CHAPTER 5

CHAPTER 5
DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

CHAPTER 6

CHAPTER 6
CONCLUSIONS

- More than one rhizobium strain can nodulate the same host plant.
- Rhizobia from different taxonomic groups can nodulate the same legume genus.
- