.*, 1996).

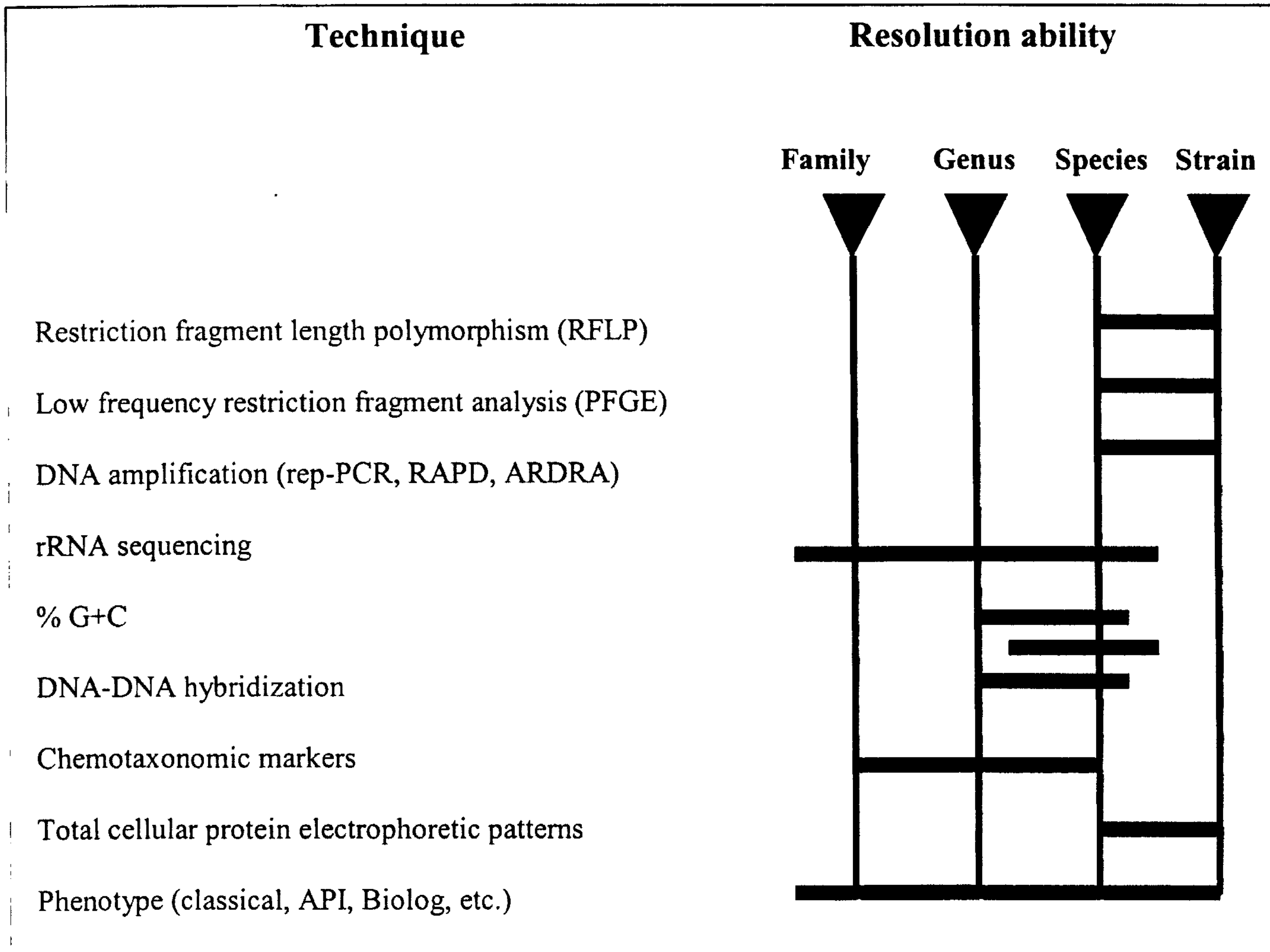

Figure 3.1 Taxonomic resolution of some of the techniques used in polyphasic taxonomy. Abridged from Vandamme *et al.*, 1996.

2.2 Numerical analysis

The primary aim of numerical taxonomy or computer-assisted classification is to assign individual bacterial strains to homogeneous groups using large sets of different types of phenotypic data (Goodfellow & O'Donnell, 1993). Numerical taxonomy arose in parallel with the development of computers and allowed the comparison of large numbers of phenotypic traits for large numbers of strains (Vandamme *et al.*, 1996). The application of

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numerical analyses subsequently led to the revision of the pre-1960 classification of many bacterial genera (Goodfellow & O'Donnell, 1993). More recently, it has found application in the taxonomy of rhizobial isolates (see Chapter 4 for more specific references). Additionally, McInroy *et al.* (1999) characterised rhizobia from African acacias and other tropical woody legumes by applying numerical taxonomy based on substrate utilisation patterns.

2.3 Chemosystematics

Chemosystematics is a discipline in which information derived from the whole organism or cell fractions is used to classify, identify or type bacteria. The development of this area of taxonomy is greatly due to the introduction and application of reliable, rapid and sensitive analytical methods, such as chromatography and electrophoresis. Using these tools, traits such as cell wall composition, cellular fatty acids, whole-cell proteins, sugars and amino acids could be used as chemotaxonomic markers. These could be used at all taxonomic levels, although the discriminatory power of these may vary between taxa (Goodfellow & O'Donnell, 1993).

2.4 Multilocus Enzyme Electrophoresis (MLEE)

It is often difficult, and sometimes impossible, to relate phenotypic variation to allelic variation at a specific locus. Hybridisation of total DNA has been widely used to define species limits (Wayne, 1987). However, this application has made little contribution to estimates of the variation within species due to the large experimental error associated with this application arising from the interference of extrachromosomal DNA and variation between laboratories (Selander *et al.*, 1986).

MLEE has long been used as a standard method in eucaryotic population genetics and although not so widely used in procaryotes, a number of reports have verified its applicability in the study of bacteria (Demezas *et al.*, 1991). In the application of the MLEE method microorganisms are characterised by electrophoretic analyses of a range of enzymes, such as glucose-6-phosphate dehydrogenase or malate dehydrogenase, which are important components of metabolic pathways. Due to their essential role in metabolism they are widely distributed across diverse genera. Furthermore, they are chromosomally encoded, present as single copies on the genome and their products are easily identified by staining following

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electrophoresis. MLEE method has the advantage over other phenotypic methods in that it provides an unbiased assessment of the population genetic structure since the presence of a specific enzyme electromorphs do not appear to provide a selective advantage for the strains in which they reside (Eardly, 1994).

The electrophoretic variation observed among the enzymes is related to allelic variation in the genes coding for these proteins. Typically, the electrophoretic variation at 15 to 30 enzyme loci are considered and can provide information on the genetic variation within and among species (Eardly, 1994; van Berkum & Eardly, 1998). Genetic distance or relatedness is usually expressed as the proportion of loci at which dissimilar alleles occur (Selander *et al.*, 1986).

The applicability of this technique in the examination of *Rhizobium* species has been described in Eardly (1994). In a recent report describing *Sinorhizobium arboris* and *Sinorhizobium kostiense*, 13 enzyme loci were analysed (Nick *et al.*, 1999b). The *S. arboris* strains revealed six and the *S. kostiense* three distinctive multilocus genotypes. Other earlier reports on rhizobial MLEE studies have indicated that species of *Bradyrhizobium* and *Rhizobium* are extremely diverse in comparison to human bacterial pathogens (Martinez-Romero & Caballero-Mellado, 1996).

3. Genotypic Methods

3.1 Determination of the DNA base ratio (moles percentage G+C)

The determination of the moles guanosine and cytosine is considered one of the classical genotypic methods forming part of the standard description of bacterial taxa. Among procaryotes it ranges between 24% and 76% and within well-defined species and genera the range is not more than 3% and 10%, respectively (Vandamme *et al.*, 1996). Differences in the moles % G+C are taxonomically useful for separating groups, however, similarities in base composition do not necessarily indicate close relationship, since the linear sequence of bases in the DNA molecule is not considered (Rosello-Mora & Amann, 2001). Conversely, organisms with widely different base composition will have few DNA sequences in common and are likely to be distantly related. Most bacterial genera have, however, comparatively narrow ranges of G+C values (Austin & Priest, 1986). It should be noted that estimates of

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G+C content must be treated with caution since variation between laboratories have been observed and is therefore always a mean value (Logan, 1994).

The G+C content of DNA may be determined by various methods which exploits the physical, chemical and optical properties of DNA. The most common method is the optical tracking of denaturing of DNA. This denaturation is associated with a higher absorbance (approximately 40%) at 260 nm. The thermal denaturation midpoint (T_m) depends on the DNA base composition and is therefore an important taxonomic feature (Grimont, 1988). This midpoint temperature increases with increased mole % G+C. When T_m is determined the mole % G+C can be determined by an established empirical formula:

mole % G+C = 2.44 T_m −169.3 (Austin & Priest, 1986)

Although a less popular method, CsCl gradients can also be used to determine the mole % G+C. In this instance the density of DNA is exploited since DNA density increases linearly with the mole % G+C. The method requires ultracentrifugation for a long period of time and reference DNA of known mol % G+C. Other methods for determining mole % G+C include DNA bromination, comparative absorbance ratio determination and the release of DNA bases by acid hydrolysis and subsequent chromatographical separation (Johnson *et al.*, 1985).

Species of *Rhizobium* usually have G+C values in the range 59 to 64 mole %, while in *Azorhizobium* the range is 66 to 68 mole % and *Bradyrhizobium* strains have an intermediate value of 61 to 65.4 mole % (Graham *et al.*, 1991). *Allorhizobium* (de Lajudie *et al.*, 1998a), *Sinorhizobium* (de Lajudie *et al.*, 1994) and *Mesorhizobium* (Jarvis *et al.*, 1997) have mole % G+C values of 60.1, 60.8 to 65.7 and 59-64, respectively.

3.2 DNA-DNA hybridisation studies

Traditional typing methods represent information of no more than 10% of the genome. In contrast, methods involved in determining DNA relatedness represent considerably more information since a larger portion of the genome is examined (Vandamme *et al.*, 1996). A characteristic of DNA and RNA is its ability for hybridisation or reassociation. Under standardised conditions, DNA from different organisms reassociate depending on the similarity of their nucleotide sequences. This allows quantification of the degree of relatedness and is usually expressed as % similarity or homology (Rosello-Mora & Amann,

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2001). The percent DNA binding or DNA-DNA hybridisation value is an indirect parameter of the sequence similarity between two entire genomes. Genetically closely related organisms will have more nucleotide sequences in common and therefore a higher degree of nucleotide binding will occur (Vandamme *et al.*, 1996). Based on DNA relatedness, genomic species (or genetic species) is defined as a group of strains showing homology of 70% or more under optimal hybridisation conditions and with 5 °C or less $\Delta\ T_m$ (Wayne *et al.*, 1987). A later description of circumscription of the genomic species was made by Grimont, (1988): strains showing 80% reassociation at optimal temperature with divergence below 5 °C belong to one genomic species and that strains showing less than 60% reassociation and more than 7 °C divergence do not belong to the same genomic species.

Many different procedures have been developed to measure DNA similarity, and can be of two types; immobilised DNA or free solution renaturation. Most of these methods require radioactively labelled reference DNA. In the immobilised assay, single stranded (ss) DNA is immobilised on a nitrocellulose filter, incubated in the presence of labelled DNA from reference organisms and the amount of reassociation is estimated by measuring the radioactivity on the membrane. In this estimation, results from heterologous reactions (involving DNA from different strains) are also included (Grimont, 1988).

In the free solution approach, DNA hybridisation relies on the renaturation rates determined spectrophotometrically at 260 nm. Rates of DNA reassociation between test and reference organism and of each DNA separately are monitored by falls in absorbance at 260 nm (Logan, 1994).

In other methods a small amount of sheared, radiolabelled denatured DNA and larger amounts of sheared unlabelled DNA are mixed in a 1: 500 ratio and allowed to reassociate under optimal conditions. Reassociated fragments are separated from non-reassociated fragments by either hydroxyapatite (HA) chromatography or selective digestion of single stranded fragments by S1 nuclease. Both single and double stranded DNA adsorbs to HA, and can be selectively eluted by raising the buffer molarity. The radioactivity of the single-stranded and double-stranded fragments is measured and the percentage reassociation calculated (Grimont, 1988).

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The S1 nuclease procedure is performed under conditions which prevent or reduce the digestion of double-stranded DNA, allowing digestion of nearly all ss-DNA. Half of the hybridisation mixture is treated with S1 nuclease, the remaining double stranded molecules precipitated and the percentage reassociation calculated by comparison with the untreated sample.

Recently, Christensen *et al.* (2001) developed a micro-well DNA-DNA hybridisation assay. This method was aimed at reducing the time and labour used to perform such hybridisations. Briefly, the method entails the covalent binding of mechanically-sheared DNA to the micro-wells and addition of photo-activatable-biotin-labelled reference DNA. The amount of biotin-labelled DNA bound to wells after stringency washes can then be determined by the addition of chemicals, which react with the biotin label, to generate a fluorescent signal.

3.3 DNA-RNA hybridisation

Bacterial cells contain several classes of RNA, including mRNA, rRNA and tRNA. Most comparative RNA hybridisation studies have been performed with either 16S and /or 23S rRNA molecules. Additionally, these two molecules account for 80% of the nucleic acid in a bacterial cell, which can readily be isolated. rRNA is now generally accepted as a target for studying phylogenetic relationships since it is present in all bacteria, functionally constant and consists of conserved and variable regions (Woese, 1987; Stackebrandt & Goebel., 1994).

In DNA-rRNA hybridisations, sequence homology between labelled 16S or 23S rRNA from a reference strain, and the rRNA cistrons within the chromosomal DNA from a second isolate is determined. The usual approach is to expose immobilised, denatured chromosomal DNA to labelled rRNA. Following the removal of unbound labelled rRNA and RNase treatment, the thermal stability of the hybrid is tested by subjecting the filter to a series of temperature increases up to 95 °C. The radioactivity eluted at each step is measured as the hybrids are denatured to give values of thermal stability (Austin & Priest, 1986; Logan, 1994). One drawback of this approach is that the G+C content of rRNA (52-54 mole % range) requires high hybridisation temperatures to be used. Such high temperatures can cause thermal degradation of the rRNA. Using formamide alleviates the problem of RNA degradation since lower temperatures may be used (Johnson, 1985). Furthermore, duplicating hybridisation

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conditions between different laboratories is difficult, making correlation of data almost impossible.

In 1988 Grimont described the relationship between DNA-DNA and DNA-RNA reassociation. It was clear that these two methods covered different domains of relatedness; DNA-DNA hybridisation being useful for species delineation where DNA-RNA lacks accuracy. On the other hand, DNA-DNA hybridisation is insufficiently accurate when organisms are distantly related, whereas DNA-RNA reassociation is fully able to determine these distant relationships.

3.4 DNA based typing techniques

DNA-directed typing methods have improved substantially during the last few years. Their suitability as a taxonomic tool includes their universal applicability, reproducibility and is relatively easy to perform.

First-generation DNA-based typing methods included restriction fragment length polymorphism (RFLP) analyses of the whole genome and plasmid DNA. The former method entails the digestion of genomic DNA with restriction endonucleases, electrophoresis and visualisation of the DNA fragments. In general, these patterns are very complex and difficult to compare. The complexity may be resolved by selecting low frequency cutting restriction enzymes or southern blotting with specific probes. Since the fragments generated with low frequency cutting enzymes are usually very large, pulsed–field gel electrophoresis (PFGE) is employed instead of conventional agarose electrophoresis (Vandamme *et al.*, 1996). Studies by Gordillo *et al.* (1993) and Tenover *et al.* (1995) have shown PFGE to be a highly discriminatory typing method. Alternatively, these complex DNA patterns may be transferred to membranes and hybridised with a specific labelled probe. Typically rRNA probes are used, which may be 16S rRNA, 23S rRNA or both (Vandamme *et al.*, 1996).

The introduction of the polymerase chain reaction (PCR) methodology (Mullis & Faloona, 1987; Saiki *et al.*, 1988) has led to the development of numerous typing methods. These PCR-based techniques have attracted much interest because of their universal applicability, simplicity and speed with which it may be carried out (Vandamme *et al.*, 1996). Subsequently, the approach to determine relationships based on restriction endonuclease sites,

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was extended to target specific genomic regions amplified by PCR. Both whole genome- and amplified gene RFLP analysis have several limitations. In both instances sequence divergence is estimated based on information from only a small portion of the genome and may not be representative of the sequence divergence across the entire genome. If southern hybridisation is performed, the applied probes may react poorly, if at all, with distantly related lineages. Furthermore, fingerprint patterns may also be influenced the presence of polymorphic insertion sequences (van Berkum & Eardly, 1998).

3.4.1 DNA fingerprinting patterns generated by PCR.

(a) Random whole-genome analysis

Whole genome analysis relies on the presence of repetitive elements, which are targeted by a number of PCR-based techniques. These include RAPD, ERIC-, BOX- and REP-PCR, and the analysis of restriction enzyme sites on the genome by PFGE and AFLP. All of these techniques recognise random sites on the genome, which cannot be predicted without the whole genome sequence.

(i) Random amplified polymorphic DNA assay (RAPD)

The RADP assay, also known as arbitrary primed PCR, was first described by Williams *et al.* (1990) and Welsh & McClelland (1990). In this technique short random sequence primers are used to initiate amplification of random regions on the bacterial genome. Since the number and site of these random priming sites differ for different strains of a bacterial species, separation of such amplification products, using agarose gel electrophoresis, will result in a banding pattern characteristic of a particular strain. However, in most cases the sequences of the RAPD primers and reaction parameters, which generate the best DNA banding pattern, need to be determined empirically (Olive & Bean, 1999). According to Vila *et al.* (1996), the RAPD assay is more discriminatory than RFLP of either the 16S rRNA genes or the 16S-23S ITS region, but less discriminatory than REP-PCR. The major drawback associated with this technique is its sensitivity; slight changes in reaction conditions and reagents make standardisation extremely difficult (Olive & Bean, 1999).

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(ii) Analyses of interspersed repetitive elements (rep-PCR)

Bacterial genomes contain various repetitive DNA elements: the repetitive extragenic palindromic (REP) element, the enterobacterial repetitive intergenic consensus sequence (ERIC) and BOX elements located within the intergenic regions. The exact function of these highly repeated and conserved elements remains unknown. Although their involvement in various cellular functions, such as mRNA stabilisation and homologous recombination have been suggested, no single function has emerged explaining their conserved and ubiquitous nature (de Bruijn, 1992).

The REP sequence consists of a 33 bp sequence which is found in approximately 500 copies dispersed around the chromosome of *Eschericia coli* and *Salmonella typhimurium* (Gilson *et al.*, 1984; Stern *et al.*, 1984). The 126 bp ERIC sequences are present in many copies on the genome of many enterobacteria; to date there is no evidence of it being present outside gram-negative enterobacterial species (Versalovic *et al.*, 1991; Hulton *et al.*, 1991). The BOX element is an inverted repeat element initially found in *Streptococcus pneumoniae*. They are mosaic repetitive elements composed of various combinations of three subunit sequences referred to as boxA, boxB and boxC (Martin *et al.*, 1992). Additionally, these BOX elements have now been found in a number of other bacterial species and have no sequence similarity to the other two repetitive elements (Olive & Bean, 1999).

Comparative sequence analyses of the REP and ERIC elements have led to the development of oligonucleotides targeting these regions (Versalovic *et al.*, 1991). These were employed to detect the presence of REP- and ERIC-like sequences in a number of eubacteria. Surprisingly these elements were found in a large variety of bacterial genera, preferable gram-negative bacteria as described by de Bruijn (1992). A pilot study by the same author showed the presence of these elements in four genera of the family *Rhizobiaceae*. The suitability of this method for classification purposes was also investigated. The results showed that these elements were highly conserved within this group and could indeed be used to distinguish closely related rhizobia. Subsequent studies by others, such as Vinuesa *et al.* (1998) led to similar conclusion. Another study of 51 fast-growing Sudanese and Kenyan rhizobial isolates by Nick *et al.* (1999a) found that rep-PCR fingerprinting could be used as a first method to rapidly classify rhizobial strains of unknown taxonomic status.

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These repetitive elements have superior discriminatory abilities over those of restriction analysis of the 16S rRNA and 16S-23S spacer region, MLEE and biochemical characterisations (Olive & Bean, 1999).

(b) Specific gene variation

Both single-locus and multilocus approaches have been used for molecular typing of bacterial isolates. The single-locus approach includes highly variable genes usually implicated in causing disease such as the neurotoxins of *Clostridium botulinum*. In contrast, the multilocus approaches include multi-locus sequence typing, the analysis of multi-gene families such as *rrn* operons and tRNA genes (Gürtler & Mayall, 2001). In 1996 Gürtler & Stanisch reported on the content and order (5' to 3') of the *rrn* operon as being: 16S rRNA, spacer, 23S rRNA, spacer and 5S rRNA sequences. This arrangement is universal for most bacteria with exceptions reported by Gürtler (1999). Some of these genomic regions, other novel regions and their specific application in polyphasic taxonomy will be discussed in later sections.

(i) The 16S or small subunit ribosomal RNA (ssu-rRNA) gene

The sequencing of the ssu rRNA gene has led to a better understanding of the relationship among the bacteria and may be considered the most useful and most used molecular chronometer (Woese, 1987). The usefulness of 16S rRNA sequences for classification purposes was summarised by Woese *et al.* (1985):

- The rRNA molecule is part of a large molecular complex central to the function of the cell, making transfer between species impossible. Phylogeny based on rRNA is therefore reflective of the true phylogeny of the whole organism.
- It is functionally constant to a degree rare among macromolecules. Consequently "non-chronometric" changes in sequence (i.e. those that are selected and so would distort phylogenetic analysis) occur rarely, making rRNA a particularly accurate molecular chronometer.
- The molecule is large and contains many functionally defined domains. Thus, the rare evolutionary redesign of one such domain (which involves non-chronometric, selective changes) amount to a smaller perturbation only on the molecule as a whole, which is not the case for smaller molecules, such as cytochrome *c* or the 5S rRNA.

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Additionally, the ssu-rRNA molecules have both conserved and variable regions making them suitable for use in the analyses of closely related and more distantly related organisms.

However, the reliability of using the ssu rRNA gene alone for estimating phylogenies, has been questioned due to the conserved nature of this molecule. The applicability of the species definition, based on the ssu rRNA similarity in relation to DNA-DNA reassociation values, was investigated by Stackebrandt & Goebel (1994). These authors concluded that ssu rRNA sequence data are most reliable at similarity values lower than 97%, whereas at values higher than 97% DNA-DNA hybridisation provides a more reliable estimate. The 16S rRNA nucleotide similarity values (Table 3.1) among members of the *Rhizobiaceae* range from 88% to 96.3%

Young & Huakka (1996) highlighted some of the limitations of the ssu rRNA as a phylogenetic and taxonomic tool when they considered the diversity and phylogeny of rhizobia. The sources of the limitations were illustrated by the following cases:

- **Recombination among ssu sequences**

  The first 300 bases of *S. meliloti* are significantly more similar to that of *R. leguminosarum* than to the *M. loti* sequence which is more likely due to recombination events than to parallel evolution.

- **Heterogeneity within species**

  Certain genotypes of *R. leguminosarum* have been found to have a 73-nucleotide insertion in the first loop of the ssu rRNA.

- **Heterogeneity within genomes**

  Most bacteria have several copies of the rRNA genes. The cloning and sequencing of PCR-amplified ssu rRNA of *Sinorhizobium saheli* revealed two different sequences in the first stem-loop structure.

Keswani & Whitman (2001) investigated the relationship between 16S rRNA sequence similarity (*S*) and the extent of DNA hybridisation (*D*) in procaryotes. These authors found that it was possible to accurately estimate the distribution of *D* from *S* when the presence of nonultrametric rRNA sequences and differences between genera or families were controlled. The relationship between *D* and *S* varied between procaryotic taxa but was not significantly

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different between procaryotic domains. The extent of DNA hybridisation also changes more rapidly for closely related organisms than for distantly related organisms. Therefore ranges of *D* assigned for intergeneric relationships should be interpreted with caution. The nonultrametric property of some rRNA led to lower *S* values than expected from the value of *D*. For such taxa, 16S rRNA sequence similarity was a poor indicator of the relatedness for closely related strains. Therefore, the ultrametric property of rRNA sequences should be tested before making taxonomic or phylogenetic conclusions based upon *S*.

(ii) The large subunit (lsu) or 23S gene

The lsu rRNA gene is used to a lesser extent for phylogenetic inferences. The topology of the lsu rRNA phylogenetic tree has been reported to be similar to that of the tree based on the ssu rRNA gene sequences (Ludwig *et al.*, 1995). However, this region is almost twice the size of the ssu rRNA molecule and contains highly variable stem-loop structures or intervening sequences which may be useful for classification and identification purposes (van Berkum & Eardly, 1998). Tesfaye *et al.* (1997) compared the partial 23S rDNA sequences from *Rhizobium* species. Comparative sequence analysis identified divergent regions that appeared to include characteristic species- or strain-specific sequences. This led to a later report (Tesfaye & Holl, 1998) of 23S-specific primers, which could differentiate between different effectiveness groups within the *Rhizobium-Trifolium* cross-inoculation group.

(iii) 16S-23S Intergenic spacer regions

The 16S-23S Intergenic spacer (IGS) region is not well conserved and exhibits a large degree of sequence, length and frequency variation (Massol-Deya *et al.*, 1995). This is particularly true for members of the α-subclass of the *Proteobacteria*, in which its length varies from 800-1200 nucleotides (Normand *et al.*, 1996). Comparing it to other genes of the *rrn* operon, the IGS region is at least twice as variable as the 23S rRNA gene and four times as variable as the 16S rRNA gene (Grundmann *et al.*, 2000). A detailed study by Gürtler (1999) showed that the IGS region was composed of highly conserved blocks of sequences while others were more variable. A more detailed analysis of these variations led the authors to conclude that these variable regions might have arisen due to recombination or selection events. Due to this variation, analysis of this region results in a higher level of discrimination, particularly between closely related species, and has been used for the identification purposes of a number

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of bacteria. Comparative sequence analyses of the IGS region were able to provide high resolution of the relatedness between members of the genus *Nitrobacter* (Grundmann *et al.*, 2000). Similarly, an IGS-RFLP study by Laguerre *et al.* (1996) was able to indicate intraspecific polymorphism between different biovars of *Rhizobium leguminosarum*.

Table 3.1. Small subunit rRNA nucleotide similarity values among the genera of the *Rhizobiaceae*.

	Mesorhizobium	***Sinorhizobium***	***Agrobacterium***	***Rhizobium***	***Azorhizobium***	***Bradyrhizobium***
Allorhizobium (X67221)	93.1	94.2	96.3	93.5	88	86.4
Mesorhizobium (X67229)		95.9	93.6	94.4	91.4	88.1
Sinorhizobium (X67222)			95.3	96	91.2	88.3
Agrobacterium (D14500)				94.5	90.6	88
Rhizobium (U29386)					91.4	88.9
Azorhizobium (X67221)						90.3

The ssu rRNA sequences used were *M. loti*, *S. meliloti*, *Agrobacterium tumefaciens*, *R leguminosarum* bv. *vicae*, *Allorhizobium caulinodans* and *B. japonicum*. Adapted from van Berkum & Eardly (1998). Comparative values for *Allorhizobium undicola* were obtained from de Lajudie *et al.*, (1998a). GenBank accession numbers are indicated in parenthesis.

(iv) Other genomic regions used as phylogenetic markers.

Several other molecules have been used as suitable phylogenetic markers such as cytochromes, ferredoxins and azurins, ATPase, elongation factor and heat shock proteins. However, these are not as easy to handle as ribosomal RNA. The reasons are:

- The degeneracy of the genetic code prevents the formation of conserved regions necessary for the development of PCR amplification primers that could cover the full range of prokaryotic diversity; material to be sequenced must be obtained following time-consuming cloning steps.
- Not all of these proteins are ubiquitously distributed, restricting analyses to only partial phylogenetic trees.

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- The occurrence of protein families, which make it difficult to decide whether proteins are truly homologous or paralogous (Stackebrandt & Rainey, 1995).

A few of these, not so widely used genomic regions, will be discussed briefly in the following sections:

❖ **Glutamine Synthetase II as a novel taxonomic marker.**

Glutamine synthetases (GS) are key enzymes in nitrogen metabolism and are ubiquitous, well-conserved enzymes. Together with glutamate synthase, GS is responsible for ammonium assimilation. Different GS's (designated GSI and GSII) differ with regard to their primary and tertiary structure and seem to have resulted from the duplication events. Very few prokaryotes have two GS isoenzymes, however, *Rhizobium* and *Agrobacterium* are among such genera. A proteomic study (isoelectric focussing and SDS-PAGE), genetic characterisation (southern blotting, PCR-RFLP) and immunochemical characterisation of the GS of various *Rhizobium tropici* and *Rhizobium etli* strains revealed the usefulness of GSII as a novel taxonomic marker. The GSII results support the separation of *R. etli* and *R. tropici* as *bona fide* species (Taboada *et al.*, 1996). An expansive study by Turner & Young (2000) showed that the GSI and GSII phylogeny of rhizobia are incongruent, while GSI phylogeny closely resembles that of 16S rRNA phylogeny.

❖ **Sigma factors of the σ^{70} family**

The sigma factor protein is a dissociable subunit of the eubacterial RNA polymerase holoenzyme conferring the promoter specificity required for transcription initiation. Two broad families of sigma factors have been identified: the σ^{70} and σ^{54}. The former (σ^{70}) is further subdivided into three groups:

- Group 1: the primary sigma factors, present in all known eubacteria and are essential for cell viability;
- Group 2: similar in sequence to the primary sigma factors, include stationary-phase-specific sigma factor RpoS, considered dispensable for cell growth.
- Group 3: varies considerably in sequence from the first two groups, although it is considered dispensable for the cell, this group includes functional groupings such as heat shock and sporulation sigma factors.

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Some of the ideal phylogenetic characteristics of Group 1 sigma factors include: it is ubiquitously distributed among eubacteria, essential for cell viability, contains highly conserved structural features and a large number of sequences are already available. Sequence analyses of Group 1 sigma factors of various eubacteria showed similar resolution as obtained with other molecules, such as the ssu rRNA (Gruber *et al.*, 1997).

❖ **The elongation factor Tu and ATP-synthetase β-subunit genes**

Eubacteria, mitochondria and chloroplasts contain a proton-translocating ATP-synthase (ATPase) complex containing two portions: F_1 and F_0. F_0 is intrinsic to the membrane forming a proton channel. The other (F_1) is an extrinsic membrane protein complex composed of five subunits. The β-subunit contains the catalytic site of the complex and its primary structure is highly conserved and is an ideal macromolecule for deducing phylogenetic relationships (Amann *et al.*, 1988).

The elongation factor (Tu) is also considered a suitable phylogenetic marker. These molecules play a central role in the translation machinery that is different from the tasks of the rRNAs, though functionally connected. Consequently, elongation factor phylogenies are only partly useful for testing the validity of rRNA-based phylogenies.

Since the ATPases are functionally independent of the components of the translation apparatus, such phylogenies are useful to evaluate rRNA and elongation factor phylogenies. A comparative study by Ludwig *et al.* (1993) found that elongation factor data were in good agreement with rRNA trees, although the elongation factor trees showed reduced resolution at certain phylogenetic levels. On the other hand, the discriminatory power of the ATPase β-subunit was less than that of the elongation factor.

❖ **Transfer RNA's as genotypic fingerprints of eubacteria.**

Höfle (1990) described a new method for rapid genotypic classification of bacteria based on the high-resolution gel electrophoresis of low molecular weight (LMW) RNA fraction of bacterial strains. This fraction is composed of the total pool of transfer RNA and the 5S rRNA. Such an electrophoresis profile consists of bands belonging to different size ranges: 5S rRNA (110-131 nucleotides), class 2 tRNAs (82-96 nt) and class 1 tRNAs (72-79 nt). A

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study of members of the family *Rhizobiaceae* by Velazquez *et al.* (1998) showed their LMW RNA profiles to be consistent with established taxonomic classification. The 5S profiles gave sufficient discrimination between the different genera of the *Rhizobiaceae*, while class 2 tRNA profile were species specific.

4. Conclusion

Considerable advances have been made in the field of bacterial systematics. To a large extent this is due to the development of molecular techniques. As discussed, each of these techniques has its specific level of discrimination and, as such, these techniques should not be applied on an exclusive basis, but rather complement each other. The choice of technique will, to a large extent, be dictated by the aims of a particular study; where diversity is the primary question one or two of these techniques will be sufficient. On the contrary, the work of a bacterial systematisist will be more extended to generate a consensus taxonomy.

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CHAPTER 4

Taxonomy of the rhizobia

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1. Introduction

Nitrogen-fixing bacteria forming symbiotic associations with members of the Leguminosae, and related pathogenic bacteria, have been ascribed to the genera *Rhizobium* (Frank, 1889), *Bradyrhizobium* (Jordan, 1982), *Azorhizobium* (Dreyfus *et al.*, 1988), *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994), *Mesorhizobium* (Jarvis *et al.*, 1997), *Allorhizobium* (de Lajudie *et al.*, 1998a), *Agrobacterium* (Conn, 1942) and *Phyllobacterium* (Knösel, 1984) within in the ∝-subdivision of the *Proteobacteria*.

The first root nodule bacterium was cultured by Beijerinck, which he named *Bacillus radicicola*. The taxonomy of these root-associated organisms however, remained in dispute for a number of years. Later these root-nodule bacteria were referred to as *Rhizobium leguminosarum*. By 1929 *Rhizobium leguminosarum, R. trifolii, R. phaseoli, R. meliloti, R. japonicum* and *R. lupini* were recognised rhizobial species. The distinction between these species was based on the formation of nodules on the roots of certain legumes. This method of species designation was later discontinued due to incongruous plant-bacterium reactions. Other characteristics such as growth rate, acid production, serology, DNA base ratios, numerical taxonomy, DNA hybridisation and phage susceptibility were also considered (van Berkum & Eardly, 1998). These early proposals were formerly adopted in the approved lists of bacterial names in 1980 (Skerman *et al.*, 1980).

Bacteria inducing crown-galls were first isolated in 1907 and were named *Bacterium tumefaciens* (van Berkum & Eardly, 1998). Conn (1942) concluded that a soil bacterium, *Alcaligenes radiobacter*, isolated previously in 1902, was similar to the crown-gall producers and legume nodule bacteria. Based on similar morphological and physiological characteristics, Conn (1942) proposed the genus, *Agrobacterium*, which would contain both the plant pathogens and related soil saprophytic bacteria. By 1980 the approved list of bacterial names (Skerman, 1980) included the following species in this genus: *A. tumefaciens, A. radiobacter, A. rhizogenes* and *A. rubi*. The other *Agrobacterium* species, *A. vitis* and *A. larrymoorei*, were described later by Ophel & Kerr (1990) and Bourzar & Jones (2001), respectively.

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The genus *Phyllobacterium* (Knösel, 1984), affecting leaves of plants of the families Myrsinaceae and Rubiaceae, was omitted from the approved list of bacterial names. However, based on DNA-rRNA hybridisation results of Gillis & de Ley (1980) two species, *P. myrsinacearum* and *P. rubiacearum*, were tentatively classified within the *Rhizobiaceae*.

There has been many improvements and amendments since the publication of the approved list of names by Skerman *et al.* (1980). This is mainly due to the application of polyphasic taxonomy (as discussed in Chapter 3) and the isolation of rhizobia from previously uninvestigated leguminous host species. These major developments in rhizobial systematics will be discussed in the following sections. Young *et al.* (2001) recently proposed a revision of the genus *Rhizobium*; the major arguments for this suggestion will be discussed at the end of this Chapter. The taxonomic outline of the ∝-*Proteobacteria*, as it will appear in the second edition of Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2001) is summarised in Appendix A.

2. The genera of the *Rhizobiaceae*

The genera *Rhizobium* and *Bradyrhizobium* are separated by several morphological and physiological characteristics. These include differences in growth rate, flagellation, symbiotic gene location, etc. (Jordan, 1984; Elkan, 1992). *Azorhizobium caulinodans* is equally distinct from the former two. Until very recently *Agrobacterium* and *Phyllobacterium* were the only other recognised genera within the *Rhizobiaceae*. The establishment of the genera *Sinorhizobium* (de Lajudie *et al.*, 1994) and *Mesorhizobium* (Jarvis *et al.*, 1997) are well supported by sequencing data of the small subunit rRNA sequences. Additionally, de Lajudie *et al.* (1998a) have also described the genus *Allorhizobium*.

Molecular data based on the SSU rRNA sequences (Fig. 4.1) indicate that rhizobia are polyphyletic, since no branch of the phylogenetic tree carries all the rhizobia and no other bacteria. Twenty four different genera are also present with this subdivision of the purple bacteria (Young, 1996).

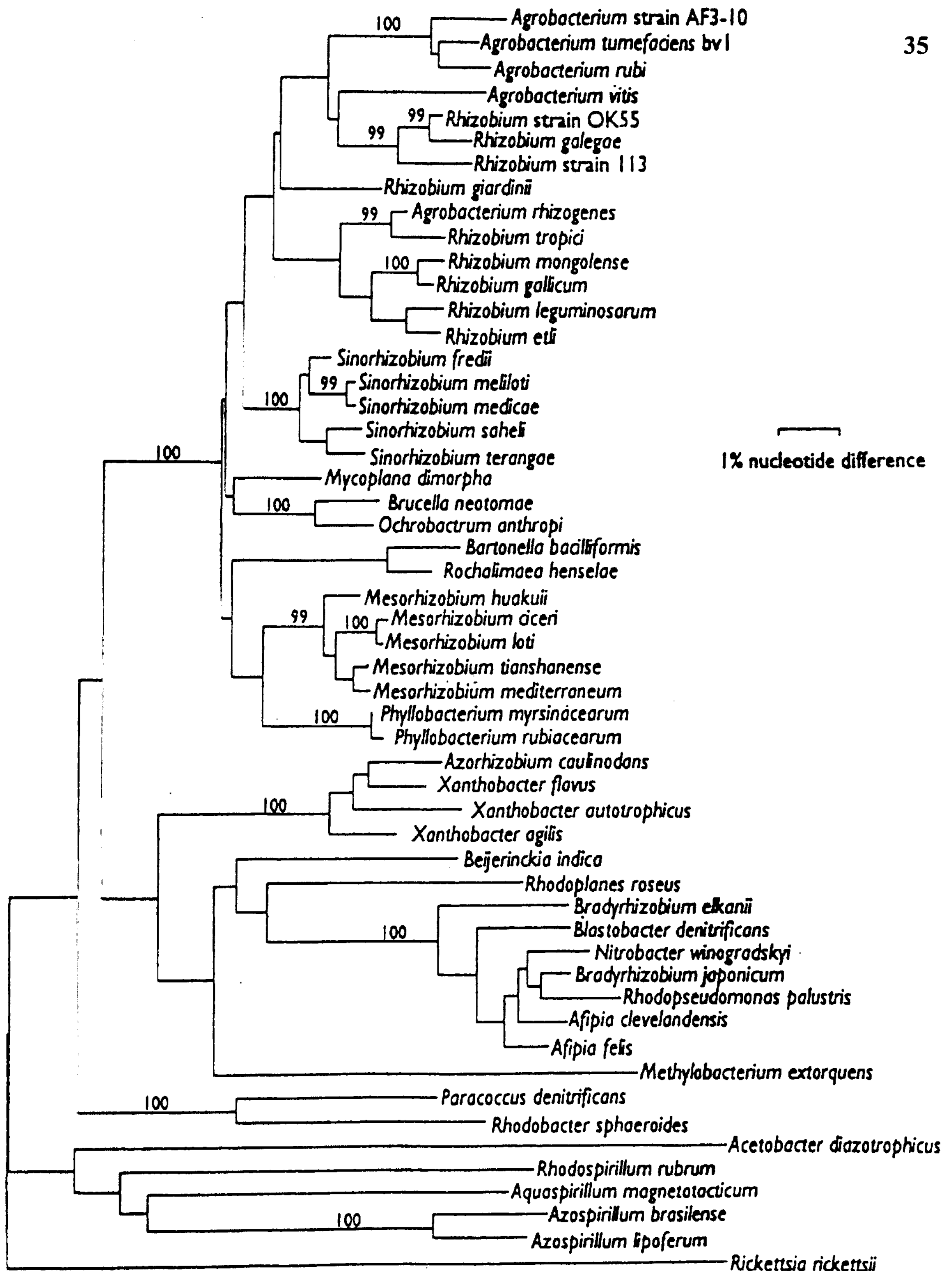

Figure 4.1. Phylogenetic relationships of the *Rhizobiaceae* within the α-subdivision of the Purple bacteria based on the SSU rRNA gene. Jukes-Cantor distances were derived from the aligned sequences to construct the unrooted tree using the unrooted Neigbour-joining method. Five hundred replicates were generated in a bootstrap analysis to derive a majority rule consensus tree. The scale bar indicates 1% nucleotide difference (van Berkum & Eardly, 1998).

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(a) The *Bradyrhizobium* genus

The genus *Bradyrhizobium* comprises slow-growing microsymbionts, producing an alkaline reaction and no serum zone in Litmus milk (van Berkum & Eardly, 1998). Currently, only two species, *B. japonicum* (Jordan 1982) and *B. elkanii* (Kuykendall *et al.*, 1992) are recognised, and a third, *B. liaoningense* (Xu *et al.*, 1995), has been suggested. Besides the genus *Phyllobacterium*, investigation of the taxonomy of bradyrhizobia has not advanced as rapidly as it has with the other rhizobial lineages. This might be attributed to their low growth rate which shifted the focus to rhizobia rather than bradyrhizobia. Nonetheless, considerable genetic diversity among the slow-growing microsymbionts has been recognised over an extended period. In evidence to this heterogeneity Jarvis *et al.* (1986) proposed a photosynthetic ancestry for *Bradyrhizobium* based on the high homology of its SSU rRNA sequence to that of *Rhodopseudomonas palustris*. This was further supported by van Berkum *et al.* (1995) with the analyses of diverse stem-nodulating bradyrhizobia on *Aeschynomene indica*. A more recent study (Molouba *et al.*, 1999) showed that these photosynthetic rhizobia formed a separate sub-branch on the *Bradyrhizobium* rRNA lineage, distinct from *B. japonicum* and *B. elkanii.*

Because of the relatively small 16S rRNA divergence among bradyrhizobia, Willems *et al.* (2001a) recently undertook an extensive DNA-DNA hybridisation study. These were performed between *Bradyrhizobium* reference strains and bradyrhizobia, isolated mainly from *Faidherbia albida* and *Aeschynomene* species. Their results showed that the genus *Bradyrhizobia* consists of at least 11 genospecies, of which genospecies I, II and III respectively represented *B. japonicum*, *B. elkanii* and *B. liaoningense*.

(i) *B. japonicum* and *B. elkanii*

B. japonicum and *B. elkanii* form symbiotic associations with soybeans. Since these crops are of agricultural importance, these symbionts have been the focus of intensive scientific investigation (van Berkum & Eardly, 1998). These slow-growing microsymbionts were previously classified into 17 groups based on serological differences. This approach has, however, been shown to have limited application (Date & Decker, 1965). *B. japonicum* was the only recognised species within the genus *Bradyrhizobium* until Hollis *et al.* (1981) characterised strains obtained from soybeans. These strains showed 30% or less DNA-DNA homology with *B. japonicum*, type strain ATCC 10324. These divergent isolates, which

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belonged to a different DNA homology group as *B. japonicum*, was suggested as representing a distinct species. Differences in genomic RFLP data, antibiotic resistance patterns, extracellular polysaccharide (EPS) composition, MLEE and differences in hemoproteins, provided additional justification for the separate species status of isolates from this divergent DNA homology group. Kuykendall *et al.* (1992) therefore proposed *Bradyrhizobium elkanii* to describe this group of strains.

(ii) *B. liaoningense*

Xu *et al.* (1995) analysed 17 extra slow-growing (ESG) isolates from root nodules of soybeans in different provinces of the People's Republic of China. Based on numerical taxonomy analyses, %G+C content, DNA-DNA hybridisation, partial 16S rRNA sequencing, nitrogen and carbon content of cell components, *Bradyrhizobium liaoningense* was proposed.

(b) The *Azorhizobium* genus

Legume species of the genera *Neptunia, Aeschynomene* and *Sesbania* bear nodules both on the roots and the stems (Dreyfus *et al.*, 1981). Regarding the nodulation of *Sesbania rostrata,* Dreyfus *et al.* (1984) found two types of strains forming a symbiotic association. The first forms nodules on both stems and roots and fix nitrogen asymbiotically. The second forms only root nodules and was unable to fix nitrogen in culture. DNA: rRNA hybridisations showed the root nodulating strains to be related to the genus *Rhizobium*, while the stem- and root nodulators showed close relation to *Xanthobacter flavus.* There were, however, sufficient morphological and biochemical differences validating separate genus status (Dreyfus *et al.*, 1988). Thus, to determine the exact taxonomic position of this group of nodulating microorganisms, Dreyfus *et al.* (1988) analysed a collection of isolates from stem- and root nodules. This investigation included numerical analyses of phenotypic characteristics, comparative protein gel electrophoresis and hybridisations, which led to the description of the new genus *Azorhizobium.* This genus contains one species, *A. caulinodans.*

(c) The genus *Allorhizobium*

Isolates from nodules of *Neptunia natans,* an indigenous stem-nodulated tropical legume from waterlogged areas of Senegal, were analysed by de Lajudie *et al.* (1998a). *N. natans* is agriculturally important since it is being considered as green manure for rice cultivation in

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India and is consumed in South-East Asia (Subbarao *et al.*, 1995). Nodule isolates from *N. natans* have been reported to be fast growers (Dreyfus *et al.*, 1984), but a detailed taxonomical analysis has not yet been reported. A polyphasic approach by de Lajudie and co-workers (1998a) described these *N. natans* isolates to represent a new genus, *Allorhizobium*, with one species, *Allorhizobium undicola*. *Agrobacterium vitis* and *Rhizobium galegae* were closely related to the new genus when considering 16S rRNA gene, but the low bootstrap values, defining those branching points, suggest these relationships are currently insignificant.

(d) The genus *Rhizobium*

(i) *R. leguminosarum*

In 1889 Frank used this name to describe the symbionts of the legume genus *Vicia* which distinguished them from the symbionts of the genera *Trifolium* (*R. trifolii*) and *Phaseolus* (*R. phaseoli*) (van Berkum & Eardly, 1998). The majority of symbionts of the latter two genera was characterised both at a phenotypic and genetic level, which led to their reclassification as *R. leguminosarum* (Jordan, 1984). Different biovars (bv.), which indicate specific plant affinities, have also been described (Jordan, 1984). These include biovars vicia, trifolii and phaseoli for nodulation of genera *Vicia*, *Trifolium* and *Phaseolus* respectively.

(ii) *R. tropici*

A revision of the classification of *Rhizobium phaseoli* led to the description *R. leguminosarum* bv. phaseoli (Jordan, 1984). However, evidence of heterogeneity in *R. leguminosarum* bv. phaseoli was clearly indicated by protein patterns, antibiotic resistance, serological type, MLEE, hybridisation data, plasmid profiles and exopolysaccharide structure (Martinez-Romero *et al.*, 1991). The authors further reported on two types of bean isolates, which differed significantly in their symbiotic plasmids. These were designated Type I and Type II. Since Type II was found to retain their plasmids after prolonged incubation at 37°C, to be heat tolerant (Karanja *et al.*, 1988) or acid and aluminium resistant (Graham *et al.*, 1982), it has received considerable attention. Additionally, it is also able to establish effective symbiosis with both *Phaseolus vulgaris* and *Leucaena* spp. Martinez-Romero *et al.* (1991) thus undertook a study to define the taxonomic position and taxonomic relatedness of Type II strains. *R. tropici* was proposed with two subgroups, Type II A and Type II B, the latter being the designated type strain. It appears that these two subgroups are sufficiently

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different to warrant two separate species status, but additional information to support this has not been reported (van Berkum & Eardly, 1998). Willems & Collins (1993) reported SSU rRNA sequences, which showed that the phylogenetic position of *R. tropici* was distinct from that of *R. leguminosarum*, supporting the proposal of *R. tropici*.

(iii) *R. etli*

The *Rhizobium* Type I strains, referred to in the discussion of *R. tropici*, was further analysed by Segovia and co-workers (1993) and *Rhizobium etli* sp. nov. was proposed. They are characterised by their ability to establish effective symbiosis with bean plants, the reiteration of the nitrogenase structural genes, the organization of *nodA* and *nodBC* into two transcriptional units, the presence of the polysaccharide inhibition gene and 16S rDNA sequences. This new species also includes at least one biovar, *Rhizobium etli* bv. phaseoli. Recently, Wang *et al.* (1999a) identified rhizobial isolates, from a small Mexican leguminous plant, *Mimosae affinis*, as *Rhizobium etli* and a new biovar *R. etli* bv. mimosae has been proposed. This biovar differs from biovar phaseoli in its ability to nodulate *Leucaena leucocephala*, non-production of melanin and variation in both *nifH* gene sequences and organisation.

(iv) *R. galegae*

Fast-growing root nodule bacteria which nodulate *Galegae orientalis* and *Galegae officinalis* were initially reported to be related to *R. leguminosarum* and *R. loti*. However, since *G. orientalis* have potential agricultural application, its symbiont has been studied more extensively. These results showed that the *Galegae* rhizobia formed a homologous group of fast-growing rhizobia, which was not related to the then, recognised *Rhizobium* species (Lindström, 1989). In a further study by Terefework *et al.* (1998), both the 16S and 23S rRNA genes were targeted in an effort to resolve contradictions from previous studies and clarify the phylogenetic position of *R. galegae*. 16S rDNA analysis showed that *R. galegae* formed a subgroup on the *Agrobacterium* branch while being part of the *Rhizobium* branch in the 23S analysis. Young & Haukka (1996) also reported that *R. galegae* is not closely related to typical members of the genus *Rhizobium*, but not close enough to *Agrobacterium* to be transferred to that genus. Terefework *et al.* (1998) thus proposed that *R. galegae* remains in the genus *Rhizobium* until more information become available.

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(v) ***R. huautlense***

The nodulation of *Sesbania* have been reported previously (de Faria *et al.*, 1989). Except, for *Azorhizobium caulinodans*, which forms stem and root nodules on *Sesbania rostrata* (Dreyfus *et al.*, 1988), very few of the *Sesbania* symbionts have been investigated extensively. Rinaudo *et al.* (1991) investigated DNA homologies among a group of 191 isolates obtained from *Sesbania.* Although *A. caulinodans* was predominanent among these isolates, a small group of isolates was also related to *Rhizobium.* A subsequent polyphasic study by Wang *et al.* (1998) of isolates from *Sesbania herbacea* led to the description of *R. huautlense.*

(vi) *R. gallicum* and *R. giardinii*

A diversity study of isolates from various locations in France revealed strains genotypically different from *R. leguminosarum.* Within this collection a second group of isolates, which could not be assigned to the former group or to *R. leguminosarum*, was also identified. Analyses, including genomic RFLP with several probes (Geniaux *et al.,* 1993; Laguerre *et al.,* 1993a), DNA homology and partial 16S rDNA sequencing (Laguerre *et al.,* 1993b) led to the recognition of two genomic species. At that stage no names were proposed for these new species since additional data based on phenotypic characteristics were required. Amarger *et al.* (1997) reported on these phenotypic features and the complete 16S rDNA sequence and subsequently proposed two new species, *R. gallicum* and *R. giardinii.* Furthermore, the authors also provided results (symbiotic traits and genotypic data) supporting the division of each species into two biovars. The first subgroup had similar symbiotic characteristics as the phaseoli biovars of *R. leguminosarum* and *R. etli*, while the second showed species-specific symbiotic phenotype and genotype. The two proposed biovars were thus *R. gallicum* bv. gallicum and *R. gallicum* bv. phaseoli; and *R. giardinii* bv. giardinii and *R. giardinii* bv. phaseoli, respectively.

(vii) ***R. hainanense***

Gao *et al.* (1994) isolated a total of 63 rhizobial strains in the Hainan Province, a tropical region of the People's Republic of China. Preliminary analyses of these isolates included numerical taxonomy, DNA hybridisation and DNA composition. The slow-growing isolates were to related to *Bradyrhizobium japonicum.* However, the fast-growers were more diverse

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since some formed unique subgroups. One of the subgroups, designated subgroup IV, was clearly distinguishable from all *Rhizobium* species known at that time. Chen *et al.* (1997) extended the analyses of this subgroup to include full 16S rDNA sequences, symbiotic performances and DNA-DNA hybridisation. This led to the proposal of the name *Rhizobium hainanense* for this species.

(viii) *Rhizobium mongolense*

Medicago ruthenica is considered a potential new forage crop since it is more tolerant to environmental stresses than the presently cultivated *Medicago sativa*. The rhizobial symbionts involved in the nodulation of *Medicago ruthenica* have, however, not been studied in great detail (van Berkum *et al.*, 1998). The symbionts of *Medicago sativa* have been reported on by Eardly *et al.* (1990) and de Lajudie *et al.* (1994) and classified as *Sinorhizobium meliloti*. In a later study Rome *et al.* (1996b) also proposed *Sinorhizobium medicae* as an additional species forming symbiosis with plants of the genus *Medicae*. In 1998 van Berkum *et al.* undertook a study in which rhizosphere samples were collected to study the diversity of the nodulating strains using *Medicago ruthenica* as trap hosts. These isolates were characterised phenotypically, genetically and phylogenetically, leading to the conclusion that at least three genomic species, within the genus *Rhizobium*, form symbiosis with *Medicae ruthenica*. One of these was *R. tropici* (Martinez-Romero *et al.*, 1991) while the second was proposed as *Rhizobium mongolense*. The third strain was provisionally included in the new species based only on MLEE data. However, neither 16S rDNA sequence nor DNA hybridisation results supports the placement and this isolate may possibly represent a species with a chimeric genotype.

(ix) *R. yanglingense*

The north-western regions of China are temperate regions with arid and semi-arid soils, where some herbaceous and woody legumes grow. The diversity of rhizobial populations associated with 11 species of these legumes were analysed by Tan *et al.* (1999) in terms of phenotypic and genetic traits. The host plants included shrubs such as *Amorphae fructicosa*, *Sophora viciifolia* and *Caraganna* spp. and a semi-shrub *Glycyrrhiza* spp. The perennial herbaceous plants included *Coronilla varia*, *Gueldenstaedtia* spp. and *Amphicarpeae trisperma*. Five different clusters were obtained, of which one (designated cluster 9) showed

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high homology to the genus *Rhizobium*. Cluster 9 isolates were all obtained from perennial herbaceous plants. More recently Tan *et al.* (2001) expanded the investigation of cluster 9 by the inclusion of more isolates from *Coronilla varia,* and *Gueldenstaedtia multiflora*. Additionally, whole-cell protein SDS-PAGE analyses, DNA base composition, DNA hybridisation and 16S rDNA sequencing were also done. Based on the low DNA homology value (42%) to other known *Rhizobium* spp. and phenotypic differences, the new species, *Rhizobium yanglingense*, was proposed.

(e) The genus *Sinorhizobium*

DNA-DNA reassociation analyses indicated the existence of distinct genetic groups of *Rhizobium*. One such group (named group 3) consisted of closely related strains nodulating *Medicago* and *Trigonella* spp. (Crow *et al.*, 1981). Wedlock and Jarvis (1986) later reported that fast-growing soybean isolates from China were also closely related to this group. Chen *et al.* (1988) followed a numerical taxonomical approach, based on phenotypic traits, and proposed that these fast-growing soybean isolates be assigned to a separate genus, *Sinorhizobium*. However, the molecular evolutionary systematic data (based on SSU rRNA genes) reported by Jarvis *et al.* (1992) and Willems & Collins (1993) did not support this separate genus assignment, discouraging the recognition of this new genus. An expanded taxonomical study (polyphasic taxonomy) by de Lajudie *et al.* (1994), which included the group 3 isolates, the fast growing Chinese soybean isolates and two isolates from Senegalese tropical trees, provided sufficient support which merits the creation of the new genus *Sinorhizobium*.

(i) *S. fredii* and *S. xinjiangense*

Fast-growing rhizobia have been recognised from soil and soybean nodules collected in China (Keyser *et al.*, 1982). A comparative study of these organisms showed that these strains had cultural traits corresponding to the fast-growing genus *Rhizobium* and symbiotic traits, which corresponded to the slow-growing *Bradyrhizobium*. Since these fast-growing strains did not correspond to any of the known species, Scholla & Elkan (1984) proposed the name *Rhizobium fredii* to describe these new species. Additionally, based on differences in serological reactions, antibiotic resistance, acid production and DNA-DNA hybridisation two new chemovars were proposed within *R. fredii*. These included *R. fredii* chemovar fredii and

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R. fredii chemovar siensis. The taxonomic position of these fast-growing isolates remained doubtful and prompted Chen *et al.* (1988) to undertake a numerical taxonomic study to extend the known information. These authors proposed the establishment of a new genus, *Sinorhizobium*, containing two species: *Sinorhizobium fredii* comb. nov. (previously *R. fredii*) and *Sinorhizobium xinjiangense* sp. nov. In 1993 Willems and Collins undertook a phylogenetic study of rhizobia and agrobacteria based on 16S rDNA sequences. Expressing the phylogenetic relationships using Fitch analyses, *R. fredii* was recovered as a subgroup in the main *Rhizobium-Agrobacterium* cluster. However, when applying the parsimony algorithm and bootstrap analyses to this data, several inconsistencies regarding branching orders and positions were evident, most notably, the positioning of *R. fredii* outside the main *Rhizobium-Agrobacterium* cluster. These results thus provided support for the establishment of the new genus *Sinorhizobium*.

(ii) *S. meliloti* and *S. medicae*

S. meliloti, formerly known as *Rhizobium meliloti* was described by Jordan (1984) and is able to form symbiotic association with *Medicago, Melilotus* and *Trigonella*. Eardly *et al.* (1990) used multilocus enzyme electrophoresis (MLEE) and RFLP analysis of the rRNA operons to determine the genetic structure of *S. meliloti* populations obtained from *Medicago* species. Two subgroups were identified and based on the magnitude of the genetic difference between them; the authors concluded that these might represent separate species. de Lajudie *et al.* (1994) also reported that *S. meliloti* could be distinguished from other *Sinorhizobium* species by its gel electrophoretic protein profiles, DNA-DNA hybridisation data and 16S rRNA gene sequence. Rome *et al.* (1996a) performed DNA-DNA hybridisation, PCR RFLP of the symbiotic regions (*nodD-K, nodDI* and *nodII*), the 16S rDNA gene and the intergenic spacer region (IGS) on a collection of isolates obtained from *Medicago truncatula*. These results supported the existence of the two genomic subspecies; the first corresponding to *S. meliloti* while the second showed a low level of homology to several species of *S. meliloti*. This same group subsequently studied the taxonomic status of the second genomic species, and proposed that these isolates belong to a new species, *Sinorhizobium medicae* (Rome *et al.*, 1996b). These species can be distinguished from *S. meliloti* since they are able to form effective nodules on *Medicago polymorpha* (*S. meliloti* forms ineffective nodules on this host plant).

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(iii) *S. saheli* and *S. terangae*

de Lajudie and co-workers (1994) studied the nodulating strains of *Sesbania* and *Acacia* from Senegal (West Africa). Initially, strains were compared based on whole-cell protein profiles and three protein electrophoretic clusters (designated S, T & U) were identified. Subsequently, representatives of these different groupings were further analysed in terms of their nodulating host range, DNA base compositions, DNA-DNA hybridisations and 16S rDNA sequences. The results indicated that clusters S and T were related to the *Rhizobium meliloti* - *Rhizobium fredii* (now *S. meliloti* - *S. fredii*) rRNA branch. They were however, also genotypically and phenotypically distinct from each other. They subsequently proposed an emendation to the genus *Sinorhizobium* to include *Sinorhizobium saheli* for species from cluster S and *Sinorhizobium terangae* for species from cluster T. The remaining cluster (cluster U) demonstrated considerable heterogeneity, but was closely related to *R. huakuii* based on 16S rDNA sequences. They concluded that more studies with more representatives of this group were necessary to clarify the taxonomic position of this group.

(iv) *S. arboris* and *S. kostiense*

Zhang *et al.* (1991) performed numerical analyses of rhizobial strains from *Acacia senegal* and *Prosopis chilensis* growing in Sudan. These strains were all found to be fast-growers and extremely diverse in terms of their cross-nodulation patterns and physiological and biochemical properties. This diversity within the tree rhizobia was later confirmed by the unique banding patterns of some strains generated by pulsed-field gel electrophoresis of restricted total DNA (Haukka & Lindström, 1994). Haukka *et al.* (1996) performed partial 16S rRNA sequencing analysis on some of these strains and found them to be related to the genera *Sinorhizobium* and *Mesorhizobium*. Nick *et al.* (1999a) performed DNA-DNA hybridisation, rep-PCR genomic fingerprinting and %G+C content determination on these isolates. These results were in good agreement with each other and results obtained previously by 16S rDNA sequencing (Haukka *et. al.*, 1996). A subsequent study by Nick *et al.* (1999b) focussed on a collection of these five Kenyan and 25 Sudanese strains. Two of these strains grouped with *S. saheli* and seven with *S. terangae*, while the rest formed phenotypically and genotypically distinct groups. Based on these differences Nick *et al.* (1999b) proposed that strains of these distinct groups be classified as new species, *Sinorhizobium arboris* and *Sinorhizobium kostiense*.

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(f) The *Mesorhizobium* genus

Jarvis *et al.* (1997) proposed the establishment of a new genus *Mesorhizobium* and suggested the transfer of *R. loti, R. huakuii, R. ciceri, R. mediterraneum* and *R. tianshanense* to this new genus. These amendments were proposed since the true phenetic and phylogenetic differences among species of the *R. loti* group were obscured by the inclusion of all fast-growing rhizobia in the genus *Rhizobium*. Moreover, further subdivision of the genus *Rhizobium* has been proposed at various meetings concerned with rhizobial taxonomy. The term "meso" refers to both growth rate and phylogenetic position. According to van Berkum & Eardly (1998), the former refers to the slower growth rate when compared with *Rhizobium* and *Sinorhizobium* but faster growth rate in comparison with *Bradyrhizobium*. However, slower growth rate is not necessarily a commonly shared characteristic shared among all the members of this genus. With regard to phylogenetic position "meso" refers the intermediate position between the *Agrobacterium-Rhizobium-Sinorhizobium* complex and the genera *Azorhizobium* and *Bradyrhizobium*.

(i) *M. loti*

A revision of the genus *Rhizobium* led to the combination of the fast-growing *R. trifolii, R. phaseoli* and *R. leguminosarum* into a single species, designated *R. leguminosarum* with different biovars. As a part of this revision, *Rhizobium loti* was proposed to describe fast-growing rhizobia nodulating *Lotus* species (Jarvis *et al.,* 1982). The *Lotus*-nodulating rhizobia were found to be widely divergent based on several traits which included: bacteroid ultrastructure, internal antigens, extracellular polysaccharide composition, enzymatic complement, SDS-PAGE of total soluble proteins, growth rate, acid production on yeast extract media, susceptibility to flavolans, isoflavonoids, phage relationships, DNA homology and plant cross-nodulation (Jarvis *et al.*, 1982). The divergence between the fast- and slow-growing *Lotus* rhizobia was further substantiated when DNA-rRNA hybridisation analyses, indicated that the rRNA of the slow-growing *Lotus* isolates were more closely related to *Bradyrhizobium* than to *Rhizobium* (Jarvis *et al.*, 1986). Within the fast-growing *Lotus* rhizobia considerable divergence was also present, since they formed part of an extensive plant cross-inoculation group. The plant species involved were from the genera *Lupinus, Ornithopus, Lotus, Anthyllis, Caragena, Astragalus, Ononis, Genista* and *Mimosa*. Crow *et al.* (1981) identified four genetic groups of fast-growing rhizobia based on DNA homologies.

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The fourth group included strains which showed a high level of DNA relatedness to two fast-growing *Lotus* rhizobia. However, low levels of similarities were found with other reference strains, suggesting that DNA homology group 4 included other species, in addition to *R. loti*.

One isolate, CC809a obtained from *Lotus marocanus*, and two others (3Hoa1 & Revadim) from chickpea (*Cicer arietinum*) were included as members of the *R. loti* group (Jarvis *et al.*, 1982). However, this strain exhibited only 47% DNA homology to the *R. loti* type strain. On the contrary, CC809a showed high levels of DNA homology (85-88%) with the two chickpea isolates. According to Wayne *et al.* (1987), strains showing less than 70% DNA homology do not belong to the same species; therefore CC809a and the chickpea isolates should not have been assigned to the *R. loti* group.

(ii) *M. ciceri*

Rhizobia associated with chickpeas have not been studied in great detail or only limited numbers were included, leading to contradictory results. On the other hand, several studies showed that chickpea rhizobia form a unique group on the basis of host-plant rhizobia relationship, serological and antigenic differences, and *nifHD* gene polymorphism (Nour *et al.*, 1995). In an effort to re-examine the diversity of the chickpea rhizobia Nour *et al.* (1994a) analysed the MLEE profiles, 16S rDNA RFLP and phenotypic characters of 16 chickpea isolates. Two phylogenetically distant groups (A and B), which correlated well with generation times (fast-slow growers), were identified. The exact taxonomic status of these two groups was then investigated further by Nour *et al.* (1994b). They concluded that group A and B belonged to the genus *Rhizobium* since they formed a tight cluster which included *R. loti* and *R. galegae*, with group B being closely related to *R. loti*. DNA-DNA homology data showed low correspondence of the chickpea rhizobia to other rhizobial genera described at that time. Furthermore, only 38% DNA homology value was observed between the two groups, demonstrating that they were different species. Nour *et al.* (1994b) therefore proposed that the group B chickpea rhizobia be assigned to a new species, *R. ciceri*.

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(iii) *M. mediterraneum*

The identity of isolates belonging to group A was revealed when Nour *et al.* (1995) characterised isolates from chickpea from diverse geographical regions. This group was highly heterogeneous since it contained three genomic species and five strains remained unclassified. Genomic species could be differentiated from *R. ciceri* with regard to 16S rDNA sequence, DNA homology, 16S intergenic spacer region, previously described MLEE results (Nour *et al.*, 1994a,b) and phenotypic characteristics. *Rhizobium mediterraneum* was thus proposed as the new species to describe strains belonging to genomic species 2.

(iv) *M. huakuii*

Chen *et al.* (1991) characterised isolates from *Astragalus sinicus*, grown in the People's Republic of China as green manure. Based on numerical taxonomy, these isolates were closely related to each other and to the *Rhizobium* genus. However, they could be distinguished from *R. meliloti* and *R. leguminosarum*. When considering SDS-PAGE data of whole cell protein profiles, similar clustering patterns were observed. Additional data, including %G+C content, DNA-DNA hybridisation, further supported the distinctness of the *Astragalus* isolates and this group was subsequently described as *Rhizobium huakuii*.

(v) *M. tianshanense*

The nodulating strains of different leguminous plants (including *Glycyrrhiza*, *Sophora*, *Caragana*, *Halimodendron* and *Swainsonia*), growing in the arid saline desert soil of the Xinjiang region of north-western People's Republic of China were characterised by Chen *et al.* (1995). Initially two groups were identified, the first of which contained the fast-growing reference strains. Further subgroups, corresponding to eight previously described *Rhizobium* spp, were also evident within this first group. The second group could be further divided into three subgroups, one corresponding to *Bradyrhizobium*, another subgroup of three fast-growers and a third of moderately slow-growers. On the basis of the extensive analyses Chen *et al.*, (1995) concluded that the moderately slow growers represent a new species, *Rhizobium tianshanense*.

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(vi) *M. plurifarium*

A previous taxonomic study of the rhizobia nodulating *Acacia* species in West Africa identified cluster U, which was closely to *R. huakuii* (now *M. huakuii*). Furthermore, cluster U, was extremely heterogeneous and the authors refrained from making any new species proposal until additional isolates were available for this group (de Lajudie *et al.*, 1994). de Lajudie *et al.* (1998b) expanded the taxonomic data of cluster U by including nodulation tests on diverse legumes and genomic typing (REP-PCR, 16S rDNA sequencing & DNA-DNA homologies). In addition, cluster U was extended with the inclusion of five Senegalese isolates obtained from *Acacia senegal, Acacia tortilis* subsp. *raddiana* and *Prosopis juliflora.* From these results, it was evident that *M. plurifarium* (referring to the fact that his species contains strains isolated in several places in East and West Africa and South America) represents a separate species in the *Mesorhizobium* genus.

(vii) *M. amorphae*

Wang *et al.* (1999b) investigated 50 isolates from *Amorpha fruticosa*, from several different sites in China. *A. fruticosa* has been cultivated for many years in Asia, and is useful as ground cover, windbreaker, green manure, food for wildlife, a source of oil in the production of glycerol, etc. A polyphasic approach showed that these isolates can be classified into five groups and was mainly associated with the genera *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium*. From more detailed analyses of three groups of isolates, mainly related to the genus *Mesorhizobium*, it was evident that these groups were distinguishable from the known species within the genus *Mesorhizobium*. However, differences among the groups were less distinct and Wang *et al.* (1999b) hesitated to separate them into three different species. Only the most distinct group (group 1) was proposed as a new species within the genus *Mesorhizobium*, for which the name *Mesorhizobium amorphae* was proposed.

(viii) *M. chacoense*

Species of the genus *Prosopis* are important indigenous trees in many ecosystems in South America and some have been introduced into Africa (Velázquez *et al.*, 2001). Reports by de Lajudie *et al.* (1998b) and Huakka *et al.* (1996) have indicated that these trees can be nodulated by the indigenous rhizobia. Except for these reports little work has been described about the American symbionts of *Prosopis*. The work by Velázquez *et al.* (2001) therefore

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focussed on rhizobial strains from *Prosopis chilensis*, growing in diverse geographical locations in central Argentina. Results from a polyphasic approach, including 16S rDNA sequencing, LMW RNA profiles, SDS-PAGE of whole-cell proteins, phenotypic traits and DNA-DNA hybridisation, led to the description of *Mesorhizobium chacoense.*

(g) The genus *Agrobacterium*

Agrobacterium (Conn, 1942) is a genus containing plant pathogenic species and is closely related to *Rhizobium*. *Agrobacterium* species were originally classified according to the phytopathogenic effects of strains, as well as their ability to produce 3-ketolactose. Those strains causing crown gall were placed in *A. tumefaciens,* those causing hairy-root in *A. rhizogenes*, those causing cane gall on *Rubus* spp. in *A. rubi* and non-pathogens in *A. radiobacter* (Holmes, 1981). Many different authors have resisted the classification based on phytopathogenicity since *A. tumefaciens* and *A. radiobacter* are indistinguishable except for presence or absence of the tumour-inducing (Ti) plasmid. These plasmids are mobile genetic elements, transferable between different species (van Larebeke *et al.*, 1975; Abe *et al.*, 1998). A taxonomy based on traits conferred by plasmids is therefore unstable and unreliable.

Results obtained from various methods (Keane *et al.*, 1970; Holmes *et al.*, 1981; etc.) investigating various *Agrobacterium* species have indicated three genetically and phenotypically distinct clusters, excluding *Agrobacterium rubi*. These groups correspond to the different biovars or biotypes. These were later recognised as different species: *Agrobacterium tumefaciens* [biovar 1] (Smith & Townsend, 1907), *Agrobacterium rhizogenes* [biovar 2] (Riker *et al.*, 1930) and *Agrobacterium vitis* [biovar 3] (Ophel & Kerr, 1990). According to Keane *et al.* (1970) it is generally agreed that in *Agrobacterium* taxonomy biovars have species status. The fourth species is *Agrobacterium rubi* is usually isolated from *Rubus* spp. Since *A. tumefaciens* and *A. radiobacter* differ in plasmid content, biotype 1 is circumscribed to include the non-pathogenic *A. radiobacter*.

(i) *Agrobacterium tumefaciens*

In 1907 Smith & Townsend described *A. tumefaciens* as plant pathogenic bacteria causing crown galls (as summarised in van Berkum & Eardly, 1998). Later authors (Holmes & Roberts, 1981; Willems & Collins, 1993) described both phenotypic and genotypic traits

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which separate *A. tumefaciens* from other *Agrobacterium* species. Based on the high DNA homology between *Agrobacterium radiobacter* and *Agrobacterium tumefaciens* (80 to 87% correspondence) and subsequent 16S rDNA sequence data, Sawada *et al.* (1993) argued that both strains belong to the same species and proposed *A radiobacter* as the type species over *A. tumefaciens*. However, key judicial elements (Opinion 33 of Judicial Commission, 1970) of *Agrobacterium* taxonomy and Rule 38 of the International Nomenclature of Bacteria (Lapage *et al.*, 1992) were not considered. Opinion 33 recognises *Agrobacterium tumefaciens* as the type species, while Rule 38 states that when two taxa of the same rank are united, the name under which they are united should be chosen according to the priority of publication. Consequently, *Agrobacterium tumefaciens* should take precedence over *A. radiobacter*.

(ii) *Agrobacterium rhizogenes*

A. rhizogenes belongs to biovar 2 and is distinguishable from other agrobacteria. Ophel & Kerr (1990) reported that *A. rhizogenes* had 28%, 22% and 47% DNA homology with *A. tumefaciens, A. rubi* and *A. vitis*, respectively. Furthermore, *A. rhizogenes* had only 94% SSU rRNA nucleotide sequence homology with the abovementioned three *Agrobacterium* species.

(iii) *Agrobacterium rubi*

Agrobacterium rubi was described as an organism causing cane gall on the fruiting canes of *Rubus* by Hildebrand (1940) and could be differentiated from *A. tumefaciens* in terms of its physiology and pathogenicity. The type strain is distinguishable from other *Agrobacterium* species by low DNA-DNA reassociation values. Ophel & Kerr (1990) reported DNA binding levels of *A. rubi* as 22%, 8% and 11% with *A. rhizogenes, A. radiobacter* and *A. tumefaciens*, respectively. Willems and Collins (1993) reported 16S rDNA sequence similarity values of 98.5%, 95.2 and 94% with *A. tumefaciens, A. vitis* and *A. rhizogenes*, respectively.

(iv) *Agrobacterium larrymoorei*

Agrobacterium strains isolated from tumours of weeping fig trees (*Ficus benjamina* L.) were found to differ from *A. tumefaciens, A. rhizogenes, A. vitis* and *A. rubi* in terms of their

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differential oxidation of carbon sources and fatty acid content. Phylogenetic data based on 16S rDNA sequences also suggested that these strains were sufficiently different and may represent a novel species (Bouzar *et al.*, 1995). This led Bourzar & Jones (2001) to determine the DNA relatedness of these isolates to other *Agrobacterium* species. Based on these values, previously described phenotypic traits and phylogenetic information, *Agrobacterium larrymoorei* was proposed as a new species.

(v) *Agrobacterium vitis*

In 1990 Ophel & Kerr undertook a study to determine the relationship of *Agrobacterium* isolates from grapevine. These were commonly referred to as biovar 3 and included in one of the heterogeneous groups of *A. tumefaciens* (Kersters & De Ley, 1984). The results of phenotypic tests, serological reaction and DNA homology studies clearly indicated these biovar type 3 isolates to be distinguishable from the other described *Agrobacterium* species and *Agrobacterium vitis* was proposed as a new species (Ophel & Kerr, 1990).

(h) The genus *Phyllobacterium* (Knösel, 1984)

Two species are recognised, *P. myrsinacearum* and *P. rubiacearum*, however, since the 16S rDNA sequences of these two species are similar (Yanagi & Yamasoto, 1993) and in the absence of DNA homology data, it is very difficult to define them as two separate species. They occur on the surfaces and hypertrophies of leaves, and one report (Lamber *et al.*, 1990) has documented their isolation from the root of sugarbeet. *Phyllobacterium* shows high homology to the genus *Mesorhizobium.*

3. Proposed amendments to the current classification of the family *Rhizobiaceae*: the new *Rhizobium* genus

The original nomenclature of *Rhizobium* was based on the specificity of symbiotic partner and host plant range. However, it soon became clear that genes for nodulation and specificity was carried on symbiotic plasmids (Martinez-Romero & Palacois, 1990). These plasmids are not essential for bacterial survival and may be transferred between species (Abe *et al.*, 1998). Therefore taxonomic classification based on symbiotic association is unreliable. The taxonomy of the family *Rhizobiaceae* has undergone major changes as described in the previous sections. The proposed amendments (Young *et al.*, 2001) to the current taxonomy

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concern the genera *Agrobacterium* (Conn, 1942) and *Allorhizobium undicola* (de Lajudie *et al.*, 1998).

Allorhizobium was described as a monospecific genus containing *Allorhizobium undicola* and based on 16S rDNA sequence data, *Agrobacterium vitis* was its closest neighbour. Phenotypic, genotypic and phylogenetic traits provide sufficient support for the separate species status of *Allorhizobium undicola* and *Agrobacterium vitis*. However, closer examination of the protein profiles obtained by SDS-PAGE, PCR-RFLP of the ITS-region and nutritional data, showed no support of a closer relationship between *Agrobacterium vitis* and *Allorhizobium undicola* than to other *Agrobacterium* species and other rhizobial genera. According to Young *et al.* (2001) the unsettled state of *Agrobacterium* nomenclature and the *Rhizobium* heterogeneity were the compelling reasons which led to the description of the genus *Allorhizobium*. *Allorhizobium undicola* shares a common ancestor with *Agrobacterium vitis*, as well as with other *Agrobacterium* and *Rhizobium* species. The acceptance of sequence data alone for the description of the genus *Allorhizobium* invites reclassification of *Agrobacterium* and *Rhizobium* related to *Allorhizobium undicola* (Young *et al.*, 2001). According to these authors four possibilities exists:

i. the genus *Allorhizobium* might include *Allorhizobium undicola, Agrobacterium vitis, R. galegae* and *R. huautlense*;
ii. amalgamation of *Allorhizobium undicola* and *Agrobacterium vitis* in *Allorhizobium* and the creation of a new genus to recognise *R. galegae* and *R. huautlense*;
iii. proposal of a new genus for *Agrobacterium vitis* in a monospecific sister genus with *Allorhizobium* and the creation of a new genus to recognise *R. galegae* and *R. huautlense*
iv. the creation of monospecific genera for *Agrobacterium vitis*, *R. galegae* and *R. huautlense*.

In their effort to reclassify these genera Young *et al.* (2001) undertook 16S rDNA sequence analyses of recognised rhizobial species and related species of the families *Phyllobacteriaceae*, *Bartonellaceae* and *Brucellaceae*. Trees were constructed using four different algorithms: Maximum likelihood (ML) (Felsenstein, 1981), Neighbour-joining method (NJ) (Saitou & Nei, 1987), Minimum-evolution (ME) (Rzhetsky & Nei, 1992) and Maximum Parsimony (MP) (Swofford, 1993). The reliability of the inferred topology was

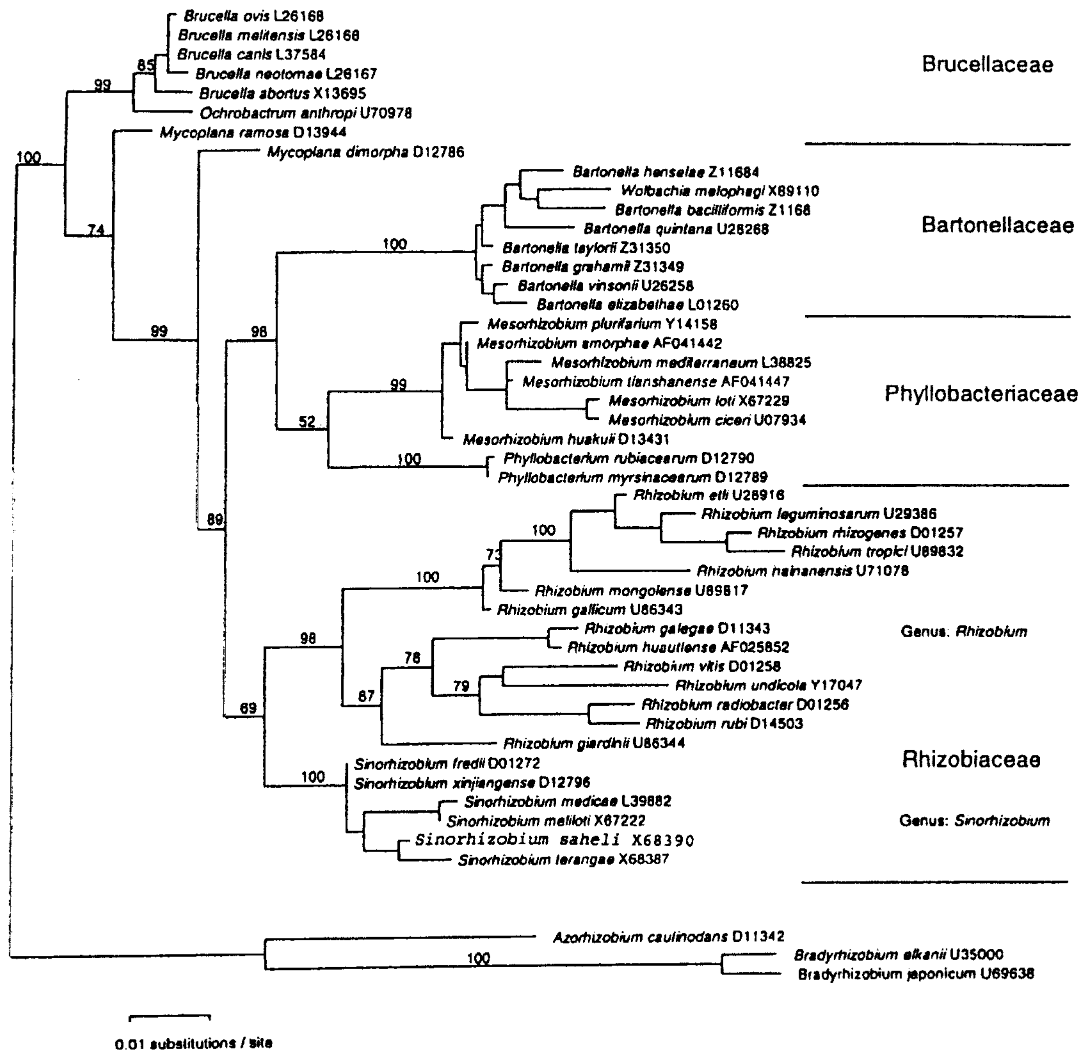

Figure 4.2. Maximum-likelihood tree expressing the relationships between the *Rhizobiaceae* and their relatives based on 16S rDNA sequences. Horizontal branch lengths are proportional to the estimated number of nucleotide substitutions and local bootstrap probabilities (as percentages) were determined for 1000 resamplings. Bacterial family names are those recorded on the website of the Bergey's Manual Trust (http://www.cme.msu.edu/Bergeys) [from Young *et al.*, 2001].

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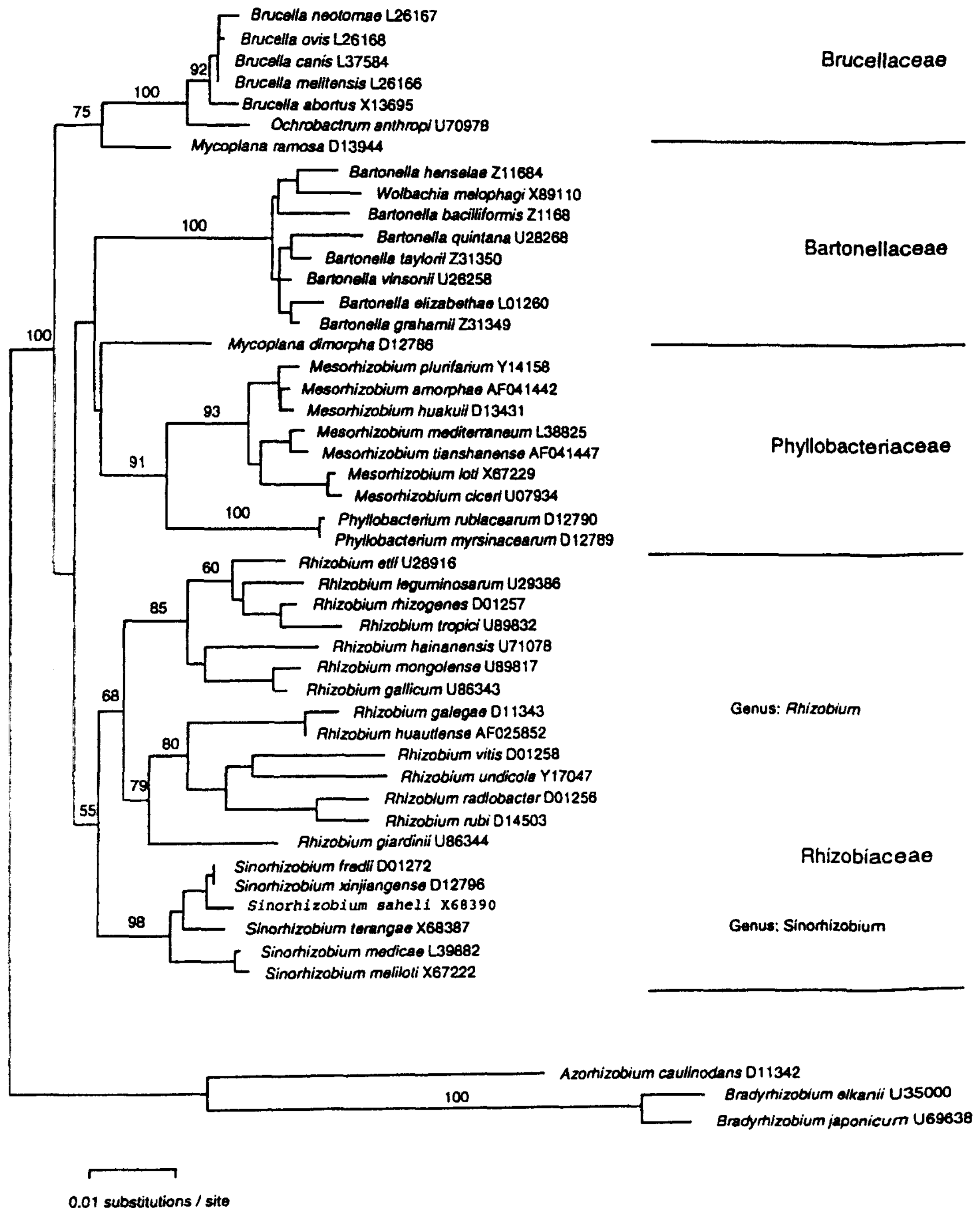

Figure 4.3. Neighbour-joining tree expressing the relationships between the *Rhizobiaceae* and their relatives, based on 16S rDNA sequences. Sites that include gaps in more than one sequence were excluded. Horizontal branch lengths are proportional to the estimated number of nucleotide substitutions and bootstrap probabilities (as percentages) are determined from 1000 resamplings [from Young *et al.*, 2001].

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evaluated by bootstrap probability (Felsenstein, 1985). The tree resulting from ML and NJ methods are shown in Fig. 4.2 and 4.3, respectively. Trees from the ML and ME methods were identical, while the MP tree was almost identical to the ML tree. The inferred phylogenetic relationships showed the family *Rhizobiaceae* to be closely related to the families *Bartonellaceae*, *Brucellaceae* and *Phyllobacteriaceae* in the ∝-*Proteobacteria*. Bootstrap values for the node linking the two *Rhizobium-Agrobacterium* clades were 98%, 68%, 49% and 74% when using the ML, NJ, MP and ME methods, respectively. Furthermore, the analyses provided no support for *Allorhizobium* as an outlying taxon.

The high sequence similarity between members of *Bartonellaceae*, *Brucellaceae* and *Rhizobiaceae* represent a problem for a coherent classification. The former two are mammalian pathogens, and obviously share very little phenotypic traits. The authors further suggested that a straight out acceptance of the phylogenetic relationships for these genera would be erroneous. It is rather suggested that the limit of accurate phylogenetic inferences for these taxa using 16S rDNA sequences alone may have been reached. Alternative sequences and more refined methods for inferences should thus be used for analysis.

Furthermore, no single or multiple phenotypic characteristics have been reported in the circumscriptions of *Agrobacterium*, *Rhizobium* and *Allorhizobium* by which these genera can be differentiated. The conclusion was thus reached that these three genera do not have unique phenotypic circumscriptions; phylogenetic differentiation is not constant and depends on the choice of algorithm and sequences included. Young *et al.* (2001) thus proposed the amalgamation of these genera into a single genus. Applying rule 38 of the International *Code of Nomenclature of Bacteria* (Lapage *et al.*, 1992) the name *Rhizobium* is proposed for the genus, the emended names of all species currently included in this genus are indicated in Fig. 4.2 and Fig. 4.3.

The 16S rDNA sequences indicate that *Mesorhizobium* may be closer related to *Phyllobacterium* than to *Rhizobium* and *Sinorhizobium*. Furthermore, slower growth rate (suggesting underlying metabolic differences) and distinct fatty acid profile as described by Jarvis *et al.* (1996) are considered sufficient justification for their separation from other members of *Rhizobium*.

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Sinorhizobium is retained as a separate genus, pending further phenotypic analyses currently underway by other researchers (personal communications made to Young *et al.*, 2001).

4. Conclusion

The main motivation for the intensive investigation of rhizobia is its ability to fix atmospheric nitrogen symbiotically. For a long time nodulation specificity was considered a basis for classification. However, this approach has been shown to be insufficient to accurately denote the diversity of these organisms. The application of molecular techniques has contributed significantly to a better insight into the phylogeny and taxonomy of all living organisms, including bacteria. The application of these techniques and the increased isolation of rhizobia from diverse leguminous plants and habitats caused dramatic changes to the established rhizobial taxonomy. This increased isolation of nodulating strains and the elucidation of their taxonomical relationships will ultimately also assist the inoculant industry to find more suitable inoculant strains.

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CHAPTER 5

Characterisation of the indigenous rhizobia by partial 16S rDNA Sequencing and PCR-Restriction Fragment Length Polymorphism of the 16S-23S rDNA Intergenic Spacer region.

ABSTRACT
A selection of rhizobial isolates associated with legumes indigenous to South Africa was characterised by partial 16S rDNA sequencing and RFLP of the 16S-23S IGS region. Although the nodule isolates were obtained from a wide range of leguminous hosts, 16S rDNA sequence analysis, showed most of them to be related to the genera *Bradyrhizobium* and *Mesorhizobium*. A few isolates were shown to be heterogeneous since no clear affiliation to a specific genus could be established. Previous research (Willems *et al*., 2001b) has shown that 16S rDNA sequences contain insufficient discriminatory ability to describe the highly diverse bradyrhizobia. IGS-RFLP analysis was therefore performed on isolates related to the genus *Bradyrhizobium*, as well as a group of isolates showing high homology to the *Mesorhizobium* genus. Using this approach it was possible to indicate the presence of additional genotypes, not clearly resolved by the highly conserved 16S rRNA gene.

Keywords: *Rhizobium*, 16S rDNA sequencing, 16S-23S IGS RFLP

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INTRODUCTION

Rhizobia are considered bacteria of agricultural and environmental significance since they are able to fix atmospheric nitrogen during their symbiotic association with leguminous plants of the family Fabaceae and the nonleguminous Parasponia. This advantageous association with these plants, many of which are important food crops, has led to numerous investigations into the diversity of these symbionts. These efforts relied heavily on modern molecular biological techniques and have led to the description of the genera *Rhizobium* (Frank, 1889), *Bradyrhizobium* (Jordan, 1982), *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994), *Mesorhizobium* (Jarvis *et al.*, 1997), *Allorhizobium* (de Lajudie *et al.*, 1998a) and *Azorhizobium* (Dreyfus *et al.*, 1988).

The taxonomy of root- and stem nodulating bacteria is directed by a set of minimal standards (Graham *et al.*, 1991), which requires phenotypic, genetic and phylogenetic traits to be considered in the description of these bacteria. These requirements are in line with the polyphasic approach (Colwell, 1970). Within this approach characteristics of various discriminatory powers are targeted to resolve the complex intra-and intergenic relationships of the bacteria (Murray *et al.*, 1990). Polyphasic taxonomy has consequently contributed to an increased stability of the rhizobial taxonomy.

The nodulation status of legumes growing in South Africa remained unknown for a long period of time until Grobbelaar and co-workers embarked on a systematic survey of approximately 1400 legume species (Strijdom, 1998). This survey stretched over two decades and included almost 1000 species growing under diverse geographical and climatological conditions. Unfortunately, the associated bacterial symbionts were not isolated. More recently, Dagutat (1995) and Dagutat & Steyn (1995) reported on the diversity of rhizobia associated with a diverse range of leguminous hosts. Their results showed most of the indigenous strains to be related to the genus *Bradyrhizobium* (Jordan, 1982), while others showed no association or correspondence with the known rhizobial genera. However, this research was limited to the analysis of growth rate characteristics, colony morphology and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of total soluble cellular proteins and did not comply with the minimal standards as

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discussed earlier. Kruger (1998) therefore undertook an expansive study to include more diverse leguminous plants and analyses of the 16S rDNA gene by RFLP using a set of four different tetrameric restriction endonucleases. Following this approach the author was able to elucidate the identity of most of the indigenous rhizobia. According to Moyer *et al.* (1996), using a combination of three or more tetrameric restriction endonucleases, more than 99% of the operational taxonomic units may be identified. However, it is not meant to replace the 16S rDNA sequencing, but rather a means to more efficiently and effectively describe microbial diversity prior to the sequencing step. Furthermore, phylogenetic relationships based on 16S rDNA sequence data show that rhizobia are polyphyletic: some being closer related to non-symbiotic, non-nitrogen fixing genera within the α-*Proteobacteria* than to each other (Young, 1996; van Berkum & Eardly, 1998). Thus, the aim of this work was to determine the partial 16S rDNA sequence of a selection of rhizobia from indigenous legumes in an effort to either confirm or establish their phylogenetic position. Furthermore, the 16S-23S intergenic spacer (IGS) region is reported to be a useful target region to characterise bacterial strains at a higher discriminating power than that sometimes achieved with 16S rDNA sequences, (Laguerre *et al.*, 1996; Selensky-Pobell *et al.*, 1996, Terefework *et al.*, 1998) and therefore the IGS regions of the indigenous strains was also characterised in an effort to show additional diversity present amongst the indigenous rhizobial isolates.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study are indicated in Table 5.1. These isolates were selected from a rhizobial culture collection established in previous studies by Dagutat (1995) and Kruger (1998). Additional rhizobial strains (Table 5.1), isolated from the root nodules of *Aspalathus linearis* were obtained from the ARC-Plant Protection Research Institute (Private Bag X134, Pretoria 0001, South Africa). Reference cultures of the different rhizobial genera were obtained from the bacterial culture collections of the Laboratorium voor Microbiologie, State University Gent, Belgium and the United States Department of Agriculture (USDA), Soybean and Alfalfa Research Laboratory, Maryland, USA. The 16S rDNA sequences of the rhizobial reference strains and other members of the α-*Proteobacteria* were obtained from

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GenBank database of the National Centre for Biotechnology (NCBI) [website address: www.ncbi.nlm.nih.gov/Genbank/]

Maintenance of bacterial cultures

Strains were maintained on yeast extract mannitol (YM) agar, containing 1% (m/v) mannitol, 0.5% (m/v) K_2HPO_4, 0.02% (m/v) $MgSO_4.7H_2O$, 0.01% (m/v) NaCl, 0.04% (m/v) yeast extract and 1.5% (m/v) bacteriological agar. For long term storage cultures were grown (with rigorous shaking) in yeast mannitol broth (YMB) at 28 °C for approximately five days. The turbid culture broth was subsequently mixed at a 1:1 ratio with sterile 50% glycerol in sterile cryotubes and copies of each stored at both -20 °C and -70 °C.

Genomic DNA extraction

Bacterial cultures were grown in YMB for four to seven days and genomic DNA extracted by the hexadecyltrimethyl ammonium bromide (CTAB) method as described by Wilson (1990). Briefly, approximately 1.5 ml cell suspension was centrifuged and the cell pellet resuspended in 567 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) followed by the addition of 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K. Sufficient cell lyses was achieved by incubating this mixture overnight at 55 °C. Nucleic acids were separated from cell debris, denatured proteins and polysaccharides by the addition of 100 µl 5 M NaCl and 100 µl CTAB/ NaCl solution (10% CTAB in 0.7 M NaCl). This was followed by incubation at 65 °C for 10 min. The CTAB-protein/polysaccharide complexes were then separated from the nucleic acids using phenol-chloroform extraction as described by Saambrook *et al.* (1989). Nucleic acids were precipitated from the aqueous phase by the addition of 0.6 volume isopropanol. The precipitated DNA was pelleted by centrifugation and excess salt removed by washing with 70% ethanol. The genomic DNA was dried in a vacuum dryer and dissolved in double distilled sterilised water. The integrity and concentration of the purified DNA samples were determined by agarose gel electrophoresis (Saambrook *et al.*, 1989).

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Table 5.1 List of indigenous rhizobia analysed in this study.

Subfamilies of the Fabaceae are indicated by **P:** Papilionoideae, **C:** Caesalpinioideae, **M:** Mimosoideae

Isolate	Host legume	Subfamily	Isolate	Host legume	Subfamily
2	*Rhynchosia nervosa*	P	**104a1**	*Chamaecrista sp.*	P
3a	*Trifolium sp.*	P	**114d**	*Pseudarthria hookeri*	P
7b	*Alysicarpus rugosus*	P	**123b**	*Desmodium repandum*	P
13b	*Lotononis bainesii*	P	**125e**	*Indigofera woodii*	P
15b	*Desmodium tortuosum*	P	**128a**	*Melolobium obcordatum*	P
28c	*Desmodium tortuosum*	P	**PL10a**	*Argyrolobium tomentosum*	P
29d	*Alysicarpus rugosus*	P	**UP20b**	*Lotus hispidus*	P
33b	*Crotalaria sp.*	P	**UP26b**	*Crotalaria damrensis*	P
36b	*Neonotonia wightii*	P	**UP27b**	*Crotalaria damrensis*	P
40a	*Seshania bispinosa*	P	**SA3**	*Trifolium africanum*	P
42bs	*Neonotonia wightii*	P	**xhm5**	*Aspalathus cordata*	P
46c2	*Acacia sieberana var woodii*	M	**xhj7**	*Aspalathus linearis*	P
48a	*Tephrosia purpurea*	P	**xhj8**	*Aspalathus linearis*	P
49b	*Indigofera melanadenia*	P	**xhj12s**	*Aspalathus linearis*	P
60	*Rhynchosia monophylla*	P	**xhj12fr**	*Aspalathus linearis*	P
66a1t1	*Acacia robusta*	M	**xhj13**	*Aspalathus linearis*	P
70a	*Crotalaria brachycarpa*	P	**xhj15**	*Aspalathus linearis*	P
70b2	*Crotalaria brachycarpa*	P	**xhj18**	*Aspalathus linearis*	P
74a	*Indigofera arrecta*	P	**xhj20**	*Aspalathus linearis*	P
82a	*Tephrosia purpurea*	P	**xhj23**	*Aspalathus linearis*	P
98d2	*Bolusanthus speciosus*	P	**xhj26**	*Aspalathus linearis*	P
103b	*Indigofera hilaris*	P	**xhj27**	*Aspalathus linearis*	P

PCR amplification of the 16S rDNA gene and the 16S-23S IGS region

The 16S rRNA gene of each isolate was amplified using universal primers fD1 and rP2 as described by Weisburg *et al.* (1991). However, since no PCR product cloning procedures were anticipated, linker sequences containing the restriction enzyme recognition sites were not included during the synthesis of these oligonucleotides. These primers were therefore designated fD1SHRT and rP2SHRT (Table 5.3). The PCR reaction was carried out in a volume of 100 µl and contained 50 pmol of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$, approximately 50 ng genomic DNA and 0.5 U Taq DNA polymerase (Southern Cross Biotechnologies). Additionally, the reaction also contained 50 mM KCl, 10 mM Tris-HCl pH 9.0 and 0.1% Triton X-100 as supplied in the reaction buffer. Amplification was carried out on a Perkin Elmer GeneAmp PCR System 2400 thermocycler using the following thermal

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Table 5.2: Primers used for amplification and/ or sequencing of the different genomic regions analysed in this study.

*Primer	Sequence	Target Region	Reference
fD1SHRT	5' AGAGTTTGATCCTGGCTCAG 3'	16 rRNA gene	Weisburg *et al.* (1991)
rP2SHRT	5' ACGGCTACCTTGTTACGACTT 3'	16 rRNA gene	Weisburg *et al.* (1991)
16SRNAII-S	5' GTGTAGCGGTGAAATGCGTAG 3'	16 rRNA gene	Kuhnert *et al.* (1996)
FGPS1490	5' TGCGGCTGGATCACCTCCTT 3'	16S-23S IGS	Laguerre *et al.* (1996)
FGPS132	5' CCGGGTTTCCCCATTCGG 3'	16S-23S IGS	Laguerre *et al.* (1996)

* All primers synthesised by Roche Molecular Diagnostics (Germany)

profile: initial denaturation step at 95 °C for 3 min, 30 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 30 sec), and extension (72 °C for 1 min). An additional extension step (72 °C for 10 min) was performed after completion of the 30 cycles. To determine the size, purity and concentration of the amplified products, aliquots (usually 5 µl) were run on a 1% agarose gel (results not shown). DNA molecular weight marker VI (Roche Molecular Diagnostics, South Africa) was included on each gel.

Since residual reaction components, such as unincorporated dNTPs, primers, etc., can interfere with subsequent DNA sequencing methodologies, 16S rDNA PCR products were purified using the High Pure PCR Product Purification kit (Roche Molecular Diagnostics, South Africa) or Qiagen PCR Purification Kit (Southern Cross Biotechnologies, South Africa) according to the manufacturer's instructions. To assess the purity and concentration of the purified product, 1 µl was subjected to electrophoresis on a 1% agarose gel [Promega] (results not shown).

DNA sequence determination of the 16S rRNA gene

The purified PCR products were sequenced directly, without any additional cloning procedures, using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (with AmpliTaq® DNA Polymerase, FS) (PE Applied Biosystems). Each sequencing reaction was carried out in a 5 µl volume containing approximately 100 ng template DNA, 12.5 pmol primer, and 2 µl ready reaction premix (supplied with the sequencing kit, containing the dye terminators, dNTP's, AmpliTaq DNA Polymerase, $MgCl_2$, and Tris-HCl

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buffer pH 9.0). For each isolate two different reactions were performed, each respectively containing primers 16SRNAII-S, an internal forward primer (Kuhnert *et al.*, 1996), and rP2SHRT (Weisburg *et al.*, 1991). The reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler and consisted of 25 cycles of denaturation (96 °C for 10 sec), annealing (50 °C for 5 sec), and extension (60 °C for 4 min). Products of the sequencing reactions were precipitated with 60% (v/v) ethanol at room temperature for 15 min, washed with 70% (v/v) ethanol, vacuum dried and stored at minus 20 °C until needed. Prior to electrophoresis, the purified products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer (PE Applied Biosystems), prepared by combining deionized formamide and 25 mM EDTA (pH 8.0) containing 50 mg/ml Blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran. The reactions were denatured for 2 min at 90 °C and loaded onto the ABI Prism model 377 automated DNA sequencer, which uses a high resolution polyacrylamide gel.

Phylogenetic analysis of the 16S rDNA sequences

The sequencing gels were analysed with the ABI Prism Sequencing Analysis 3.1 and the ABI Prism Sequencing Navigator 1.0.1 computer programmes (PE Applied Biosystems). For each isolate, the sequence of both strands was determined using the rP2SHRT and 16SRNAII-S primer pair. These sequences were aligned, producing a layout from which ambiguous sites could be resolved. The ClustalX programme (Thompson *et al.*, 1997) was used to analyse the nucleic acid sequences. A distance matrix was constructed by pairwise alignment of the sequences. The phylogenetic tree was constructed from the distance matrix using the neighbour-joining method of Saitou & Nei (1987). All branch lengths were proportional to the estimated divergence along each branch. The bootstrap method (Felsenstein, 1985) was used in combination with the neighbour-joining method to estimate confidence levels of the phylogenies. The phylogenetic trees were displayed using NJplot (Perrière & Gouy, 1996).

16S-23S rDNA IGS RFLP analysis

The 16S-23S rDNA IGS region was amplified using the primer set FGPS1490 and FGPL132 (Laguerre *et al.*, 1996) and using the same set of conditions as those described for the amplification of the 16S rRNA gene. The PCR products were used directly in a restriction

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reaction, without any prior purification. The digestion reactions were performed using 10 U of each of the following restriction enzymes *Hae*III, *Cfo*I, *Taq*I and *Nde*II (Roche Molecular Diagnostics, South Africa) in optimal buffers as prescribed by the supplier. Electrophoresis of the restricted DNA was carried out on a 3% agarose gel in a Hybaid Maxi Gel System at 80 V for 90 min. DNA molecular weight marker VI (Roche Molecular Diagnostics, South Africa) was included at multiple positions on gels as a standard. Restriction patterns were identified visually for each of the restriction enzymes used. A matrix, describing the presence (indicated by 1) or absence (indicated by zero), of a particular pattern within an isolate was generated. In this way a specific profile for each isolate was compiled. The Dice coefficient (Nei & Li, 1979) within the Bionum computer programme (Applied Maths, Kortrijk, Belgium) was used to construct a distance matrix from this one (1)-zero (0) profiles. These distance values were subsequently analysed to generate a tree using the unweighted pair group method of arithmetic mean algorithm (UPGMA) in GelCompar 4.0 (Applied Maths, Kortrijk, Belgium).

RESULTS

16S rDNA amplification and sequence determination

PCR amplification of the 16S rRNA gene using primers fD1SHRT and rP2SHRT resulted in the detection of an amplified fragment of about 1500 bp for all rhizobial isolates tested. This corresponds to the expected size of the 16S rRNA genes as previously determined for the *rrn* operon of *E. coli* (Brosius *et al.*, 1978).

A partial sequence (about 700 bp) of the 16S rRNA gene was determined using the primer set 16SRNAII-S (an internal forward primer) and rP2SHRT. These sequences correspond approximately to positions 750 and 1450 (numbering according to the *E. coli* 16S rDNA gene sequence). Sequence data, especially at the extremities of the fragment, usually contained ambiguous site, which the automated sequencer was unable to resolve and therefore DNA sequences from both strands was particularly useful to accurately determine the relevant nucleotide residue at such sites. The sequences were submitted to GenBank and their respective accession numbers are shown in Figure 5.1.

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Phylogenetic position of the indigenous rhizobia as indicated by partial 16S rDNA sequence analyses

Phylogenetic relationships of the indigenous rhizobia were investigated by comparative sequence analyses of a 700 bp fragment of the 16S rRNA gene. Nucleotide sequences of the rhizobial reference strains were obtained from the GenBank database, suitably edited to correspond to this 700 bp region and included for comparative purposes. A phylogenetic tree (Figure 5.1) was constructed by the neighbour-joining algorithm (Saitou & Nei, 1987) using the ClustalX computer programme.

The phylogenetic tree was composed of two main lineages: the first containing the genera *Mesorhizobium*, *Sinorhizobium*, *Rhizobium*, *Agrobacterium* and *Allorhizobium*, while the second indicated the phylogenetic position of the slow-growing *Bradyrhizobium*. *Azorhizobium undicola* had an intermediate position between these two lineages. The indigenous rhizobia were related to all of the rhizobial genera except *Allorhizobium* and *Azorhizobium*. Two isolates, PL10a and 36b, showed no clear generic affiliation.

Isolates obtained from the root nodules of *Aspalathus linearis,* a shrublike legume, part of the fynbos ecosystem in South Africa, showed high homology to members of the genus *Mesorhizobium*. Isolates xhj26, xhj20, xhj13, xhj23, xhj18, xhj7, xhj15, xhj8, xhj27 (Cluster A) shared almost identical sequence with *M. loti*. Isolate xhm5, obtained from *Aspalathus cordata*, was also closely related to *M. loti*. The other *A. linearis* isolate, xhj12fr, was clearly separated from the "mesoloti-group", sharing 97% sequence homology with *M. ciceri*. Isolates 98d2 and 128a were the only other symbionts showing relatedness to the genus *Mesorhizobium*, showing sequence correspondence to *M. plurifarium* and *M. chacoense,* respectively.

Species of the *Sinorhizobium* genus formed a phylogenetic coherent group, with two of the indigenous isolates, xhj12s and UP27b, related to this group. Both xhj12fr and xhj12s were obtained from the same nodule, but were shown here to belong to different genera.

The phylogenetic branch carrying the genera *Rhizobium*, *Allorhizobium* and *Agrobacterium* contained the isolates UP26b, 66a1t1, SA3, 28c and 7b. Isolates UP26b and 66a1t1 were

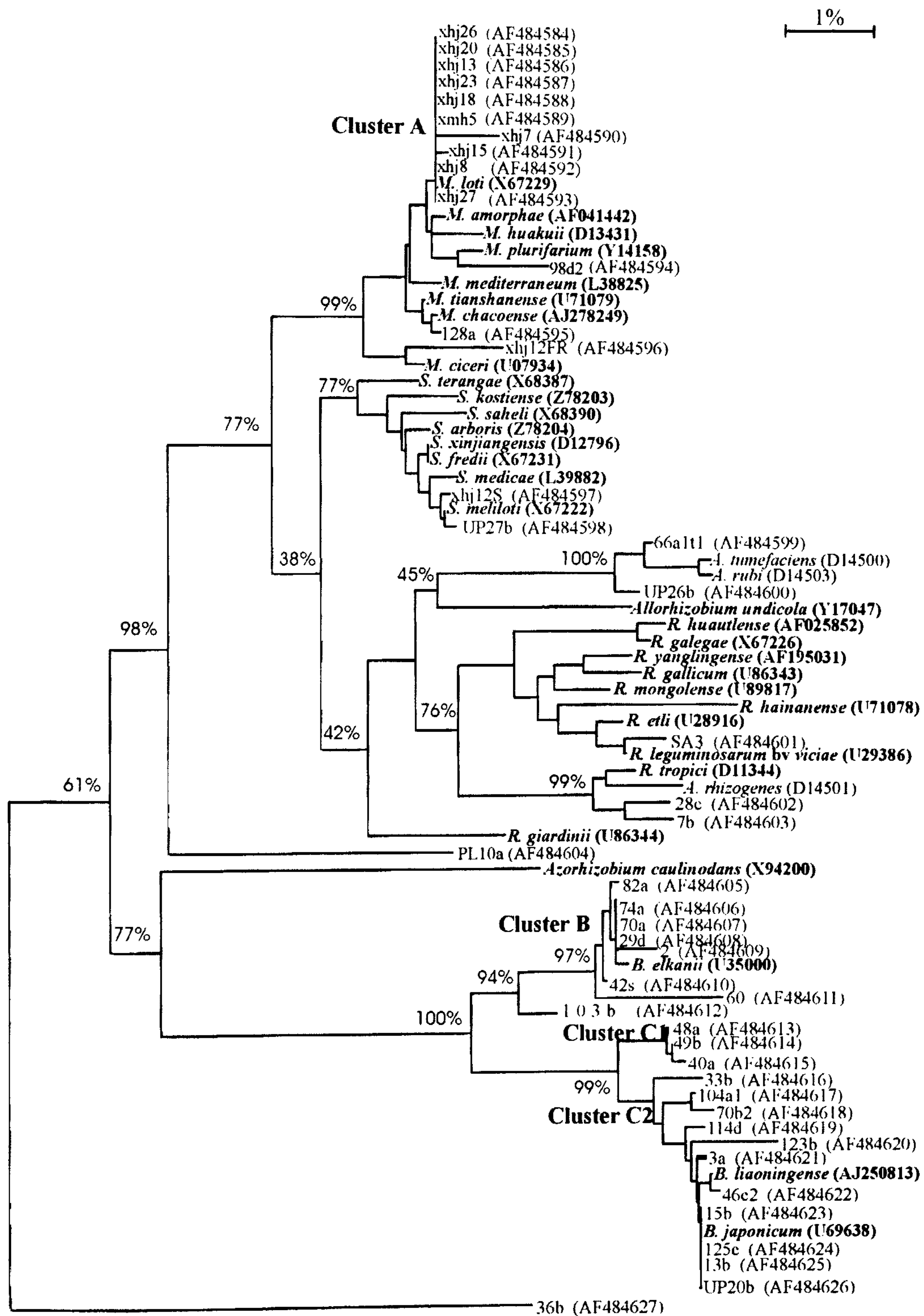
1%
xhj26 (AF484584)
xhj20 (AF484585)
xhj13 (AF484586)
xhj23 (AF484587)
xhj18 (AF484588)
Cluster A
xmh5 (AF484589)
xhj7 (AF484590)
xhj15 (AF484591)
xhj8 (AF484592)
M. loti (X67229)
xhj27 (AF484593)
M. amorphae (AF041442)
M. huakuii (D13431)
M. plurifarium (Y14158)
98d2 (AF484594)
M. mediterraneum (L38825)
M. tianshanense (U71079)
M. chacoense (AJ278249)
128a (AF484595)
xhj12FR (AF484596)
M. ciceri (U07934)
99%
77%
S. terangae (X68387)
S. kostiense (Z78203)
S. saheli (X68390)
S. arboris (Z78204)
S. xinjiangensis (D12796)
S. fredii (X67231)
S. medicae (L39882)
xhj12S (AF484597)
S. meliloti (X67222)
UP27b (AF484598)
77%
38%
66a1t1 (AF484599)
A. tumefaciens (D14500)
A. rubi (D14503)
UP26b (AF484600)
100%
45%
Allorhizobium undicola (Y17047)
98%
R. huautlense (AF025852)
R. galegae (X67226)
R. yanglingense (AF195031)
R. gallicum (U86343)
R. mongolense (U89817)
R. hainanense (U71078)
76%
R. etli (U28916)
SA3 (AF484601)
R. leguminosarum bv viciae (U29386)
42%
R. tropici (D11344)
99%
A. rhizogenes (D14501)
61%
28c (AF484602)
7b (AF484603)
R. giardinii (U86344)
PL10a (AF484604)
Azorhizobium caulinodans (X94200)
82a (AF484605)
74a (AF484606)
Cluster B
70a (AF484607)
29d (AF484608)
77%
(AF484609)
97%
B. elkanii (U35000)
94%
42s (AF484610)
60 (AF484611)
103b (AF484612)
100%
Cluster C1
48a (AF484613)
49b (AF484614)
40a (AF484615)
33b (AF484616)
99%
104a1 (AF484617)
Cluster C2
70b2 (AF484618)
114d (AF484619)
123b (AF484620)
3a (AF484621)
B. liaoningense (AJ250813)
46c2 (AF484622)
15b (AF484623)
B. japonicum (U69638)
125c (AF484624)
13b (AF484625)
UP20b (AF484626)
36b (AF484627)

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Figure 5.1 (Previous *page*) Phylogenetic relationships of the indigenous rhizobial isolates and other rhizobial reference strains. This analysis was based on comparative sequence analysis of approximately 700 bp fragment of the 16S rRNA gene. The tree was constructed using the Neighbour-joining method of Saitou & Nei (1987). Horisontal branch lengths reflect the phylogenetic distances, while vertical branch lengths are non-informative and set for clarity only. The scale bar indicates 1% nucleotide difference and bootstrap values of some of the major branching points are shown. GenBank accession numbers are indicated in brackets. *M: Mesorhizobium, S: Sinorhizobium, R: Rhizobium, A: Agrobacterium & B: Bradyrhizobium*.

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related to *Agrobacterium tumefaciens* and *Agrobacterium rubi*, while 28c and 7b were present in the group containing *R. tropici* and *Agrobacterium rhizogenes.* In this analysis *Rhizobium giardinii* was also recovered as lineage, clearly separable from the one carrying *Rhizobium, Agrobacterium* and *Allorhizobium* reference strains.

The second main phylogenetic lineage consisted of the bradyrhizobia. Within this lineage two subgroups (Clusters B & C) were evident and most of the indigenous isolates were distributed between these subgroups. The first subgroup (B) contained *Bradyrhizobium elkanii* and isolates 42bs, 82a, 74a, 70a, 2, and 29d. Also related to this subgroup was isolates 103b and 60, which appeared to be part of a separate group that diverged prior to the branching of *B. elkanii.* Bootstrap values of 94% and 97%, respectively, supported this separate branching of 103b and 60. The second subgroup contained the closely related *B. japonicum* and *B. liaoningense* reference strains together with 48a, 49b, 40a, 33b, 104a1, 70b2, 114d, 123b, 3a, 46c2, 15b, 125e, 13b and UP20b. The branches within this group were poorly resolved which is due to the small sequence divergence (no more than 1.5%) among isolates within this group.

Phylogenetic position of the indigenous rhizobia within the α-*Proteobacteria*

The genera of the family *Rhizobiaceae* do not form a cluster distinctly separated from other genera in the α-*Proteobacteria* (Young, 1996; van Berkum & Eardly, 1998). This means that the rhizobia are closer related to other non-symbiotic bacteria than they are to each other. The 16S rDNA sequence analysis was therefore extended (Fig. 5.2) to include representative members of the α-*Proteobacteria* in an effort to establish an improved understanding of the phylogenetic position of the indigenous isolates.

The *Rhizobium-Agrobacterium-Allorhizobium* (RAA) cluster was related to *Brucella neotomae, Ochrobactrum anthropi* and *Bartonella hensellae*, while the *Phyllobacterium* species showed high relatedness to the *Mesorhizobium, Sinorhizobium* and RAA cluster. According to several authors (Young & Haukka, 1996; de Lajudie *et al.*, 1998a,b) strains representing the genera *Bartonella, Brucella, Phyllobacterium* are sometimes interspersed between members of the RAA-cluster. Additionally, the chosen algorithm and selection of

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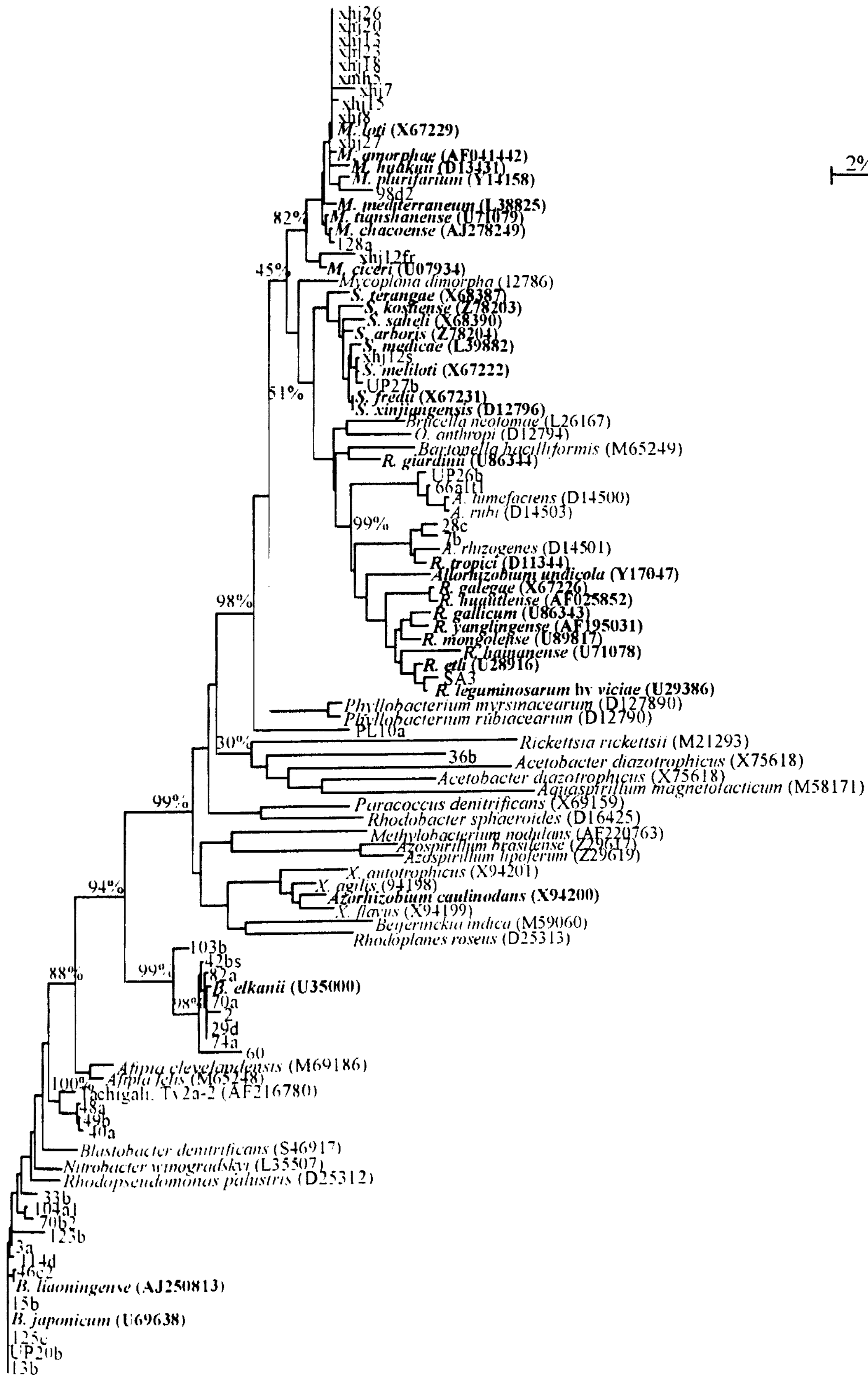

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Figure 5.2. (Previous page) Phylogenetic relationships of the indigenous rhizobial isolates and other alpha-*Proteobacteria*. This analysis was based on comparative sequence analysis of approximately 700 bp fragment of the 16S rRNA gene. The tree was constructed using the Neighbour-joining method of Saitou & Nei (1987). Horisontal branch lengths reflect the phylogenetic distances, while vertical branch lengths are non-informative and set for clarity only. The scale bar indicates 2% nucleotide difference and bootstrap values of some of the major branching points are shown. GenBank accession numbers of the different reference strains are indicated in brackets, while those of the indigenous isolates are indicated in Fig 5.1. *Methylobacterium nodulans* was included, although it is not yet validly accepted as a new species. *M: Mesorhizobium, S: Sinorhizobium, O: Ochrobactrum, R: Rhizobium, A: Agrobacterium, X: Xanthobacter, B: Bradyrhizobium.*

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included sequences may also influence the phylogenetic groupings (Amarger *et al.*, 1997; Chen *et al.*, 1997; Jarvis *et al.*, 1997).

The genus *Bradyrhizobium* showed a high relatedness to species of the genera *Afipia, Blastobacter, Nitrobacter, Rhodopseudomonas.* The indigenous isolates, previously resolved into the *Bradyrhizobium japonicum-Bradyrhizobium liaoningense* subcluster was again recovered within this group, except isolates 48a, 49b and 40a. These isolates shared high sequence identity with the divergent *Bradyrhizobium* symbiont, Tv2a-2, as described by Parker (2000). Tv2a-2 was isolated from the root nodule of a tree, *Tachigali versicolor* of the Caesalpinioideae subfamily. Additionally, these isolates shared a recent common ancestor with *Blastobacter denitrificans.*

No clear generic affiliation could be established for PL10a, while 36b was associated with the *Rhodospirillum*, *Acetobacter* and *Aquaspirillum* subgroup.

Analyses of combined 16S-23S IGS RFLP analyses of closely related 16S phylogenetic clusters.

The IGS-PCR products of different rhizobial genera are indicated in Figure 5.3. In general the length of the intergenic region of the different genera corresponded to approximately 1000 bp. A significant smaller product (~500 bp) was obtained for members of the genus *Agrobacterium*, while the IGS for *Mesorhizobium* was in the region of 1500 bp. Due to the significant variation in length and number of amplified products, only rhizobial reference strains, related to the observed clusters A, B, C1and C2 (see Figure 5.2), were included in the analyses. Each cluster was also analysed separately and dendrograms, describing the heterogeneity within each cluster, were generated using the UPGMA algorithm with the GelCompar software. The observed restriction fragment patterns for each isolate are shown in Table 5.3. The IGS-RFLP profiles of isolates not present in closely related clusters, such as 128a, 98d2, xhj12fr, UP26b, 66a1t1, SA3, 28c, 7b, xhj12S, UP27b, 36b and PL10a were not analysed. Isolates 60 and UP20b consistently gave amplified products outside the size range obtained with the other isolates and were also not included.

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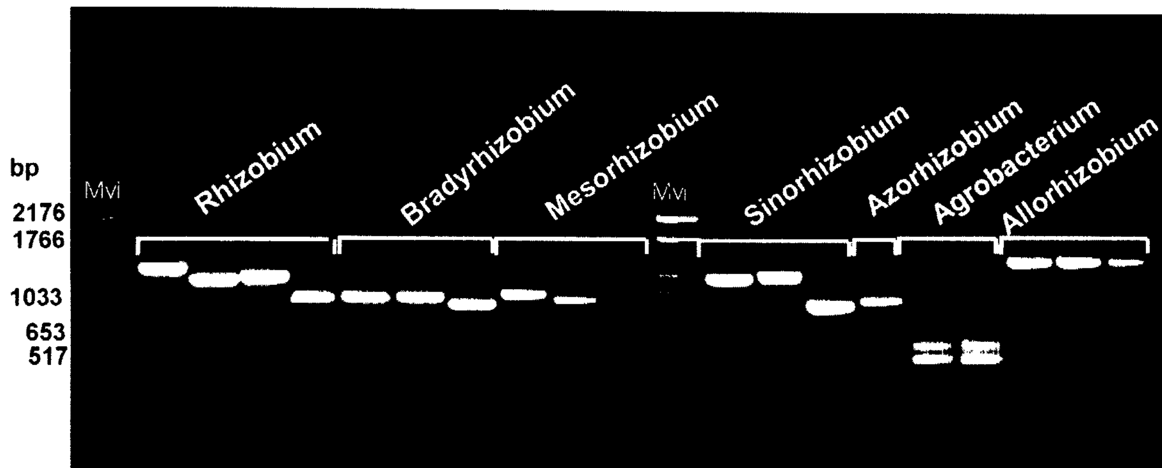

Figure 5.3. Agarose gel electrophoresis of 16S-23S IGS PCR products of representatives of the different rhizobial genera. Lane1 & 12: DNA molecular weight marker VI (Roche Molecular Biochemicals (SA). Lane2: *R. mongolense* (LMG 19141), Lane 3: *R. hainanense* (USDA 3588), Lane 4: *R. tropici* (LMG 9503), Lane 5: *R. leguminosarum* bv. *trifoli* (LMG 8820), Lane 6: *B. elkanii* (LMG 6134), Lane 7: *B. japonicum* (LMG 6138), Lane 8: *Bradyrhizobium* sp. (LMG 8319), Lane 9: *M. ciceri* (LMG 14989), Lane 10: *M. huakuii* (14107), Lane 11: *M. tianshanense* (LMG 18976), Lane 13: *S. saheli* (LMG 7837), Lane 14: *S. fredii* (LMG 6217), Lane 15: *S. terangae* (LMG 7834), Lane 16: *Azorhizobium caulinodans* (USDA 4892), Lane 17: *Agrobacterium rhizogenes* (LMG 150), Lane 18: *Agrobacterium radiobacter* (LMG 140), Lane 19: *Allorhizobium undicola* (USDA 4902), Lane 20: *Allorhizobium undicola* (USDA 4903), Lane 21: *Allorhizobium undicola* (USDA 4904).

An amplified product of about 970 bp was observed for most of the indigenous rhizobia related to clusters B, C1 and C2. In some instances a smaller, 500 bp product was observed. These bands were, however, less prominent in comparison to the 970 bp band. For the isolates present in cluster A, an amplified product of 1000 bp was obtained, as well as an additional less prominent band of about 650 bp.

Cluster A

This cluster comprised most of the isolates obtained from *Aspalathus linearis* and one from *Aspalathus cordata* (xhm5). The four restriction enzymes had equal discriminatory ability within this group, each generating, on average, 5 IGS RFLP types. Only reference strains *M. huakuii*, *M. plurifarium* and *M. loti* grouped close to the *Aspalathus* isolates. Isolates xhj7 and xhj8 (subgroup 1A) had identical *Hae*III, *Cfo*I, and *Taq*I profiles. However, *Nde*II digestion showed that the isolates had different genotypes. Such difference could not be resolved by 16S rDNA sequencing since the observed difference was only 1%. The *Hae*III

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Table 5.3 : Restriction patterns obtained of *Mesorhizobium*, *Bradyrhizobium* spp. and indigenous rhizobial strains after digestion of IGS PCR products with the indicated enzymes. Clusters refer to those obtained in 16S rDNA phylogenetic tree (See Fig 5.1). NS indicates the absence of a restriction site. Numerical and alphabetical designations are used to differentiate between the different RFLP patterns. Alphabetical subscript annotations (a, b, c1 and c2) are used to indicate the RFLP type of a particular cluster. Distinctive patterns obtained from rhizobial reference strains are indicated by the following subscripts: *loti*: *M. loti*; *h*: *M. huakuii*; *tia*: *M. tianshanense*, *am*: *M. amorphae*; *plu*: *M. plurifarium*; *c*: *M. ciceri*; *med*: *M. mediterraneum*, *ml*: *M. mediterraneum* & *M. loti*; *h/am*: *M. huakuii* & *M. amorphae*, *Be*: *B. elkanii*; *Bjap*: *B. japonicum*, *Bliaon*: *B. liaoningense*.

Strain	Patterns obtained following restriction of 16S-23S IGS PCR products with:			
	*Hae*III	*Cfo*I	*Nde*II	*Taq*I
Cluster A				
xhj7	$H1_a$	$C1_a$	$N1_a$	$T1_a$
xhj8	$H1_a$	$C1_a$	N_h	$T1_a$
xhj26	$H4_a$	$C1_a$	N_h	$T1_a$
M. huakuii (LMG 14107)	H_h	$C_{h/am}$	N_h	T_h
xhj15	$H3_a$	C_{loti}	$N4_a$	$T3_a$
xhj18	$H3_a$	C_{loti}	$N4_a$	$T3_a$
xhj23	$H3_a$	C_{loti}	$N4_a$	$T3_a$
xhj20	H_{loti}	$C_{h/am}$	$N6_a$	$T4_a$
xhj27	$H5_a$	$C_{h/am}$	N_{tia}	$T5_a$
M. plurifarium (LMG 11892)	H_{plu}	C_{plu}	N_{plu}	T_{plu}
xhm5	H_{loti}	$C_{h/am}$	$N7_a$	$T4_a$
M. loti (LMG 6125)	H_{loti}	C_{loti}	N_{loti}	T_{loti}
M. ciceri (LMG 14989)	H_c	C_c	N_c	T_c
M. mediterraneum (LMG 17148)	H_{med}	C_{med}	N_{med}	T_{med}
M. tianshanense (LMG 18976)	H_{tia}	C_{tia}	N_{tia}	T_{tia}
M. amorphae (LMG 18977)	H_{am}	$C_{h/am}$	N_{am}	T_{am}
xhj13	H10(NS)	$C_{h/am}$	N_{tia}	T2 (NS)
Cluster B				
103b	$H1_b$	$C1_b$	$N1_b$	$T1_b$
42bs	$H2_b$	$C2_b$	$N2_b$	$T2_b$
82a	H_{Be}	C_{Bliaon}	N_{Be}	$T3_b$
70a	H_{Be}	C_{Bliaon}	N_{Be}	$T3_b$
29d	H_{Be}	C_{Bliaon}	N_{Be}	$T3_b$
2	H_{Be}	C_{Bliaon}	N_{Be}	$T4_b$
Cluster C1				
49b	$H1_{c1}$	$C1_{c1}$	$N1_{c1}$	$T1_{c1}$
48a	$H1_{c1}$	$C1_{c1}$	$N1_{c1}$	$T2_{c1}$
40a	$H1_{c1}$	$C1_{c1}$	$N1_{c1}$	$T2_{c1}$
Cluster C2				
104a1	$H1_{c2}$	$C1_{c2}$	$N1_{c2}$	$T1_{c2}$
70b2	$H1_{c2}$	$C1_{c2}$	$N1_{c2}$	$T1_{c2}$
123b	$H2_{c2}$	$C2_{c2}$	N_{Bjap}	$T3_{c2}$
46c2	$H2_{c2}$	$C2_{c2}$	$N2_{c2}$	$T4_{c2}$
13b	$H3_{c2}$	$C4_{c2}$	N_{Bjap}	$T2_{c2}$
15b	$H3_{c2}$	$C4_{c2}$	N_{Bjap}	$T2_{c2}$
3a	$H3_{c2}$	$C4_{c2}$	N_{Bjap}	$T2_{c2}$
114d	$H2_{c2}$	$C3_{c2}$	N_{Bliaon}	$T5_{c2}$
33b	$H5_{c2}$	$C3_{c2}$	N_{Bliaon}	$T6_{c2}$
125e	$H4_{c2}$	C_{Bjap}	N_{Bliaon}	$T1_{c2}$

and *Nde*II patterns also separated xhj26 from the closely related xhj7 and xhj8. *M. huakuii* is related to this cluster due to its common *Nde*II profile with xhj8 and xhj26. Isolates xhj15, xhj18 and xhj23 (subgroup 1B) had identical enzyme profiles, with their *cfo*I profile being similar to that of *M. loti.* Although xhj20 and xhj27 were also present in this subgroup, they were clearly divergent from the other members of this subgroup and each other. The *Cfo*I profile of *M. huakuii* and *M. amorphae* was the only common RFLP pattern between these isolates. The clustering of xhm5 and *M. loti* (subgroup 1C) is due to a common *Hae*III profile, while the position of xhj13 (in group 2), almost as an "outgroup" is owed to the absence of *Hae*III and *Taq*I. *M. ciceri, M. mediterraneum, M. tianshanense, M. amorphae* were also present in group 2, showing no relatedness to any of the symbionts of *Aspalathus.* This in contrast to the results obtained by partial 16S rDNA sequencing, since no more than 2% sequence divergence was observed among the *Mesorhizobium* reference strains and the *Aspalathus* isolates

Cluster B

This cluster comprised isolates (82a, 70a, 29d and 2) showing high homology to *B. elkanii.* Isolate 2 differed only from the rest of the group due to a distinct *Taq*I pattern, while the *Hae*III and *Nde*II profiles were that of *B. elkanii.* It is however, noteworthy that these isolates had a characteristic *B. liaoningense-Cfo*I profile, while results of the 16S rRNA gene indicated that these isolates were related to *B. elkanii.* No correspondence was noted between the patterns of isolates 103b and 42bs among themselves or any of the bradyrhizobial reference strains.

Cluster C1

The different IGS patterns of isolates within this cluster do not correspond to any of the bradyrhizobial patterns. However, *B. liaoningense* appears to be the closest neighbour to these isolates. A closer examination of all the restriction types within this group indicates that restriction of the IGS-PCR products yielded on average four fragments of which two

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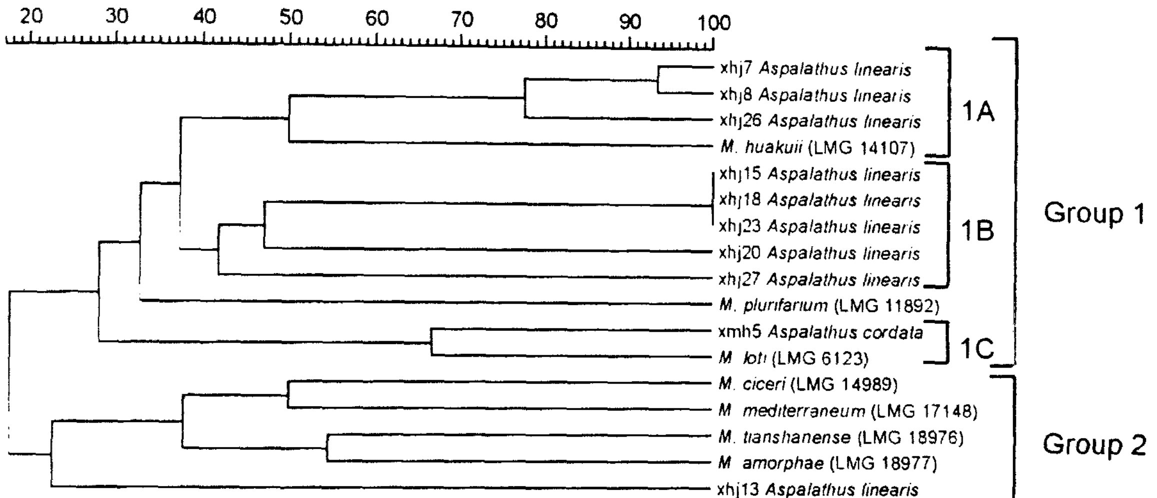
Cluster A



Cluster B

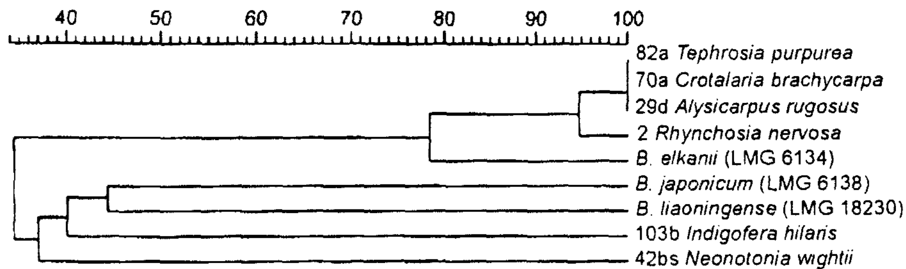


Figure 5.4: Dendrograms based on the combined *Hae*III, *Cfo*I, *Nde*II and *Taq*I restrictions of amplified 16S-23S IGS PCR products. The dendrograms of clusters (A, B, C1 & C2) are shown separately as described in text. Clusters were identified by 16S rDNA sequencing. Dendrograms were constructed using UPGMA. The x-axes represent the correlation between the isolates.

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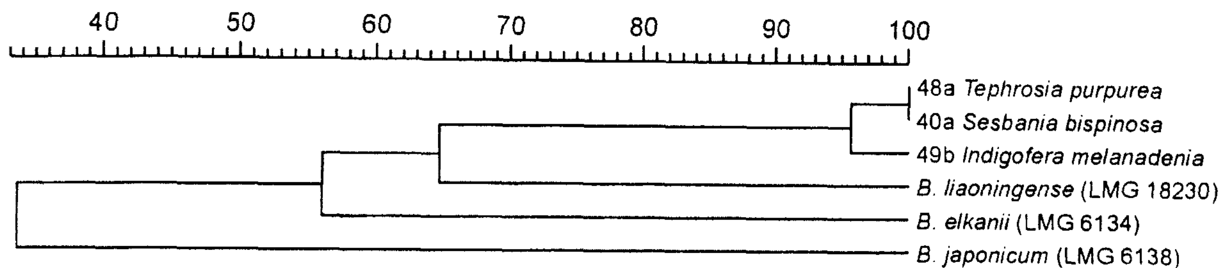
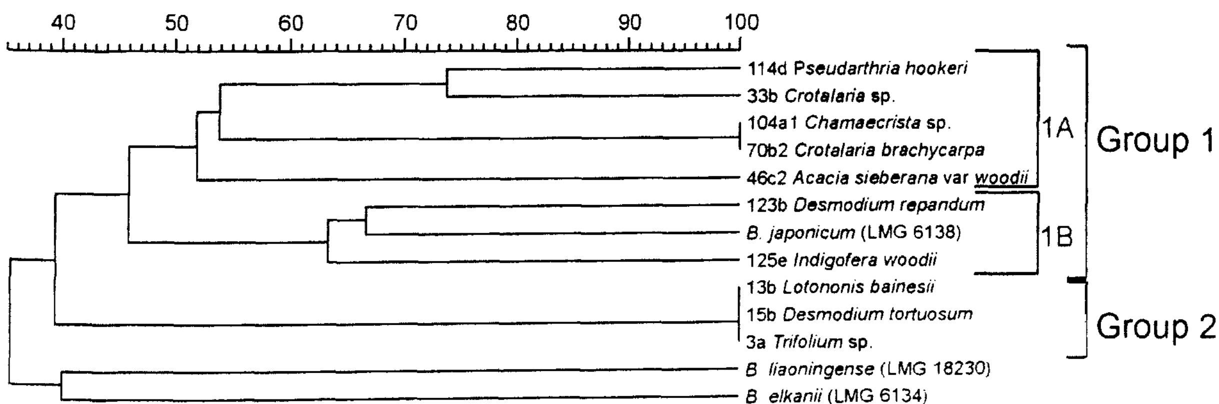
Figure 5.4. *continues*

Cluster C1



Cluster C2



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were common in *B. liaoningense* patterns. A distinct *Taq*I profile for 49b is responsible for its separate placement within this group.

Cluster C2

Two main groups were identified within this cluster; group 1was related to *B. japonicum* and group 2 showing no clear generic affiliation. The genomic diversity within this group is evident from the five *Hae*III and six *Taq*I patterns, none of which corresponded to any bradyrhizobial reference pattern. Equally, of the five different *Cfo*I patterns, only one corresponded to the *B. japonicum* pattern, while one of each *B. japonicum* and *B. liaoningense* patterns were part of the *Nde*II profile for this cluster. Isolates 114d and 33b share common *Cfo*I and *Nde*II profiles, while isolate 46c2 only shares a common *Hae*III pattern with members of this subgroup. Although, there appears to be little correspondence between IGS-RFLP patterns of members of the subgroup 1A, individual common bands are present in some of the *Cfo*I, *Nde*II and *Taq*I patterns. Isolates in subgroup 1B, have a common *B. japonicum-Nde*II pattern and similar, yet unique patterns for the other enzymes.

DISCUSSION

A broad range of rhizobial species have been isolated from different leguminous species occurring in South Africa. These included mostly isolates from nodules of legumes of the subfamilies Papilionoideae and Mimosoideae, while only isolate, 104a1, was obtained from the root nodules of *Chamaechrista*, which belongs to the Caesalpinioideae subfamily. Except for *Azorhizobium* and *Allorhizobium*, the indigenous strains were related to all of the described genera. Most of these were however, related to the *Bradyrhizobium* genus. Previous morphological and preliminary phylogenetic investigations (Dagutat, 1995; Kruger, 1998) indicated the diversity of the indigenous rhizobial strains, with some isolates showing no apparent affiliation to the known rhizobial genera. Since no single method can produce a perfect classification, the polyphasic approach (Vandamme *et al.*, 1991) to bacterial taxonomy has been emphasized and used for the description of both generic and specific taxa. The aim of this study was therefore to analyse this apparent diversity of the indigenous strains, targeting both conserved (16S rRNA) and variable (16S-23S IGS) genomic regions.

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Sequence analyses of the 16S rRNA gene remain one of the most reliable indicators of organismal phylogeny (Woese, 2000) allowing the rapid identification of a large number of strains. The 16S rRNA phylogeny of rhizobia has indicated them to be heterogeneous since some genera are closer related to other non-rhizobial genera of the α-*Proteobacteria* than to each other (Willems and Collins, 1993). Thus, the absence of such genera in any analyses could obscure the true phylogenetic identity of any putative rhizobial symbiont isolated from the indigenous legumes. Our comparative 16S rDNA sequence analyses were therefore subsequently extended to include the other genera of the α-*Proteobacteria*.

On the basis of 16S rDNA sequence analyses, the indigenous strains had two main generic affiliations: *Mesorhizobium* and *Bradyrhizobium*. Thirteen isolates, most of which were obtained from *Aspalathus* spp., were related to the *Mesorhizobium* genus, showing almost 98% 16S rDNA sequence identity among themselves and with *Mesorhizobium* spp.. Analysis of the more variable IGS region (Gűrtler & Stanisich, 1996), provided better resolution of this group, indicating the existence of at least seven genotypes which showed correspondence to only *M. huakuii*, *M. loti* and *M. plurifarium*. However, isolates xhj15, xhj18 and xhj23 exhibited the same RFLP pattern with all four enzymes used, indicating their similar genotype.

The *Bradyrhizobium* genus is clearly very diverse, with *B. japonicum* and *B. liaoningense* being closer related to *Afipia*, *Blastobacter*, *Nitrobacter* and *Rhodopseudomonas* than to *B. elkanii* (Willems *et al.*, 2001a). According to several authors (Barrera *et al.*, 1997; Willems *et al.*, 2001b), the highly homologous 16S rDNA sequences of bradyrhizobia contain insufficient discriminatory ability to describe this diverse group of symbionts. Alternatively, a recent report by Willems *et al.* (2001c) on sequence analyses of the IGS region of bradyrhizobia, indicated that this genomic region has a discriminatory ability comparable to that of DNA-DNA hybridisation analysis. The bradyrhizobial isolates identified in this study were obtained from a diverse group of host species. Based on their 16S rDNA sequences, three clusters (B, C1 and C2) were identified. The IGS-RFLP results indicate isolates 82a, 70a, 29d of cluster B, to be the same genotype. Although isolate 2 was related to the former isolates, it was clearly separable from them. Cluster C2, which was closely related to *B.*

japonicum and *B. liaoningense*, was a heterogeneous group containing seven genotypes among 10 isolates.

The grouping of 48a, 49b, 40a based on IGS-RFLP data suggest the existence of two genotypes. Their high homology with the divergent *Tachigali versicolor* symbiont, Tv2a-2 further suggests them to represent a separate phylogenetic lineage. According to Doyle *et al.* (1997), *Tachigali* was the most basal known genus of a clade within the Caesalpinioideae that gave rise to the subfamily Mimosoideae, where almost all species participate in root-nodule symbioses. The divergent status of the *Tachigali*-symbiont and these three indigenous isolates remains intriguing, especially since they were obtained from host species within the Papilionoideae subfamily. Initially Doyle *et al.* (1997) hypothesised that the evolution of nodulation within the Papilionoideae occurred in parallel with the origin or origins of the trait in the Caesalpinioideae/ Mimosoideae. However, in the light of evidence suggesting that nodulating flowering plants are more closely related than previously thought, the view has shifted towards a single origin for a predisposition for nodulation (Doyle, 1998). The exact origin of nodulation can however, only be refined in the presence of more phylogenetic sampling of both plant and symbiotic partners.

The failure of isolate PL10a, to group with any of the rhizobial genera was also evident in the whole cell protein profile from an earlier study by Kruger (1998) and a more detailed analysis of this isolate is necessary.

The isolation of different symbionts from the same nodule was previously reported (Dreyfus & Dommergues, 1981; Jenkins *et al.*, 1987; Dagutat, 1995) and is illustrated in this study since xhj12FR and xhj12S was isolated from the same root nodule of *Aspalathus linearis*.

Despite the lack of suitable discriminatory ability of 16S rDNA sequence analyses in some instances, it has proven sufficient to determine the diversity of the rhizobial symbionts associated with leguminous species in South Africa. Previous studies of only morphological traits could by no means have provided insight into the complex intra- and intergenic relationships among this diverse group of microorganisms. Although, most of the indigenous isolates showed good agreement with the known rhizobial genera, others demonstrated

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extreme divergence from such genera. It is therefore reasonable to expect that more diverse rhizobia may be present in South African soils which may also be correlated to the diverse range of leguminous plants in the country.

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CHAPTER 6

Phylogenetic relationship of a selection of indigenous South African rhizobia based on partial *nifH* gene sequencing and RFLP of the *nodC* gene.

ABSTRACT

The diversity and phylogeny of *nodC* and *nifH* genes were studied using a selection of rhizobial isolates, associated with indigenous legumes. Following restriction of nodC amplified products, 19 different nodC-genotypes were identified among 28 isolates investigated. Some of these nodC-genotypes correlated well with host species, while in other instances, symbionts, with the same nodC-genotype, were obtained from different leguminous hosts, indicating their broad host range. The RFLP method was, however, too robust and sequence analyses of representatives of these genotypes are necessary. When considering sequence variation within the *nifH* gene, the genetic distances between isolates were larger than that observed for the 16S rRNA gene. The *nifH* and 16S rDNA-based classifications were well correlated.

Keywords: *Rhizobium*, *nodC*, *nifH*, 16S rDNA classification

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INTRODUCTION

The symbiotic bacteria, associated with leguminous plants, represent a diverse group of microorganisms. The taxonomical classification of these bacterial symbionts have undergone major revisions and are currently split into the genera, *Rhizobium* (Frank, 1889), *Sinorhizobium* (Chen *et al.*, 1988), *Mesorhizobium* (Jarvis *et al.*, 1997), *Allorhizobium* (de Lajudie *et al.*, 1998a), *Bradyrhizobium* (Jordan, 1982) and *Azorhizobium* (Dreyfus *et al.*, 1988). The establishment of the symbiosis is accompanied by profound developmental changes in both partners: on the roots new organs or root nodules are formed while the rhizobia inhabit these nodules as nitrogen-fixing endosymbiont bacteroids (Mergaert *et al.*, 1997).

Several classes of specific genes in rhizobia are required to establish an effective symbiosis. These include *nod* genes, responsible for the production of Nod-factors, which stimulate the host plant to produce symbiotic nodules, and *nif* genes, which produce the nitrogen fixing nitrogenase enzyme (Dixon & Wheeler, 1986). A number of genetic and biochemical mechanisms by which regulatory and structural nodulation genes control host specificity, have been identified (Roche *et al.*, 1996). Structural nodulation (*nod*) genes comprise both species-specific genes (such as *nodFEHSUZ*) and those that are common (*nodABC*) in all rhizobia (Zhang *et al.*, 2000). However, work done by Roche *et al.* (1996) suggests that *nodA* and *nodC* are also components of host-specific nodulation since NodA varies in its specificity for various fatty acid substrates, while NodC is a determinant of the length of the Nod-factor backbone. These Nod-factors, which are lipochitooligosaccharides, are the main signal molecules during the nodulation process (Perret *et al.*, 2000). Nodule development is initiated by the regulatory protein, NodD, which activates the transcription of other *nod*-genes in the presence of flavonoids, produced by the host plant (van Rhijn & Vanderleyden, 1995; Denarie *et al.*, 1996). The *nodD* genes determine the first level of host specificity since the nature and abundance of the plant flavonoids may vary according to the plant host (Schultze & Kondorosi, 1998). The common *nodABC* gene products are responsible for the formation of the core of the Nod factor, which is decorated by specific substitutions as determined by the host-specific *nod* gene products. These specific substitutions determine the plant specificity (Roche *et al.*, 1996). However, according to Perret *et al.* (2000), no

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strict correlation can be drawn between the types of Nod-factors produced by rhizobia and the plants which they nodulate. As an example, *Rhizobium etli* and *Mesorhizobium loti* produce identical Nod-factors, despite them having two different host ranges (Cardenas *et al.*, 1995). Conversely, two rhizobia nodulating the same plant may produce different Nod-factors. This is the case for *R. tropici* and *R. etli*, both of which effectively nodulate the common bean plant, *Phaseolus vulgaris* (Perret *et al.*, 2000). Broad host range can also be attributed to rhizobia possessing many copies of NodD that may associate with several plant-excreted flavonoids. These flavonoid-NodD complexes are then able to activate the expression of *nod* genes. The enzymes encoded by such genes can synthesise small to large families of Nod-factors (Broughton *et al.*, 2000)

In most instances, the symbiotic genes are located on transmissible plasmids, and therefore lateral gene transfer may play an important role in the evolution of symbiosis and host range (Mergaert *et al.*, 1997). Phylogenetic studies of Nod proteins suggest that *nod* genotypes co-evolved with host plant divergence and do not correspond to 16S rRNA or *nif* phylogeny (Dobert *et al.*, 1994; Ueda *et al.*, 1995).

The phylogeny of the *nifH* gene, which codes for the Fe-protein subunit of the nitrogenase enzyme, has been reported to closely resemble that of the 16S rRNA gene, and thus these genes may share a common evolutionary history (Hennecke *et al.*, 1985; Ueda *et al.*, 1995). Instances of discordance have also been reported. Haukka *et al.* (1998) showed that bacteria with a *Sinorhizobium* chromosomal background did not necessarily share the same *nifH* genes (Eardly *et al.*, 1992).

Symbiotic performance with selected hosts is a requirement for the description of new rhizobial species (Graham *et al.*, 1991). As discussed, the genes involved in nodulation are, in most instances, located on transmissible plasmids. For this reason, phylogenies based on plant infection are considered unreliable. The study of the diversity among the symbiotic genes is still valuable to provide insight into the evolution of the *Rhizobium*-legume symbiosis. The diversity of the rhizobia, associated with indigenous leguminous plants, have been characterised by 16S rDNA sequencing and 16S-23S IGS RFLP (Chapter 5). These results indicated correspondence of the indigenous isolates to known rhizobial genera,

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whereas others were more diverse and not related to the known rhizobial genera. The aim of this work was therefore to characterise the symbiotic genome of the indigenous isolates by analysing variation within the *nodC* and *nifH* genes.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study are indicated in Table 6.1. Previously, Dagutat (1995) and Kruger (1998) established a culture collection of rhizobia associated with a range of legumes occurring in South Africa. Additionally, strains obtained form the root nodules of *Aspalathus linearis* were provided by the ARC-Plant Protection Research Institute (Private Bag X134, Pretoria 0001, South Africa). The 16S rRNA gene phylogeny of a selection of these isolates was determined in Chapter 5. For the purpose of this study, strains were selected to include representatives of most of the phylogenetic groupings as determined by 16S rDNA sequencing. Additionally, the *nifH* sequences of isolates 108a1 and 55 (not analysed in Chapter 5) were also included in the analyses.

Maintenance of bacterial cultures

Strains were maintained on yeast extract mannitol (YM) agar, containing (w/v): 1% mannitol, 0.5% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.01%, NaCl, 0.04% yeast extract and 1.5% bacteriological agar. For long term storage cultures were grown (with rigorous shaking) in yeast mannitol broth (YMB) at 28°C for approximately five days. The turbid culture broth was subsequently mixed at a 1:1 ratio with sterile 50 % (v/v) glycerol in sterile cryotubes and copies of each stored at both -20°C and -70°C.

Genomic DNA extraction

Bacterial cultures were grown in YM broth for four to seven days and genomic DNA extracted by the hexadecyltrimethyl ammonium bromide (CTAB) method as described by Wilson (1990). The integrity and concentration of the purified DNA samples was determined by agarose gel electrophoresis (Saambrook *et al.*, 1989).

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PCR amplification

The PCR reactions, for both *nifh* and *nodC* amplification were carried out in a volume of 100 µl and contained 50 pmole of each primer pair, 250 µM of each dNTP, 1.5 mM $MgCl_2$, approximately 50 ng genomic DNA and 0.5 U *Taq* DNA polymerase (Southern Cross Biotechnologies). Additionally, the reaction also contained 50 mM KCl, 10 mM Tris-HCl pH
9.0 and 0.1% Triton X-100 as supplied in the reaction buffer. All amplification reactions were carried out on a Perkin Elmer GeneAmp PCR System 2400 thermocycler.

A 750 bp fragment of the *nifH* gene was amplified using universal primers nifH-F and nifH-R (Table 6.2). In most instances the *nifH* primer set, as described by Laguerre *et al.* (2001) was unable to satisfactorily amplify the *nifH* gene of the indigenous rhizobial isolates. Subsequently a new set of *nifH* primers were designed by comparing *nifH* sequence data of rhizobial reference strains with the following GenBank accession numbers: K10620 (*B. japonicum*) J01781 (*S. meliloti*), Z95228 (*Mesorhizobium* sp.), M15942 (*R. etli* bv *phaseoli*), L16503 (*Sinorhizobium* sp.) and M55226 (*R. leguminosarum* bv. *phaseoli*). The following thermal profile were found suitable for the amplification of the *nifH* gene: An initial denaturation step for 3 min at 95 °C; followed by 35 cycles of denaturation (94 °C for 30 sec), annealing (50 °C for 45 sec) and extension (72 °C for 1 min) with a final extension at 72 °C for 10 min.

For *nodC* amplification, primers nodCF, nodCFu and nodCI (Laguerre *et al.*, 2001), indicated in Table 6.2, were used. The thermal profile used for the amplification of the *nodC* gene was similar to that of the *nifH* gene, except in instances where no amplification products were obtained, the annealing temperature was adjusted to 37 °C. This led to the appearance of non-specific bands. This was resolved by cutting the desired band from the agarose, spinning it through a glasswool (Merck, South Africa) column (packed in a 0.5 ml Eppendorf tube), and the eluent, which was recovered in a 1.5 ml tube, used as template in a new PCR.

Sequencing of nifH **DNA**

For sequencing, *nifH* PCR products were purified using Qiagen PCR Purification Kit (Southern Cross Biotechnologies. South Africa). where multiple amplification products were

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observed, the desired band was gel-purified using the Geneclean™ kit (Bio 101 Inc.) according to the manufacturer's instruction. Sequencing reactions were performed on purified *nifH* PCR products using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). Each sequencing reaction was carried out in a 5 µl volume containing approximately 100 ng template DNA, 12.5 pmole primer, and 2 µl ready reaction premix (supplied with the sequencing kit, containing the dye terminators, dNTP's, AmpliTaq DNA Polymerase, $MgCl_2$, and Tris-HCl buffer pH 9.0). The reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler and consisted of 25 cycles of denaturation (96 °C for 10 sec), annealing (50 °C for 5 sec), and extension (60 °C for 4 min). The ClustalX programme (Thompson *et al.,* 1997) was used to analyse the nucleic acid sequences. Additional sequence data of related α-*Proteobacteria* obtained from GenBank and accession numbers are indicated in the relevant figures. The *nifH* gene sequences of the gamma-proteobacteria *Klebsiella pneumoniae* and *Azotobacter chroococcum* were also included. A distance matrix was constructed by pairwise alignment of the sequences. The phylogenetic tree was constructed from the distance matrices using the neighbour-joining method of Saitou & Nei (1987). All branch lengths were proportional to the estimated divergence along each branch. The bootstrap method (Felsenstein, 1985) was used in combination with the neighbour-joining method to estimate confidence levels of the phylogenies. The phylogenetic tree was displayed using NJplot (Perrière & Gouy, 1996).

Table 6. 2. Primers used in this study

Primer	Sequence*	Target Region	Reference
NifH-F	5' CGGGAAGGGCGGAATCGGCAAG 3'	*nifH*	This work
NifH-R	5' GCATGTCCTCGAGCTC(AT)TCCAT 3'	*nifH*	This work
nodCF	5' AYGTHGTYGAYGACGGTTC 3'	*nodC*	Laguerre *et al.* (2001)
nodCFu	5' AYGTHGTYGAYGACGGITC 3'	*nodC*	Laguerre *et al.* (2001)
nodCI	5' CGYGACAGCCANTCKCTATTG	*nodC*	Laguerre *et al.* (2001)

*Abbreviations: Y = C or T H = A, C or T I = inosine N = A, C, G or T K = G or T.

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nodC RFLP analysis

The *nodC*-amplified products were used directly in a restriction reaction, without any prior purification. The digestion reactions were performed using 10 U of each of the following restriction enzymes *Hae*III, *Cfo*I, *Hinf*I, *Msp*I and *Rsa*I (Roche Molecular Diagnostics, South Africa) in optimal buffers as prescribed by the supplier. Electrophoresis of the restricted DNA was carried out on a 3% agarose gel in a Hybaid Maxi Gel System at 80 V for 90 min. DNA molecular weight marker VIII (Roche Molecular Diagnostics, South Africa) was included at multiple positions on gels as a standard. Restriction patterns were identified visually for each of the restriction enzymes used. A matrix, describing the presence (indicated by 1) or absence (indicated by zero), of a particular pattern within an isolate was generated. In this way a specific profile for each isolate was compiled. The Dice coefficient (Nei & Li, 1979) within the Bionum computer programme (Applied Maths, Kortrijk, Belgium) was used to construct a distance matrix from this one (1)-zero (0) profiles. These distance values were subsequently analysed to generate a tree using the UPGMA algorithm in GelCompar 4.0 (Applied Maths, Kortrijk, Belgium). Restrictions patterns of the *Mesorhizobium* and *Sinorhizobium* were obtained from Laguerre *et al.* (2001), while the RFLP patterns of the *Bradyrhizobium* spp. were determined in this study. Other rhizobial genera were not considered in the final dendrogram construction since initial inspection of the restriction pattern showed no correspondence to such genera.

RESULTS and DISCUSSION

The collection of isolates investigated here was obtained from root nodules from a range of diverse leguminous plants. However, the collection comprised isolates mainly from host plants of the Papilionoideae subfamily, with the Mimosoideae and the Caesalpinioideae represented by one and two isolates, respectively. In general each of the isolates was obtained from a different host plant. Isolates, obtained from the same host plant were the eight symbionts obtained from *Aspalathus linearis*, isolates 70a and 70b2 from *Crotalaria brachycarpa* and 82a, 48a and 48b from *Tephrosia purpurea*.

PCR amplification of the *nodC* and *nifH* gene fragments

A 900 bp fragment of *nodC* gene, which determines the length of the Nod-factor backbone, was obtained for most isolates. Isolates, SA3 and 66a1t1, repeatedly gave an amplification

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product of approximately 1100 bp, irrespective of the forward primer used. The nodCFu-nodCI primer pair was successfully used in most of the amplification reactions. For some isolates, as indicated in Table 6.1, no *nodC*-amplification product could be obtained. In an effort to find a basis for this lack of amplification, their phylogenetic positions based on the conserved 16S rRNA gene and the variable 16S-23S intergenic spacer region (IGS), as described in Chapter 5 (Fig. 5.1 & Fig. 5.4) were considered. Based on IGS phylogeny, isolates 103b, 3a, 13b, 15b, and xhj13 were divergent genotypes and not closely related to any of the reference species of the 16S rDNA subgroups to which they belonged. Laguerre *et al.* (2001) reported that *nodC* primers, similar to those used in this study, were unable to amplify the *nodC* fragment of *Sinorhizobium saheli* bv. acaciae. The 16S rDNA sequences of isolates xhj12FR and UP27b were closely related to *Sinorhizobium meliloti* and a *nodC* fragment for these isolates could not be obtained as well. It therefore appears that the current primers are not sufficient to cover the heterogeneity associated with the *nodC* gene. For the other strains, which also failed to amplify (as indicated in Table 6.1) no obvious causes were evident for this lack of visible amplified products.

A 750 bp *nifH* fragment was obtained for most isolates, although some showed non-specific bands smaller than the expected size fragment.

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Table 6.1. Isolates investigated in this study and a summary of RFLP data following digestion of *nodC*-amplified products with indicated enzymes.

Alphabetical denotations refer to the first letter of the relevant enzyme. Distinctive patterns are indicated by numerical designations. Strains, where no amplified product was obtained indicated by (nd). The *Hinf*I pattern, Hi2, refers to no restriction site. Subfamilies of the Fabaceae indicated in brackets: P: Papilionoideae, M: Mimosoideae, C: Caesalpinioideae

Isolate number	Host legume/ Reference strain	*nodC* restriction patterns after digestion with				
		***Hae*III**	***Hinf*I**	***Cfo*I**	***Msp*I**	***Rsa*I**
2	*Rhynchosia nervosa* (P)	H3	Hi2	C2	M2	R2
29d	*Alysicarpus rugosus* (P)	H3	Hi2	C2	M2	R2
42bs	*Neonotonia wightii* (P)	H1	Hi1	C1	M1	R1
70a	*Crotalaria brachycarpa* (P)	H3	Hi2	C2	M2	R2
82a	*Tephrosia purpurea* (P)	H3	Hi2	C2	M2	R2
103b	*Indigofera hilaris* (P)	nd	nd	nd	nd	nd
40a	*Sesbania bispinosa* (P)	H2	Hi2	C3	M3	R3
48a	*Tephrosia purpurea*(P)	H2	Hi2	C3	M3	R3
48b	*Tephrosia purpurea* (P)	H2	Hi2	C3	M3	R3
49b	*Indigofera melanadenia* (P)	H2	Hi2	C3	M3	R3
33b	*Crotalaria* sp. (P)	nd	nd	nd	nd	nd
123b	*Desmodium* repandum (P)	H2	Hi2	C3	M4	R4
114d	*Pseudarthria hookeri* (P)	H4	Hi3	C4	M5	R4
46c2	*Acacia sieberana* var *woodii* (M)	H5	Hi4	C5	M4	R5
125e	*Indigofera woodii* (P)	H6	Hi2	C3	M4	R3
70b2	*Crotalaria brachycarpa* (P)	H2	Hi2	C6	M6	R3
104a1	*Chamaecrista* sp. (C)	H2	Hi2	C6	M6	R3
3a	*Trifolium* sp.(P)	nd	nd	nd	nd	nd
13b	*Lotononis bainesii* (P)	nd	nd	nd	nd	nd
15b	*Desmodium tortuosum* (P)	nd	nd	nd	nd	nd
LMG 18230	*Bradyrhizobium liaoningense*	H2	Hi3	C6	M7	R6
LMG 6138	*Bradyrhizobium japonicum*	H2	Hi3	C6	M7	R6
LMG 6134	*Bradyrhizobium elkanii*	H10	H5	C3	M8	R6
xhj15	*Aspalathus linearis* (P)	nd	nd	nd	nd	nd
xhj13	*Aspalathus linearis* (P)	nd	nd	nd	nd	nd
xhm5	*Aspalathus cordata* (P)	nd	nd	nd	nd	nd
xhj18	*Aspalathus linearis* (P)	H6	Hi2	C7	M9	R7
xhj23	*Aspalathus linearis* (P)	H6	Hi2	C7	M9	R7
xhj7	*Aspalathus linearis* (P)	H6	Hi2	C8	M10	R8
xhj8	*Aspalathus linearis* (P)	H6	Hi2	C8	M10	R8
xhj20	*Aspalathus linearis* (P)	H6	Hi2	C7	M10	R7
xhj27	*Aspalathus linearis* (P)	H7	Hi2	C8	M9	R14
xhj26	*Aspalathus linearis* (P)	H6	Hi2	C8	M10	R8
xhj12FR	*Aspalathus linearis* (P)	nd	nd	nd	nd	nd
98d2	*Bolusanthus speciosus* (P)	nd	nd	nd	nd	nd
128a	*Melolobium obcordatum* (P)	H1	Hi2	C8	M12	R7
MSDJ 2184	*Mesorhizobium* sp.	H7	Hi7	C9	M13	R9
ACCC19665	*Mesorhizobium amorphae*	H6	Hi8	C10	M14	R10
7653R	*Mesorhizobium huakuii*	H1	Hi9	C11	M15	R11
USDA3233	*Mesorhizobium ciceri*	H8	H10	C12	M16	R12
xhj12s	Aspalathus *linearis* (P)	H8	Hi6	C6	M11	R14
USDA1002	*Sinorhizobium meliloti*	H9	Hi6	C13	M17	R13
M1	*Sinorhizobium medicae*	H10	Hi11	C14	M18	R14
36b	*Neonotonia wightii* (P)	H2	Hi12	C6	M6	R15
UP27b	*Crotalaria* damrensis (P)	nd	nd	nd	nd	nd
SA3	*Trifolium africanum* (P)	H11	Hi13	C15	M19	R16
UP26b	*Crotalaria* damrensis (P)	H12	Hi14	C16	M20	R17
66a1t1	*Acacia robusta* (M)	H13	Hi2	C7	M21	R17
7b	*Alysicarpus rugosus* (P)	nd	nd	nd	nd	nd
60	*Rhynchosia monophylla* (P)	nd	nd	nd	nd	nd
PL10a	*Argyrolobium tomentosum* (P)	nd	nd	nd	nd	nd

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Diversity of *nodC* genotypes and host specificity

The different restrictions patterns obtained after digestion with five restriction enzymes are indicated in Table 6.1. Among the 28 isolates analysed, 19 different *nodC* genotypes were present. This correlated well with results obtained by Laguerre *et al.* (2001), who also found 45 nod types among 82 strains investigated. These authors also concluded that the *nodC* gene was highly polymorphic. According to Mergaert *et al.* (1997) the *nodABC* genes were previously thought to be functionally conserved in all rhizobia. However, it has been demonstrated that different alleles of the *nodA* and *nodC* genes are present in the rhizobial gene pool and that this allelic variation contributes to host range determination. The inability of the current primers to generate an amplification product in some instances might also be related to the heterogeneity of the *nodC* gene. Of the different *nodC* types present among the selected indigenous isolates, none showed 100% correspondence to the included rhizobial reference strains.

The different restriction patterns were used to construct a dendrogram (Fig. 6.1), which could be divided into two sections. Within section 1, different clusters were present, some of which (clusters A and B) were clearly host species-specific clusters. The *nodC* genotype of isolates associated with *Aspalathus linearis* was found to be polymorphic since two related clusters (A and B) were observed, while xhj27 only had a *Hinf*I restriction pattern in common with members of clusters A and B and xhj12s was distantly related to *S. meliloti* (section 2). According to Dobert *et al.* (1994) and Ueda *et al.* (1995) different *nod* genotypes that are found in rhizobia have evolved to allow optimal nodulation of the corresponding host legumes.

Members of clusters C, E and F individually displayed identical *nodC* types despite the fact that they were obtained from different host plants. A previous investigation (Chapter 5) showed similar clustering of these isolates into highly homologous groups based on 16S rDNA sequences. Subsequent analysis of their 16S-23S intergenic spacer (IGS) regions, which have higher discriminatory ability (Gurtler *et al.*, 1996), also showed that the isolates within these groups are closely related. In the absence of DNA homology data, which is an important criterion for species delineation, it is difficult to speculate whether strains in these groups are the same species or not. However, according to Willems *et al.* (2001c), IGS

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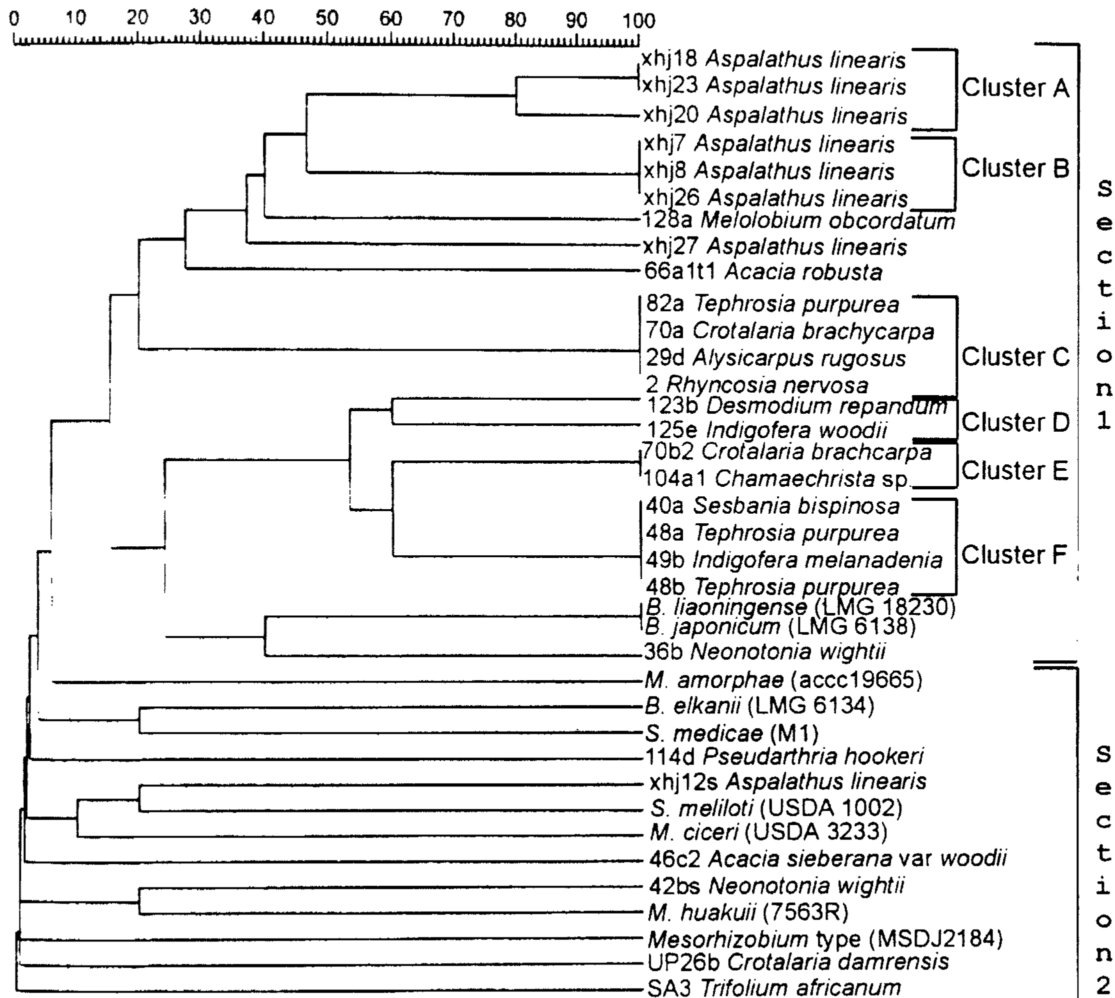

Figure 6.1. Dendrogram based on the combined *Hae*III, *Hin*fI, *Cfo*I, *Msp*I and *Rsa*I restriction patterns of *nodC* amplified product. The host species from which these isolates were obtained are shown in italics. Information of the restriction patterns of the genera *Mesorhizobium* and *Sinorhizobium* were obtained from Laguerre *et al.* (2001). The *x*-axis represents the correlation between strains.

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variability sometimes correlates well with DNA homology data for the *Bradyrhizobium* genus. Preliminary chromosomal evidence (16S rDNA sequence and IGS) therefore suggests that the respective isolates in clusters C, E and F, which share similar *nodC* types, may represent three species. In the light of this evidence, it would appear that these strains have a broad host range since they were isolated from different host legumes.

The *nodC* types of isolates 36b, 114d, 46c2, 42bs, UP26b and SA3 showed no close relation to any of the other nod types obtained in this study. Laguerre *et al.* (2001) concluded that the RFLP method was insufficient to determine the true phylogenetic relationships of the *nodC* gene since few restriction sites, within this genomic region, were conserved among species or biovars. In an effort to determine the true phylogenetic relationships between these *nodC* types, nucleotide sequencing of representatives of such types will be necessary.

Phylogeny based on partial *nifH* gene sequences and comparison with 16S rDNA classification

Partial *nifH* sequence data were used to construct a phylogeny of a selection of the indigenous strains. The *nifH* sequence of rhizobial reference strains: *B. liaoningense* (LMG 18230), *M. loti* (LMG 6125), *M. amorphae* (LMG 18977), *M. plurifarium* (LMG 11892), *S. xinjiangensis* (LMG 17930) were also determined in this study since they were not available in the GenBank sequence database. In general, the constructed phylogenetic tree (Fig. 6.2) correlated well with the 16S rRNA phylogeny (Chapter 5) in accordance with previous reports (Hennecke *et* al., 1985; Ueda *et al.*, 1995). The genetic distances, based on *nifH* data, were larger than that observed among 16S rDNA sequences. As an example, isolate 70b2 showed almost 99% sequence homology with *B. japonicum*, while it showed only 83% sequence identity based on *nifH* sequence data. 16S rDNA sequences also showed that isolates such as 40a, 48a, 49b and 60, were divergent bradyrhizobial strains. The *nifH* phylogeny confirms their divergent nature because, although they were present in the same cluster as *B. japonicum* and *B. liaoningense*, they were carried on a separate lineage. The *nifH* sequences of isolates from *Aspalathus linearis*, which showed highest 16S rDNA homology to *M. loti*, were compared to *M. loti nifH* sequences from two different sources: the *M. loti* (LMG 6125) *nifH* sequence, as determined in this study and *M. loti* genome

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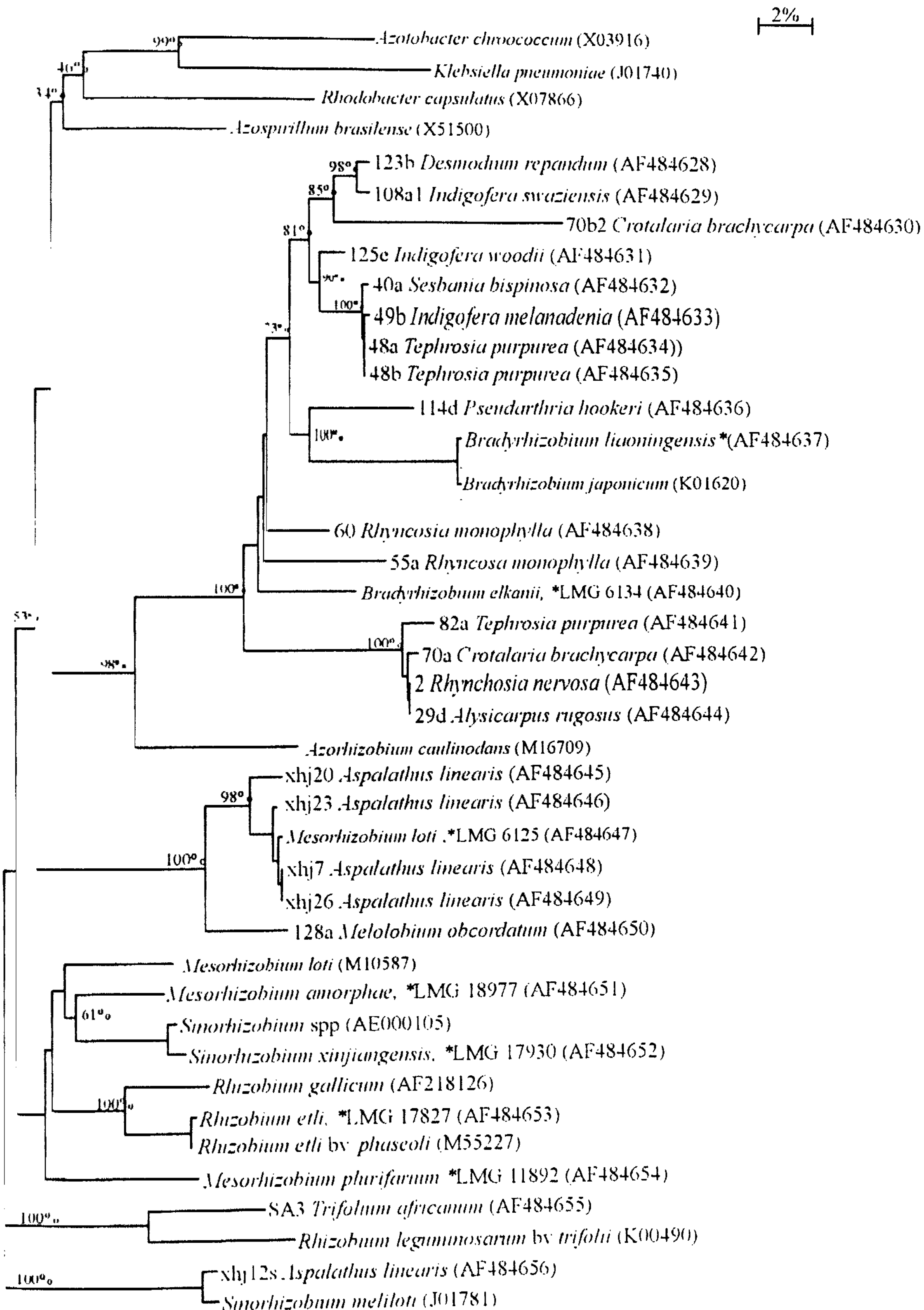

Figure 6.2. Phylogenetic tree, based on a 460 bp fragment of the *nifH* gene, expressing the relationship of a selection of the indigenous rhizobia to other rhizobial genera and other nitrogen fixing strains of the alpha- and gamma-proteobacteria. The tree was generated using the Neighbour-joining method of Saitou & Nei (1987). Horisontal branches are equal to the phylogenetic distances of which the scale indicate 2%. Bootstrap values of some of the major branching points are indicated. GenBank accession numbers are given in brackets. The *nifH* sequences of reference strains (indicated by *) were not available and were determined in this study.

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sequencing project (GenBank accession number M10587). A discrepancy was observed between these two sequences, since they had different positions on the phylogenetic tree, with the *Aspalathus linearis* isolates being closer related to LMG 6125.

When Haukka *et al.* (1998) investigated the diversity of a group of strains from leguminous trees growing in Africa and Latin America, *Sinorhizobium* strains with different *nifH* sequence were found. In this case the *nifH* tree was not consistent with the tree based on 16S rDNA sequences, suggesting the occurrence of lateral gene transfer. Among the indigenous isolates investigated here, no evidence of lateral gene transfer could be indicated.

Finally, the results presented here showed that the bacterial symbionts, forming symbiotic associations with the indigenous leguminous plants, harbour a diverse range of *nodC* genotypes. The extent of this diversity will be reflected more accurately once sequence analyses are performed on representatives of such genotypes and when more isolates are investigated from a common host species. The *nifH* phylogeny reflects the 16S rRNA gene phylogeny. However, it is unlikely that this would be true for symbionts from all leguminous plants.

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CHAPTER 7

Characterisation of methylotrophic bacteria which nodulate *Lotononis bainesii*.

ABSTRACT

Nodule isolates from a shrubby legume, *Lotononis bainesii*, was characterised by 16S rRNA gene sequencing and morphologically by substrate utilisation patterns. The symbiotic genome of these isolates was analysed by partial sequencing of the *nifH* gene. Based on the results of numerical taxonomy, the isolates formed a closely related cluster, showing no correspondence to any of the known rhizobial clusters. Analysis of nearly full-length 16S rDNA sequences demonstrated that these isolates were related to *Methylobacterium nodulans* (Sy *et al.* 2001). In the absence of *nifH* sequence data for the genus *Methylobacterium*, the *nifH* phylogeny showed these isolates to be related to *Azospirillum brasilense*. The facultative methylotrophic nature of these isolates was also demonstrated by their ability to grow in the presence of methanol as a sole carbon source.

Keywords: *Lotononis bainesii*, 16S rDNA sequencing, *nifH* gene, *Methylobacterium*

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INTRODUCTION

Symbiotic nitrogen fixing bacteria, commonly referred to as rhizobia, are able to establish a symbiotic association with most leguminous plants. As a result of this symbiotic association, specialised organs, called nodules, are induced on the host plant. Within such nodules atmospheric nitrogen is reduced to ammonia the benefit of the host plant. These nitrogen-fixing nodulating rhizobia have been assigned to different genera within the α- subclass of the *Proteobacteria* and include: *Rhizobium* (Frank, 1889), *Sinorhizobium* (Chen *et al.*, 1988), *Mesorhizobium* (Jarvis *et al.*, 1997), *Allorhizobium* (de Lajudie *et al.*, 1998), *Bradyrhizobium* (Jordan, 1982) and *Azorhizobium* (Dreyfus *et al.*, 1988). These rhizobial genera are very diverse with some being phylogenetically closer related to other non-symbiotic genera, than to each other (Young, 1996; van Berkum & Eardly, 1998; Young, 2001). Recently, Sy *et al.* (2001) reported the existence of an additional rhizobial branch involving bacteria of the genus *Methylobacterium*. These rhizobia were isolated from *Crotalaria* species and were able to grow facultatively on methanol, a common trait for *Methylobacterium* species, but unique to the known rhizobial species. Analysis of the 16S rDNA gene, nodulation ability, as well as amplification of the *nodA* gene confirmed these isolates to be nodulating *Methylobacterium* species for which the name *Methylobacterium nodulans* was proposed. According to Holland (1997), *Methylobacterium* species are usually isolated from water and leaf surfaces and are known as pink-pigmented facultative methylotrophs. However, the presence of the photosynthetic pigment, bacteriochlorophyll *a*, was not detected in *M. nodulans* (Sy *et al.*, 2001).

Lotononis species are herbs and shrubs of the subfamily Papilionoideae with more than 140 species commonly occurring under diverse climatological and geographical conditions. Their distribution is chiefly in southern Africa extending to the Mediterranean, with a few species in southern Europe and central Asia (van Wyk, 1991). *Lotononis bainesii* has proven its value as a pasture legume in regions in Australia. In addition to *L. bainesii*, other *Lotononis* species such as *L. divaricata*, *L. tenella* and *L. laxa* also have potential value as grazing plants since many are well adapted to the arid regions (Shearing, 1994). In 1958 Norris described a pigmented nodulating strain obtained from the roots of *Lotononis bainesii*. The chemical structure of this pigment was subsequently determined by Kleinig & Broughton (1982) and found to be similar to that of *Pseudomonas* species. Dagutat (unpublished results) compared

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the protein profiles of a collection of bacterial isolates obtained from the root nodules of *Lotononis bainesii*. These isolates formed a closely related cluster, clearly separated from the rhizobial reference strains. Initial partial sequence 16S rDNA sequencing performed as part of this study revealed that these isolates were indeed a group of unknown taxonomic status. However, with the report of the methylotrophic nodulating bacteria (Sy *et al.*, 2001) it became evident that these pigmented *Lotononis* isolates were related to the genus *Methylobacterium*, showing high homology to *Methylobacterium nodulans*.

This primary objective of this study was therefore to characterise the nine isolates from the root nodules of *Lotononis bainesii* obtained from different localities in southern Africa. Using nearly full length 16S rDNA and partial *nif*H sequencing and substrate utilisation patterns, it was possible to show that these pigmented nodulating strains were facultative methylotrophs, related to the genus *Methylobacterium*, more specifically *M. nodulans*.

MATERIALS AND METHODS

Bacterial strains

Strains analysed (Table 7.1) in this study was obtained from the rhizobial collection of the ARC-Plant Protection Research Institute (Private Bag X134, Pretoria 0001, South Africa). These isolates were previously obtained from the root nodules of *Lotononis bainesii*. Reference cultures (Table 7.2) of the different rhizobial genera were obtained from the bacterial culture collection of the Laboratorium voor Microbiologie (LMG), State University Gent, Belgium and the United States Department of Agriculture (USDA), Soybean and Alfalfa Research Laboratory, Maryland, USA.

Maintenance of bacterial cultures

Strains were maintained on yeast extract mannitol (YM) agar, containing (w/v): 1% mannitol, 0.5% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.01%, NaCl, 0.04% yeast extract and 1.5% bacteriological agar. For long term storage cultures were grown (with rigorous shaking) in yeast mannitol broth (YMB) at 28°C for approximately five days. The turbid culture broth was subsequently mixed at a 1:1 ratio with sterile 50% (v/v) glycerol in sterile cryotubes and copies of each stored at both -20°C and -70°C.

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Table 7.1. List of isolates analysed in this study.

Isolate	Host plant	Isolate	Host plant
xct7	*Lotononis bainesii*	xhm4	*Aspalathus linearis*
xct8	*Lotononis bainesii*	xhj7	*Aspalathus linearis*
xct9	*Lotononis bainesii*	xhj8	*Aspalathus linearis*
xct10	*Lotononis bainesii*	xhj12s	*Aspalathus linearis*
xct12	*Lotononis bainesii*	xhj15	*Aspalathus linearis*
xct13	*Lotononis bainesii*	xhj18	*Aspalathus linearis*
xct14	*Lotononis bainesii*	xhj20	*Aspalathus linearis*
xct16	*Lotononis bainesii*	xhj26	*Aspalathus linearis*
xct17	*Lotononis bainesii*	xhj27	*Aspalathus linearis*

Table 7.2. List of rhizobial reference strains analysed in this study.

Reference strains	**Strain number**	**Host plant**
Rhizobium leguminosarum	LMG 4260	*Vigna unguiculata*
Rhizobium leguminosarum	LMG 6294	*Lathyrus sp.*
R. leguminosarum bv. *trifolii*	LMG 6119	*Trifolium repens*
Rhizobium galegae	USDA 4128^T	*Galega orientalis*
Rhizobium tropici IIB	USDA 9030^T	*Phaseolus vulgaris*
Rhizobium etli	USDA 9032	*Phaseolus vulgaris*
Rhizobium sp.	LMG 6463	*Sesbania rostrata*
Bradyrhizobium japonicum	LMG 6138^T	*Glycine max*
Bradyrhizobium elkanii	LMG 6134^T	*Glycine max*
Bradyrhizobium sp.	LMG 8319	*Macrotyloma africanus*
Azorhizobium caulinodans	LMG 6465	*Sesbania rostrata*
Allorhizobium undicola	USDA 4903	*Neptunia natans*
Allorhizobium undicola	USDA 4904	*Neptunia natans*
Sinorhizobium meliloti	LMG 6133^T	*Medicago sativa*
Sinorhizobium fredii	LMG 6217^T	*Glycine max*
Sinorhizobium saheli	LMG 7837^T	*Sesbania cannabina*
Mesorhizobium loti	LMG 6125^T	*Lotus corniculatus*
Mesorhizobium huakuii	USDA 4778^T	*Astragalus sinicus*
Agrobacterium radiobacter	LMG 140^T	NS
Agrobacterium tumefaciens	LMG 187^T	*Lycopersicon lycopersicum*
Agrobacterium rhizogenes	LMG 150^T	NS
Agrobacterium aggregatum	LMG 122^T	NS

^{T} Type strain   NS not stated

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Numerical taxonomy

Substrate utilisation patterns of the isolates were assessed using a commercially available Biolog MicroPlate™ (Biolog, Hayward, California). These plates were specific for Gram-negative microorganisms and contained a preselected panel of 95 different carbon sources (Appendix B). Growth conditions and inoculation of the microplates were performed as prescribed by the suppliers. The utilisation of a specific carbon source was indicated by the development of a purple colour in the wells which was due to the presence of a redox dye, tetrazolium violet. Such wells were scored as one (1), while negative wells were scored as zero (0). The Dice coefficient (Nei & Li, 1979) within the Bionum computer programme (Applied Maths, Kortrijk, Belgium) was used to construct a distance matrix from this one (1)-zero (0) profiles. These distance values were subsequently analysed to generate a tree using the unweighted pair group method of arithmetic mean algorithm (UPGMA) in GelCompar 4.0 (Applied Maths, Kortrijk, Belgium). Substrate utilisation patterns of the rhizobial reference strains were obtained from a previous study (Kruger, 1998). Isolates obtained from the nodules of *Aspalathus linearis* was also included as further references.

To test the ability of the *Lotononis bainesii* isolates to utilise methanol as a sole carbon source, cells were grown on YM agar for five days and transferred to medium 72 (Appendix C), which contained 10 ml of filter-sterilised methanol per liter medium.

Genomic DNA extraction

Bacterial cultures were grown in YM broth for 4-7 days and genomic DNA extracted by the hexadecyltrimethyl ammonium bromide (CTAB) method as described by Wilson (1990). The integrity and concentration of the purified DNA samples were determined by agarose gel electrophoresis (Saambrook *et al.*, 1989).

PCR amplification, sequencing and analyses

The PCR reactions for the amplification of the 16S rRNA gene and a fragment of the *nifH* gene were carried out in a volume of 100 µl. Each reaction contained 50 pmole of each respective primer pair, 250 µM of each dNTP, 1.5 mM $MgCl_2$, approximately 50 ng genomic DNA and 0.5 U Taq DNA polymerase (Southern Cross Biotechnologies). Additionally, the reaction also contained 50 mM KCl, 10 mM Tris-HCl pH 9.0 and 0.1% Triton X-100 as

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supplied in the reaction buffer. All amplification reactions were carried out on a Perkin Elmer GeneAmp PCR System 2400 thermocycler.

The 16S rRNA gene was amplified using universal primers fD1 and rP2 (Table 7.3) as described by Weisburg *et al.* (1991). However, since no PCR product cloning procedures were anticipated, linker sequences containing the restriction enzyme recognition sites were not included during the synthesis of these oligonucleotides. These primers were therefore designated fD1SHRT and rP2SHRT. The following thermal profile was used: initial denaturation step at 95 °C for 3 min, 30 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 30 sec) and extension (72 °C for 1 min). An additional extension step (72 °C for 10 min) was performed after completion of the 30 cycles. The PCR products were purified using the Qiagen PCR Purification Kit (Southern Cross Biotechnologies) according to the manufacturer's instructions.

The primers (Table 7.3) used to amplify a 750 bp fragment of the *nifH* gene were designed by comparing *nifH* sequence data of rhizobial reference strains with the following GenBank accession numbers: K10620 (*B. japonicum*) J01781 (*S. meliloti*), Z95228 (*Mesorhizobium* sp.), M15942 (*R. etli* bv *phaseoli*), L16503 (*Sinorhizobium* sp.) and M55226 (*R. leguminosarum* bv. *phaseoli*). The following thermal profile was found suitable for the amplification of the *nifH* gene: An initial denaturation step: 3 min at 95 °C; followed by 35 amplification cycles denaturation (94 °C for 30 sec), annealing (37 °C for 45 sec) and extension (72 °C for 1 min) with a final extension at 72 °C for 10 min.

Due to the difficulties experienced with the amplification of the *nifH* gene and the degeneracy of the primers used, it was necessary to clone the *nifH* PCR products prior to sequencing. The PCR product of isolate xct 7 was cloned into the pDrive cloning vector supplied in the Qiagen PCR cloning kit (Southern Cross Biotechnologies) according to the manufacturer's instructions. Plasmids were introduced into competent *E. coli* DH5α cells and recombinants isolated according to standard protocols (Saambrook, *et al.*, 1989).

Sequencing reactions were performed using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). Each sequencing reaction was

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Table 7.3. Primers used for PCR amplification and sequencing in this study

Primer	Sequence	Target Region	Reference
fD1SHRT	5' AGAGTTTGATCCTGGCTCAG 3'	16 rRNA	Weisburg *et al.* (1991)
rP2SHRT	5' ACGGCTACCTTGTTACGACTT 3'	16 rRNA	Weisburg *et al.* (1991)
16SRNAII-S	5' GTGTAGCGGTGAAATGCGTAG 3'	16 rRNA	Kuhnert, *et al.* (1996)
16SRNAVII-S	5' CTTGCGACCGTACTCCCCAGGC 3'	16 rRNA	Kuhnert, *et al.* (1996)
NifH-F	5' CGGGAAGGGCGGAATCGGCAAG 3'	*NifH*	This work
NifH-R	5' GCATGTCCTCGAGCTC(AT)TCCAT 3'	*NifH*	This work

carried out in a 5 µl volume containing approximately 100 ng template DNA, 12.5 pmole primer, and 2 µl ready reaction premix (supplied with the sequencing kit, containing the dye terminators, dNTP's, AmpliTaq DNA Polymerase, $MgCl_2$, and Tris-HCl buffer pH 9.0). The reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler and consisted of 25 cycles of denaturation (96 °C for 10 sec), annealing (50 °C for 5 sec), and extension (60 °C for 4 min). PCR primers (Weisburg *et al.*, 1991) as well as two internal primers, 16SRNAII-S and 16S RNAVII-S (Kuhnert *et al.*, 1996), were used to obtain nearly full-length 16S rDNA sequence data.

To obtain the *nifH* sequence data of xct7, recombinant plasmids were sequenced using the M13 reverse and M13 forward (-20) primers.

The ClustalX programme (Thompson *et al.*, 1997) was used to analyse the nucleic acid sequences. Additional sequence data of related α-*Proteobacteria* was obtained from GenBank and accession numbers are indicated in the relevant figures. A distance matrix was constructed by pairwise alignment of the sequences. The phylogenetic trees were constructed from the distance matrices using the neighbour-joining method of Saitou & Nei (1987). All branch lengths were proportional to the estimated divergence along each branch. The bootstrap method (Felsenstein, 1985) was used in combination with the neighbour-joining method to estimate confidence levels of the phylogenies. The phylogenetic trees were displayed using NJplot (Perrière & Gouy, 1996).

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The *nifH* sequence of isolate xct 7 was also compared with *nifH* nucleotide sequences of other α- and γ-*Proteobacteria*. A distance matrix (Table 7.5) expressing these genetic distances was generated using DNAdist from Phylip version 3.5c (Felsenstein, 1989).

RESULTS

Numerical taxonomy

Substrate utilisation patterns of 95 different carbon sources were used to establish a numerical taxonomy for bacteria isolated from the root nodules of *Lotononis bainesii*. The results of the range of substrates utilised by this group of symbionts are recorded in Table 7.4. These carbon sources were previously divided into 11 categories by Garland &Mills (1991) as indicated in Table 7.4. The highest number of carbon sources tested for included carboxylic acids, amino acids and carbohydrates. Amongst the *Lotononis* isolates the full range of carboxylic acids assayed for was utilised, while only 12 out of 20 amino acids were used by the isolates. The isolates were more specific with regard to their carbohydrates as sole carbon sources since only 5 out of possible 28 sources were used.

The metabolic fingerprints generated using the Biolog system were subsequently used to generate a dendrogram (Figure 7.1) expressing the phenotypic similarities among the *Lotononis* isolates, rhizobial reference strains and other indigenous isolates obtained from *Aspalathus linearis*. Two major sections could be distinguished within this dendrogram. The first contained members of the genera *Rhizobium, Sinorhizobium, Mesorhizobium, Agrobacterium* and *Allorhizobium*, while section 2 contained the genus *Bradyrhizobium* and the *Lotononis* isolates. The overall similarity for groups within the sections is 63% and 67% for section 1 and 2 respectively. Species of the genera *Agrobacterium* and *Allorhizobium undicola* were exclusively present in clusters 2 and 3, while *Rhizobium* and *Sinorhizobium* spp. were intermixed between clusters 1 and 4. Isolates from *Aspalathus linearis* (in cluster 5) were related to *Mesorhizobium huakuii*.

The *Lotononis* isolates were distributed among three clusters with an overall similarity of 77%. Cluster 6 contained xct 14, xct 13, xct 17, xct 8, while clusters 7 and 8, contained xct 10, xct 12 and xct 7 and xct 16, respectively. Isolate xct 9 was separate from the other clusters harboring the *Lotononis* isolates. A closer examination of the substrate utilisation

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Table 7.4 Oxidation patterns of the different carbon sources utilised by *Lotononis bainesii* isolates.

Carbon sources not utilised by any of the isolates are not listed. Those showing reaction indicated by positive (+) sign and no reaction by negative (-). Categories of substrates as determined by Garland & Mills, (1991).

Carbon Sources	Isolates								
	xct7	xct8	xct9	xct10	xct12	xct13	xct14	xct16	xct17
Polymers									
Tween-40	+	+	+	-	-	+	+	+	-
Tween-80	+	+	+	-	+	+	+	+	-
Carbohydrates									
L-arabinose	-	+	+	+	+	+	+	+	-
L-fucose	-	-	-	-	-	+	+	-	-
A-D-glucose	-	-	+	-	-	+	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-
xylitol	-	-	+	-	-	-	-	-	-
Esters									
methylpyruvate	+	+	+	+	+	+	+	+	+
mono-methylsuccinate	+	+	+	+	+	+	+	+	+
Carboxylic acids									
acetic acid	+	+	+	+	+	+	+	+	+
cis-aconitic acid	+	+	-	+	+	+	+	+	-
citric acid	-	+	+	+	+	+	+	+	-
formic acid	+	-	-	-	-	+	+	+	+
D-galactonic acid lactone	-	+	+	+	-	+	+	+	+
D-galacturonic acid	-	-	-	-	-	+	+	+	-
D-gluconic acid	+	+	+	+	+	+	+	+	+
D-glucosaminic acid	-	-	-	+	-	-	-	+	-
D-glucoronic acid	-	+	-	-	-	-	-	-	-
A-/ β-/ γ-hydroxybutyric acid	+	+	+	+	+	+	+	+	+
itaconic acid	-	-	-	-	-	-	-	+	-
A-keto-butyric acid	+	+	+	+	+	+	+	+	+
A-keto-glutaric acid	+	+	+	+	+	+	+	+	+
A-keto-valeric acid	+	+	+	+	+	+	+	+	+
D, L lactic acid; malonic acid	+	+	+	+	+	+	+	+	+
propionic acid, quinic acid	+	+	+	+	+	+	+	+	+
D-saccharic acid, sebacic acid	+	+	+	+	+	+	+	+	+
succinic acid	+	+	+	+	+	+	+	+	+
Bromonated chemicals									
bromo-succinic acid	+	+	+	+	+	+	+	+	+
Amides									
succinamic acid	+	+	+	+	+	+	+	+	+
alaninamide	+	+	+	+	+	+	+	+	+
Amino acids									
L-Asparagine	+	+	+	+	+	+	+	+	+
D-alanine	-	-	-	-	-	+	+	-	-
L-alanyl-glycine	-	-	+	-	-	+	-	-	-
L-aspartic acid	+	+	+	+	+	+	+	+	+
L-glutamic acid	+	+	+	+	+	+	+	+	+
glycyl-L-glutamic acid	-	-	+	+	+	+	+	-	-
L-leucine	-	-	-	-	-	-	-	-	+
L-proline	+	-	+	+	+	+	+	+	-
L-pyroglutamic acid	+	-	+	-	-	+	+	+	+
D-serine; L-serine	-	-	+	-	-	-	-	-	-
L-threonine	-	-	+	-	+	+	+	-	-
Aromatic chemicals									
urocanic acid	-	-	-	-	-	+	+	-	-
Amines									
2-amino-ethanol	-	-	-	-	-	-	-	-	-
Alcohols									
2,3- butanediol	-	-	+	-	-	-	-	-	-

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pattern revealed that this isolate could uniquely use the following substrates: xylitol, D-serine, L-serine, 2-amino-ethanol and 2, 3-butanediol.

16S rDNA sequence analysis

Nearly full length (1180 bp) nucleotide sequence of the 16S rRNA gene was determined for the nine isolates obtained from the root nodules of *Lotononis bainesii*. The phylogenetic position of these isolates was inferred from comparative 16S sequence analysis and is indicated in the Figure 7.2. Representatives of the other genera of the α-*Proteobacteria* were also included in this analysis. The polyphyletic nature of the rhizobial genera, as described previously (Young, 1996) was again clearly illustrated in this analysis. The *Lotononis bainesii* isolates were clearly distinct from the other known rhizobial genera and showed high sequence homology to the *Methylobacterium* lineage of the α-*Proteobacteria*. The branching point leading to the branches, which carry the *Methylobacterium* species, was also supported by a bootstrap value of 100%. The *Lotononis* isolates had almost identical 16S rDNA sequences; with only xct 17 showing approximately 2.6% sequence difference from the other isolates. Sequence similarities of the *Lotononis* group and other described *Methylobacterium* species was on average 94%. The closest phylogenetic neighbor of the *Lotononis* isolates was *Methylobacterium nodulans* (Sy, *et al.*, 2001), showing sequence similarity values of close to 98%.

Analysis of the *nifH*-gene sequences.

A fragment of the *nifH* gene of isolate xct 7 was cloned and sequenced. Most of the available *nifH* sequence data, obtained from GenBank, were considerably shorter fragments of sequence and therefore only a 560 bp fragment of the gene was used for comparative analysis and construction of a phylogenetic tree (Fig. 7.3). 16S rDNA sequence analyses have already indicated the *Lotononis* isolates to be related the genus *Methylobacterium*, however, no *nifH* sequence data was available for members of this genus. In the absence of sufficient sequence data, the phylogenetic position of xct 7, was therefore assessed in the presence of other genera of the α- and γ– *Proteobacteria*, as well as the *nifH* sequences of other indigenous rhizobial isolates (indicated by # in Fig. 7.3). The indigenous isolates maintained their genetic affiliations as determined by 16S rDNA sequence analyses. No close relationship was evident between the *Lotononis* isolate xct 7 and any member of the known rhizobial genera included in this analysis. *Azospirillum brasilense* and xct 7 shared almost 90% *nifH*

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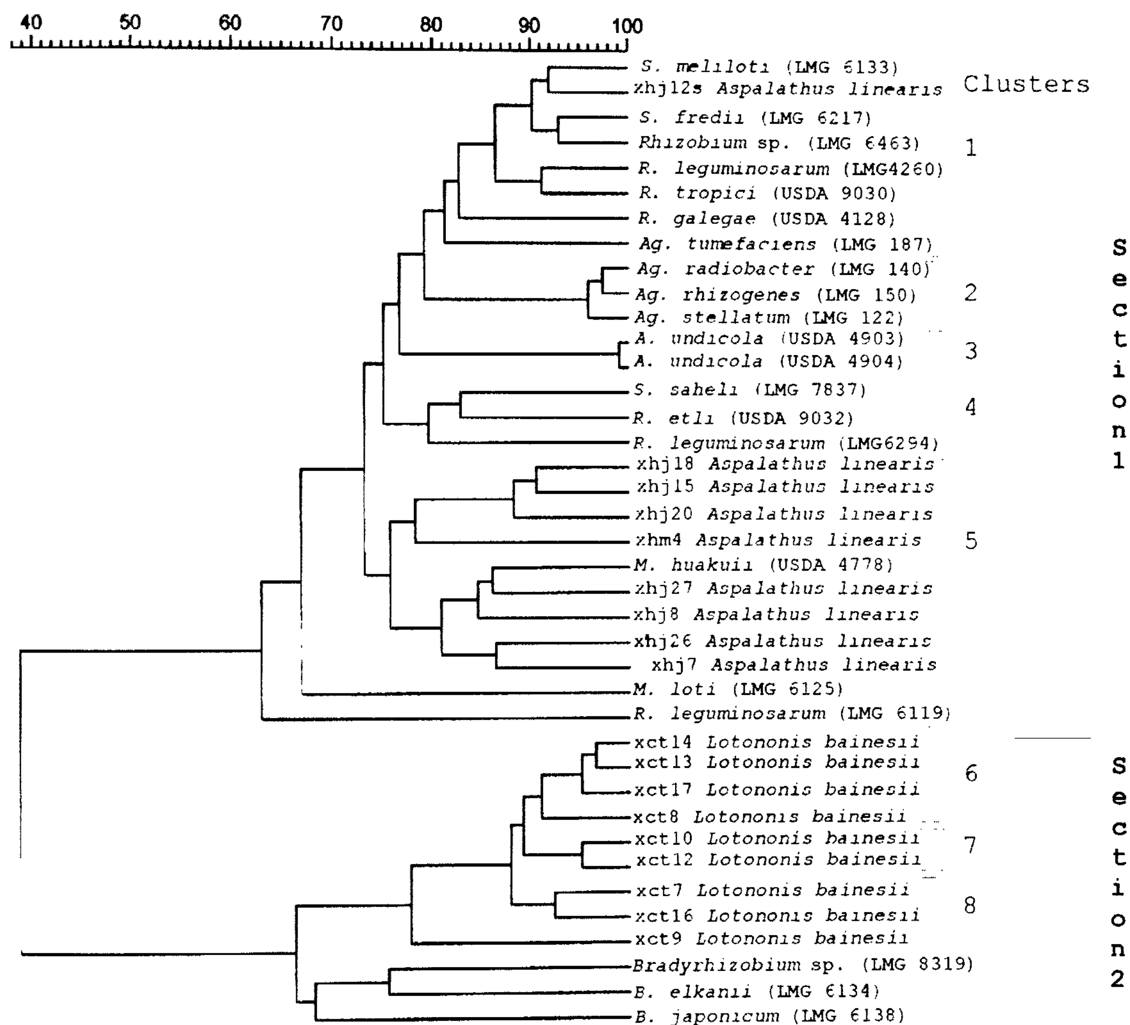

Figure 7.1. Dendrogram showing the phenotypic similarities among *Lotononis bainesii* isolates as determined by substrate utilisation patterns using the Biolog system. Rhizobial strains and isolates from Aspalathus linearis were included as references. The UPGMA method was used for cluster analysis (Sneath & Sokal, 1973). The x-axis shows the correlation between strains. *S: Sinorhizobium*; *R: Rhizobium*; *Ag: Agrobacterium*, *A: Allorhizobium*; *M: Mesorhizobium*, *B: Bradyrhizobium*. LMG strain 6119: *Rhizobium leguminosarum* bv. *trifolii.*

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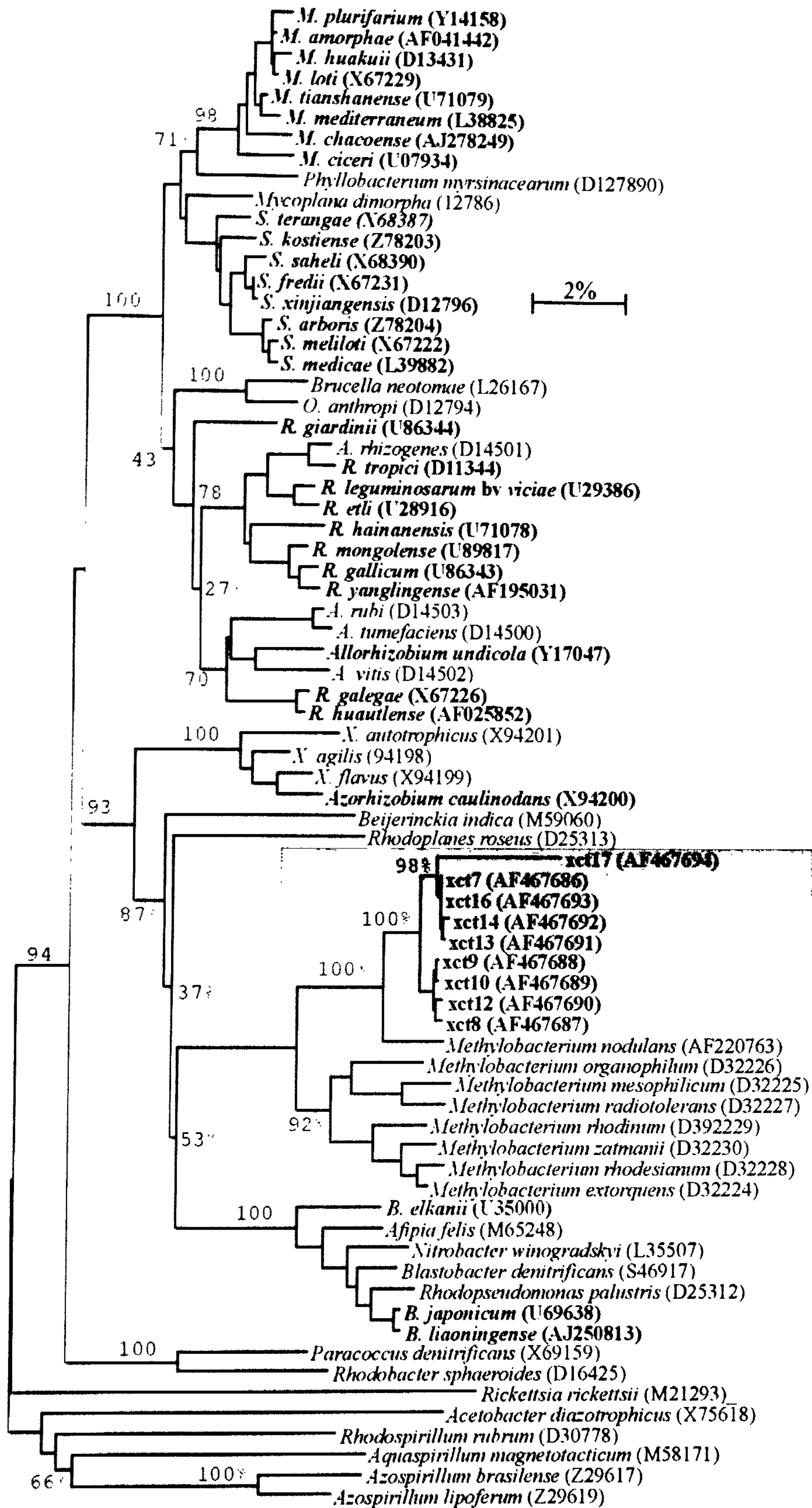

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Figure 7.2 (previous page). Phylogenetic relationships of isolates nodulating *Lotononis bainesii* (shaded box) in comparison to other rhizobial genera and other α-*Proteobacteria*. This analysis was based on comparative sequence analysis of approximately 1200 bp fragment of the 16S rRNA gene. The tree was constructed using the Neighbour-joining method of Saitou & Nei (1987). Horisontal branch lengths reflect the phylogenetic distances, while vertical branch lengths are non-informative and set for clarity only. The scale bar indicates 2% nucleotide difference and bootstrap values of some of the major branching points are shown. GenBank accession numbers are indicated in brackets. *M: Mesorhizobium, S: Sinorhizobium, O: Ochrobactrum, R: Rhizobium, A: Agrobacterium, X: Xanthobacter, B: Bradyrhizobium.*

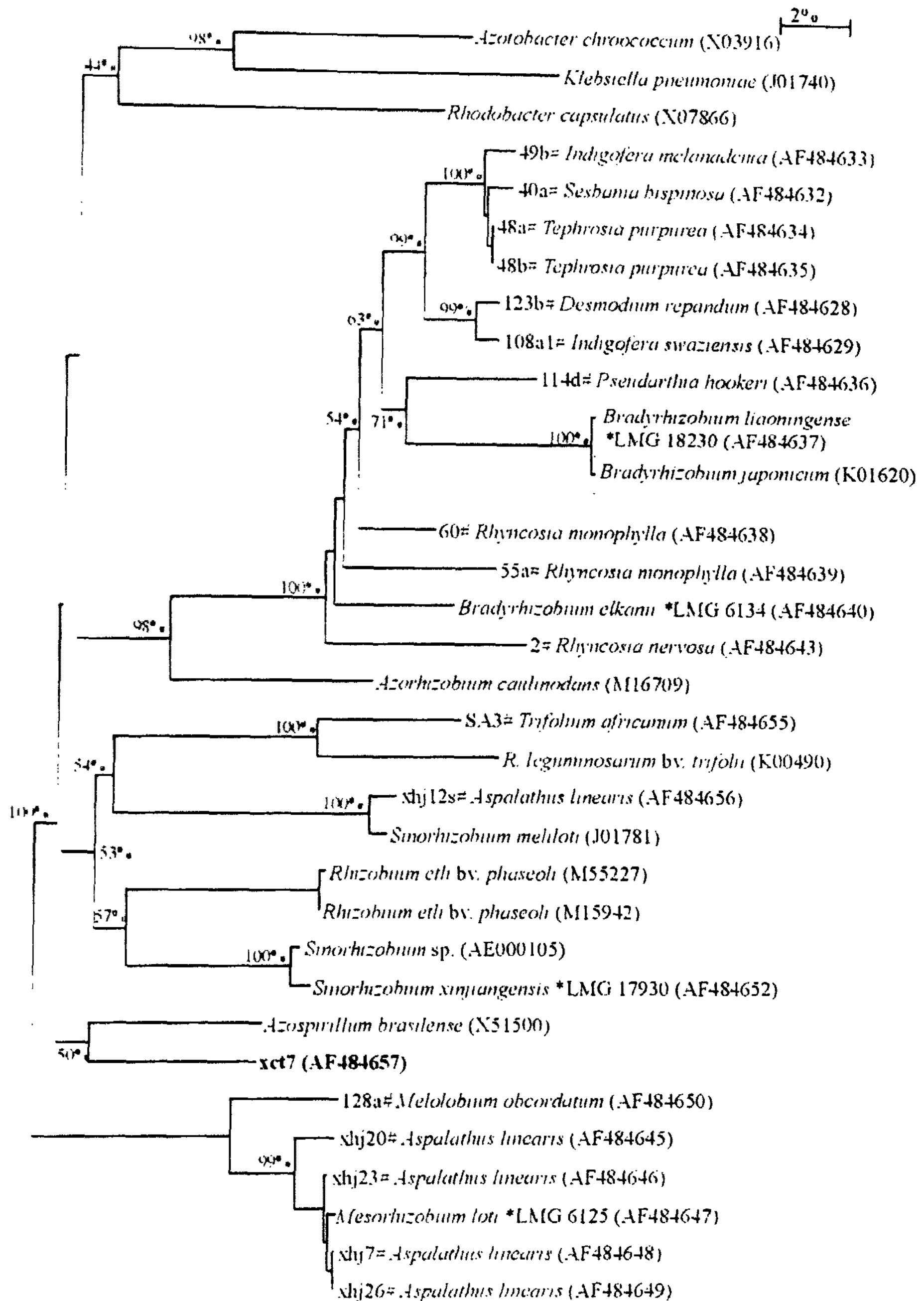

Figure 7.3. Phylogenetic tree, based on a 560 bp fragment of the *nifH* gene, expressing the relationship of *Lotononis bainesii* nodulating strain, xct7, to other rhizobial genera and other nitrogen fixing strains of the alpha- and gamma-*Proteobacteria*. The tree was generated using the Neighbour-joining method of Saitou & Nei (1987). Horisontal branches are equal to the phylogenetic distances of which the scale indicates 2%. Bootstrap values of some of the major branching points are indicated. GenBank accession numbers of the reference strains and indigenous rhizobia are indicated in brackets. The *nifH* sequences of reference strains (marked *) were determined in a related study. Rhizobial isolates (indicated by #) obtained from indigenous legumes in South Africa were included as additional references.

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sequence similarity. However, the common ancestory branching point is supported by only 50% bootstrap confidence value. Therefore more *nifH* sequence data of the genus *Methylobacterium* is needed to establish the true *nifH* phylogeny of the *Lotononis* isolates.

Since *nif* genes are found in many bacteria, besides rhizobia, the *nifH* sequence of xct 7 was compared with a few representatives of the α- *Proteobacteria* which included the known rhizobial genera, *R. capsulatus* and type II methanotrophs: *Methylocystis* and *Methylosinus* species (Auman, *et al.*, 2001). The following γ- *Proteobacteria* were included: *P. stutzeri*, *M. purpuratum*, *V. diazotrophicus* and *K. pneumoniae.* These similarity values are indicated in Table 7.5. Similarity values within the rhizobial genera ranged from 59% to 96%, while the type II methanotrophs share at least 90% sequence similarity. Sequence similarity values of xct7 and the rhizobial genera ranged from 72% to 83%. When comparing the xct7 *nifH* sequence with *M. thrichosporium* (type II methanotroph) and *M. purpuratum* (γ-*Proteobacteria*), similarity values of 84% and 81% were obtained respectively. In contrast, xct7 had a lower similarity value (72%) with *B. japonicum* and *B. liaoningense.*

Table 7.5 Sequence identities, based on a 244 bp fragment of the *nifH* gene of isolate xct7 isolated from *Lotononis bainesii* and other related bacteria from the α- & γ-*Proteobacteria*.
Members of the γ-*Proteobacteria* are shaded. The rest all belong to the α –group.

	1. *Bradyrhizobium japonicum*	2. *Bradyrhizobium liaoningense**	3. *Bradyrhizobium elkanii*	4.. *Azorhizobium caulinodans*	5 *Rhizobium etli* bv. *phaseoli*	6. *Rhizobium leguminosarum*	7. *Mesorhizobium loti*	8. *Methylosinus* sp. LW4	9. *Methylosinus thrichosporium*	10.. *Methylosinus* LW3	11. *Methylocystis* LW5a	12. *Azospirillum brasilense*	13. xct7	14. *Rhodobacter capsulatus*	15. *Pseudomonas stutzeri*	16. *Marichromatum purpuratum*	17. *Vibrio diazothrophicus*	18. *Klebsiella pneumoniae*
1. *B.japonicum* **(K01620)**	100	96	87	77	69	60	66	70	72	72	73	74	72	60	66	68	56	66
2. *B. liaoningense**		100	87	77	69	59	66	70	72	72	73	74	72	59	66	68	56	66
3. *B. elkanii**			100	84	77	65	69	77	80	74	82	80	82	70	74	71	60	70
4. *A. caulinodans* **(M16709)**				100	74	66	73	84	84	78	85	80	81	70	77	75	65	75
5. *R. etli* bv.*phaseoli* **(M15942)**					100	79	76	73	77	74	77	83	80	76	77	75	58	69
6. *R. leguminosarum* **(K00490)**						100	65	59	61	60	62	73	73	68	73	70	56	66
7. *Mesorhizobium loti*							100	70	72	71	74	79	83	73	70	71	57	64
8. *Methylosinus* sp. LW4 **(AF378721)**								100	95	90	90	83	82	73	74	79	60	72
9. *M. thrichosporium* **(AF378724)**									100	89	92	85	84	75	79	81	60	70
10. *Methylosinus* LW3 **(AF378720)**										100	85	81	79	75	74	80	60	70
11. *Methylocystis* LW5 **(AF378719)**											100	83	83	74	78	77	64	71
12. *Azospirillum brasilense* **(X51500)**												100	90	84	83	81	57	72
13. xct7													100	76	80	81	55	68
14. *Rhodobacter capsulatus* **(X07866)**														100	73	71	58	71
15. *Pseudomonas stutzeri* **(AJ297529)**															100	87	73	78
16. *M. purpuratum* **(AF059648)**																100	67	79
17. *Vibrio diazothrophicus* **(AF111110)**																	100	76
18. *Klebsiella pneumoniae* **(J1740)**																		100

* Sequence determined in a related study (unpublished results)

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DISCUSSION

Rhizobia described thus far belong to three distinct 16S rDNA-based phylogenetic branches within the α- subclass of the *Proteobacteria*. The first main branch comprises *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium* and the plant pathogen *Agrobacterium*. The second branch contains the genus *Bradyrhizobium*, while the third branch contains the stem nodulating *Azorhizobium*. Recently, the polyphyletic origin of the rhizobia within the α-*Proteobacteria* was yet again confirmed when Sy *et al.* (2001) described nodulating *Methylobacterium*, recovered from the root nodules of *Crotalaria* spp.. The genus *Methylobacterium* (Patt, *et al.*, 1976) is a group of strictly aerobic, facultatively methylotrophic, gram-negative bacteria and are usually pink to red due to the presence of carotenoids. They are distributed in a wide range of natural habitats, including soil, dust, air, fresh water and aquatic sediment (Hiraishi, *et al.*, 1995). Regardless of their ubiquitous nature in soil and water reservoirs, the recent description of *Methylobacterium nodulans* provided the first evidence of symbiotic nature of these microorganisms. However, *M. nodulans* did not exhibit the characteristic pink pigment of the methylobacteria.

In South Africa almost 100 different genera of leguminous plants are found, growing under diverse geographical and climatological conditions (Strijdom, 1998). In this study isolates obtained from the root nodules of *Lotononis bainesii* were characterised in terms of phenotypic features as well as 16S rDNA and *nifH* phylogeny. These symbionts were pink-pigmented and 16S rDNA sequencing proved them to be closely related to *M. nodulans*. Their methylotrophic nature was indicated by growth on medium 72 (results not shown), with methanol as sole carbon source. However, the maximum methanol tolerance values were not determined. Methylotrophy is not a common trait among rhizobia since none of the rhizobial reference strains were capable of utilising methanol.

Nitrogen fixing genes (*nif* genes) are found in many bacteria besides rhizobia (Haukka *et al.*, 1998). Although it has been reported that the *nifH* phylogeny closely resembles that of the 16S rRNA gene (Hennecke, *et al.*, 1985; Ueda, *et al.*, 1995), a report by Eardly *et al.* (1992) presented evidence of phylogenetic discordance that could be due to the lateral transfer of *nif* genes. In this report it was difficult to determine the exact phylogenetic position of xct 7 based on *nifH* sequence due to a lack of corresponding sequence data for the

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Methylobacterium genus. However, the *nifH* sequence of xct7 was closely related to that of *Azospirillum brasilense*.

Recently, Moulin, *et al.* (2001) described the isolation of a nodulating *Burkholderia* sp. (β-subclass of *Proteobacteria*) from the legume *Aspalathus carnosa*. This finding showed the range of bacteria able to nodulate legumes is more widespread than previously anticipated. Two novel features (nodulation by β-*Proteobacteria* and methylotrophy) of legume symbiosis are now known. It is however, interesting to note that the plant genera (*Crotalaria, Aspalathus* and *Lotononis*) involved have the same tribal affiliation (Tribe Crotalarieae). Further investigations of the symbionts associated with plant species within this tribe and other uninvestigated legumes are therefore warranted. It should now be evident that our understanding of the true bacterial diversity involved in legume symbiosis is very limited and can only increase as more host species are investigated.

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CHAPTER 8

Polyphasic evaluation of a selection of the indigenous rhizobia

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INTRODUCTION

Polyphasic taxonomy has become a widely applied approach in bacterial classification and has led to numerous revisions of the stem- and root nodulating bacteria (van Berkum & Eardly, 1998). Within such an approach, various traits of the organism are considered in an effort to generate a consensus taxonomy (Colwell, 1970; Murray *et al.*, 1990; Vandamme *et al.*, 1996). Polyphasic taxonomy therefore aims to prevent too much emphasis being placed on one method, which may lead to possible erroneous conclusions of generic relationships. This is amply illustrated by the report of Young *et al.* (2001), which indicated that results obtained by 16S rDNA sequence analyses depended on the chosen algorithm and the selection of the included sequences. Based on these observations and other phenotypic data, Young *et al.* (2001) subsequently proposed the amalgamation of the genera *Agrobacterium, Allorhizobium* and *Rhizobium* into a single genus, *Rhizobium.*

An extensive rhizobial collection has been established by the previous investigations of Dagutat (1995) and Kruger (1998). These rhizobial isolates were characterised phenotypically by comparison of whole cell protein profiles and their ability to utilise different carbon sources. In this study, the genotypic variation of a selection of the indigenous isolates was investigated. From these results it was clear that the indigenous rhizobial microflora of South Africa comprised a heterogeneous group related to the genera *Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium* and *Methylobacterium.* The generic status of some isolates, however, remained uncertain. It should be evident that a substantial amount of information has accumulated on the diversity of the indigenous rhizobia. The aim of this section is therefore to briefly summarise results obtained thus far. Genera such as *Rhizobium* and *Sinorhizobium*, who were represented by only a few isolates, will not be discussed. Furthermore, only isolates for which the full range of both phenotypic and phylogenetic data is available will be discussed. All discussions on 16S rDNA and IGS-RFLP groupings refer to those presented in Figures 5.1, 5.2, 5.4 & 7.2 in Chapters 5 & 7.

The mesorhizobia

Except for one isolate (xhj12s), all isolates obtained from *Aspalathus linearis* and *Aspalathus cordata* formed the major component of isolates related to the genus *Mesorhizobium* (Fig. 5.1 & 5.2). Numerical analysis based on substrate utilisation patterns as well as whole cell protein profiles showed that these isolates were present in a homogeneous cluster with an

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overall similarity of almost 80%. The overall 16S rDNA sequence similarity (based on a 1300 bp fragment) among the different *Aspalathus* strains ranged from 96.7% to 99.9%. With the exception of xhj12FR, which shared 98% sequence homology with *M. ciceri,* all the isolates were closely related to *M. amorphae, M. plurifarium M. huakuii* and *M. tianshanense.* Considering the clustering obtained by 16S-23S IGS-RFLP more heterogeneity was evident. A comparative study performed by Willems *et al.* (2001c) indicated that sequence analysis of the IGS region sometimes provided taxonomic information similar to DNA-DNA hybridisation for the bradyrhizobia. Although similar evidence for the genus *Mesorhizobium* is still lacking, this is a more variable region that gives better resolution of strains with closely related 16S rDNA sequences. Isolates xhj7 and xhj8 were found to have almost identical IGS-RFLP patterns and near identical protein profiles and substrate utilisation patterns. These isolates were also distinct from all the recognised *Mesorhizobium* species. Based on IGS variation, isolate xhj26 was closely related to the former two, but differed significantly with regard to its whole cell protein profiles.

Isolates xhj15, xhj18 and xhj23 were indistinguishable when considering their IGS-RFLP and Biolog patterns. The isolates also showed near identical protein profiles (correlation of higher than 80%).

In view of the current available data, it appears possible that there are at least two new *Mesorhizobium* species present within this group. The first is represented by two strains, xhj7 and xhj8, and the second by xhj15, xhj18 and xhj23. Since these isolates demonstrated almost identical 16S rDNA sequences, DNA-DNA homology analyses of representatives of these two groups will be necessary to accurately describe their species status. The IGS-RFLP patterns of xhj13, xhj20, xhj27 and xhm5, showed no close association with any mesorhizobial reference strains or any of the other *Aspalathus linearis* isolates. This divergence might be due to minor nucleotide differences, which might have affected the restriction recognition sites, or they in fact are new species. The heterogeneity of these four isolates and one such as xhj26 can only be suitably resolved when more related strains become available and DNA-DNA homologies are determined.

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The bradyrhizobia

The high 16S rDNA sequence similarity among the different bradyrhizobia makes it difficult to adequately describe the relationships within this group. On the basis of 16S rDNA sequences and DNA homology values, *B. elkanii* is clearly a distinct species from *B. japonicum* and *B. liaoningense*. The latter two species are phenotypically distinct, but genotypically closely related (Willems *et al.*, 2001b). The fact that *B. japonicum* is phylogenetically closer related to other genera (such as *Afipia, Agromonas, Blastobacter, Nitrobacter* and *Rhodopseudomonas*) than to *B. elkanii* indicates that the current bradyrhizobial taxonomy is unsatisfactory.

SDS-PAGE of total protein has a limited ability to delineate groups in bradyrhizobia (Doignon-Bourcier *et al.*, 1999). Among the reasons stated for this inability are total protein profiles with fewer bands, variation in band intensities and high background. Moreover generation times of the various bradyrhizobia may vary and the starting material may not be comparable. Grouping of *Bradyrhizobium* obtained from protein profiles should therefore be regarded with reservation. Similarly Kruger (1998) also indicated that the stability of substrate utilisation patterns depended on the standardisation of the length of incubation time. In general the clustering of the indigenous bradyrhizobia by whole cell protein profiles and variation of the 16S rDNA sequences showed little correspondence. In view of the obvious instability of the phenotypic methods in bradyrhizobial taxonomy, only the groupings obtained by 16S rDNA sequencing and 16S-23S IGS-RFLP will be discussed here. Willems *et al.* (2001c) observed that the IGS sequences of *Bradyrhizobium* sometimes give similar taxonomic information as DNA-DNA homology values. Although no IGS sequences are available for the indigenous South African bradyrhizobia, the RFLP patterns of this genomic region will be considered to describe the diversity of this group.

The overall 16S rDNA sequence similarity among the indigenous bradyrhizobial isolates ranged from 94.7 to 100%. Additionally, the indigenous bradyrhizobia also shared high homology to the different bradyrhizobia genospecies as proposed by Lafay & Burdon, (1998) [results not shown]. The variation of the IGS region was, however, suitably able to indicate further variation. Among the 19 indigenous bradyrhizobia, 14 different IGS-RFLP types were observed. These types did not show any correspondence to the IGS-RFLP patterns of the three known *Bradyrhizobium* species. Therefore DNA homology studies should be

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performed and differentiating phenotypic data described for representatives of the 14 IGS-RFLP types.

The methylobacteria

Recently, Sy *et al.* (2001) described a group of root nodulating strains from *Crotalaria* which were shown to belong to the *Methylobacterium* genus. These strains were facultative methylotrophs, an uncommon trait among rhizobia, and represented the fourth rhizobial lineage. The genomic and symbiotic properties of these methylotrophic nodulating symbionts were investigated and subsequently assigned to a new species, *Methylobacterium nodulans* (Sy *et al.*, 2001).

A group of pink-pigmented strains was isolated from the root nodules of *Lotononis bainesii*. An investigation of their whole cell protein profiles showed that these isolates were a homogeneous group (correlation of more than 80%). With the exception of isolate, xct9, these isolates also showed very similar substrate utilisation patterns. However, none of these strains showed any relation to the known rhizobial genera. These phenotypic characterisations were performed before the description of *M. nodulans* and their generic status was only resolved once 16S rDNA sequences analyses (Fig. 7.2) of these isolates were expanded to include that of *M. nodulans* and other methylobacteria. Most of the *Lotononis bainesii* isolates shared at least 99% 16S rDNA sequence homology with *M. nodulans*. Pending DNA homology values, it appears that isolates xct7, xct8, xct9, xct10, xct12, xct13, xct14 and xct16 are *M. nodulans* species. On the other hand, the sequence similarity between isolate xct17 and other *Methylobacterium* species, including *M. nodulans*, was around 95%. Based on this low sequence similarity value, it appears likely that xct17, represents an additional *Methylobacterium* nodulating species.

Our phenotypic characterisations did not include *M. nodulans* since it only became available after our phenotypic analyses were completed. It is therefore impossible to relate phenotypic differences between the *Lotononis bainesii* isolates and *M. nodulans*.

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CONCLUSION

The rapid and reliable identification of strains remains one of the most important tasks in taxonomy. The range of methods to perform this task has been extended significantly by the introduction of molecular techniques. The extensive analyses of the diversity of the indigenous rhizobia, have indicated possible new species within existing rhizobial genera and strains of known species. DNA-DNA hybridisation levels are still considered to be an important criterion for species delineation (Stackebrandt & Goebel, 1994) and therefore representatives of these possible novel South African rhizobial isolates will have to be evaluated by this procedure. DNA homology studies require good quality DNA, which in the case of *Bradyrhizobium* is not always easily obtained. DNA homology determinations currently fall beyond the scope of the laboratory where this work was performed, but the results obtained should provide a suitable basis for selection of isolates for this procedure.

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CHAPTER 9

Concluding Remarks

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The value of the symbiotic relationship between rhizobia and leguminous plants is widely recognised and has led to intensive characterisation of the involved bacterial symbionts. As a consequence, the taxonomy of these bacteria has changed dramatically since Skerman *et al.* published the Approved list of bacterial names in 1980. The most recent revision was suggested by Young *et al.* (2001) who proposed the emendment of the genus *Rhizobium* to include all species of the genus *Agrobacterium* (Conn, 1942) and *Allorhizobium undicola* (de Lajudie *et al.*, 1998a). The description of new genera and species of stem- and root-nodulating bacteria is, however, governed by a set of rules or criteria (Graham *et al.*, 1991). These criteria therefore compel the taxonomist to follow a polyphasic approach in the description of new genera and species and have significantly contributed towards a stable and reliable classification of the rhizobia.

Initial studies (Dagutat, 1995) have shown that a diverse range symbionts are associated with the leguminous plant species occurring in South Africa. Although this study presented a valuable first step towards determining the diversity of the indigenous rhizobia, a single method cannot be relied upon to accurately describe the complex inter- and intragenic relationships of the rhizobia. The aim of this work, which also extends that of Kruger (1998), was therefore to further investigate the diversity of the rhizobia associated with various leguminous plants.

The inferred 16S rRNA phylogeny showed that the indigenous rhizobia were related to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium*. Most of the indigenous rhizobia, related to the *Bradyrhizobium* genus, showed little sequence divergence from the described bradyrhizobial species. However, the 16S rDNA sequence divergence is small among *Bradyrhizobium* strains, offering limited capacity to assess the diversity among closely related strains (Willems *et al.*, 2001a). Subsequently, 16S-23S IGS-RFLP analyses were performed on such homogeneous bradyrhizobial isolates. The same approach was followed for another homogeneous cluster of isolates closely related to the genus *Mesorhizobium*. In both instances the resolution of the 16S rDNA-based phylogeny of these isolates was noticeably improved and therefore analyses of the IGS region is a valuable and complementary tool to be used in combination with 16S rDNA sequencing.

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The diverse nature of the indigenous rhizobia was further indicated by isolates whose generic affiliation remained indeterminate. Furthermore, isolates related to the genus *Methylobacterium* was also identified among the indigenous rhizobia. This was in agreement with a recent report of nodulating methylobacteria (Sy *et al.* 2001).

A limited investigation of the symbiotic genomes of the indigenous isolates focussed on partial *nifH* sequencing and RFLP of the *nodC* gene. The *nifH* phylogeny agreed with the 16S rDNA-based phylogeny. Conversely, only a few of the *nodC* genotypes showed a strong host-specific association. Our inability to define the other *nodC* genotypes may be attributed to insufficient representation in the number of the different legume hosts and the robustness of the RFLP method. Future investigations should therefore include more representatives of the various host plants and sequencing of the *nodC* genes.

The bacterial symbionts of a large number of indigenous legumes have not been studied systematically. The isolation of such symbionts is a promising strategy to increase our knowledge of the systematics and evolution of the rhizobia. Moreover, many of these unexamined rhizobia may have the ability to establish symbiotic relationships with legumes that are of economic and ecological importance. This can be achieved effectively only if knowledge regarding the diversity of the indigenous rhizobial population is known. This study has contributed significantly in this regard. In compliance with the minimal standards for description of the rhizobial symbionts, both phenotypic and phylogenetic traits which include the work of Dagutat (1995) and Kruger (1998) have been investigated for most of these strains. The requirement of DNA homology analyses is, however, still lacking and should be investigated.

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CHAPTER 10

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APPENDIX A

An abridged taxonomic outline of the α-*Proteobacteria* as described in the second edition of Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2001). Detail is provided only for the families where rhizobia are relevant. Outline may be obtained from website: http://www.cme.msu.edu/Bergeys/

Phylum BXII. *Proteobacteria*
- Class I: *Alphaproteobacteria*
  - Order I: *Rhodospirillales*
    - *Family I:* *Rhodospirillaceae*
    - *Family II:* *Acetobacteraceae*
  - Order II: *Ricketsiales*
    - *Family I:* *Ricketsiaceae*
    - *Family II:* *Ehrlichiceae*
    - *Family III:* *Holosporaceae*
  - Order III: *Rhodobacterales*
    - *Family I:* *Rhodobacteraceae*
  - Order IV: *Sphingomonadales*
    - *Family I:* *Sphingomonadaceae*
  - Order V: *Caulobacterales*
    - *Family I:* *Caulobacteraceae*
  - Order VI: ***Rhizobiales***
    - *Family I:* ***Rhizobiaceae***
      - *Genus I:* ***Rhizobium***
      - *Genus II:* ***Agrobacterium***
      - *Genus III:* *Carbophilus*
      - *Genus IV:* *Chelatobacter*
      - *Genus V:* *Ensifer*
      - *Genus VI:* ***Sinorhizobium***
    - ***Family II:*** ***Bartonellaceae***
    - ***Familiy III:*** ***Brucellaceae***
    - ***Family IV:*** ***Phyllobacteriaceae***
      - *Genus I:* *Phyllobacterium*
      - *Genus II:* ***Allorhizobium***
      - *Genus III:* *Aminobacter*
      - *Genus IV:* *Aquamicrobium*
      - *Genus V:* *Defluvibacter*
      - *Genus VI:* ***Mesorhizobium***
      - *Genus VII:* *Pseudaminobacter*
    - *Family V:* *Methylocystaceae*
    - *Family VI:* *Beijerinckiaceae*
    - *Family VII:* ***Bradyrhizobiaceae***
      - *Genus I:* ***Bradyrhizobium***
      - *Genus II:* *Afipia*
      - *Genus III:* *Agromonas*
      - *Genus IV:* *Blastobacter*
      - *Genus V:* *Bosea*
      - *Genus VI:* *Nitrobacter*
      - *Genus VII:* *Oligotropha*
      - *Genus VIII:* *Rhodopseudomonas*
    - *Family VIII:* ***Hyphomicrobiaceae***
      - *Genus I:* *Hyphomicrobium*
      - *Genus II:* *Ancalomicrobium*
      - *Genus III:* *Ancylobacter*
      - *Genus IV:* *Angulomicrobium*
      - *Genus V:* *Aquabacter*
      - *Genus VI:* ***Azorhizobium***
      - Including 14 other genera
    - *Family IX:* *Methylobacteriaceae*
      - *Genus I:* *Methylobacterium*
      - Including 2 other genera
    - *Family X:* *Rhodobiaceae*
      - *Genus I:* *Rhodobium*

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APPENDIX B

Graphical representation of the 95 different carbon sources as supplied in the Gram negative Biolog plate.

A1 water	A2 α-cyclodextrin	A3 dextrin	A4 glycogen	A5 tween 40	A6 tween 80	A7 N-acetyl-D-galactosamine	A8 N-acetyl-D-glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 i-erythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentiobiose	B6 α-D-glucose	B7 m-inositol	B8 α-D-lactose	B9 lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-methyl D-glucoside	C3 D-psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turanose	C10 xylitol	C11 methyl pyruvate	C12 mono-methyl succinate
D1 acetic acid	D2 cis-aconitic acid	D3 citric acid	D4 formic acid	D5 D-galactonic acid lactone	D6 D-galacturonic acid	D7 D-gluconic acid	D8 D-glucosaminic acid	D9 D-glucuronic acid	D10 α-hydroxybutyric acid	D11 β-hydroxybutyric acid	D12 γ-hydroxybutyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutaric acid	E5 α-keto valeric acid	E6 D,L-lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D-saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucuronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl-glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl-L-aspartic acid	F12 glycyl-L-glutamic acid
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenylalanine	G6 L-proline	G7 L-pyroglutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 D,L-carnitine	G12 γ-amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3-butanediol	H9 glycerol	H10 D,L-α-glycerol phosphate	H11 glucose-1-phosphate	H12 glucose-6-phosphate

APPENDIX C

Medium 72:

K_2HPO_4	1.2 g
KH_2PO_4	0.62 g
$CaCl_2.2H_2O$	34 mg
$MgSO4.7H_2O$	0.2 g
NaCl	0.1 g
$FeCl_3.6H_2O$	1 mg
$(NH4)_2SO_4$	0.5 g
Trace element solution	1 ml
Agar	15 g
Distilled water up to	1 L
PH	7.0

Trace element solution

$CuSO_4. 5H_2O$	5 mg
$MnSO_4. H_2O$	7 mg
$Na_2MoO_4. 2H_2O$	10 mg
H_3BO_3	10 mg
$ZnSO_4.7H_2O$	70 mg
$CoCl_2.6H_2O$	5 mg

After sterilisation cool down and aseptically add 10 ml filter-sterilised methanol per liter medium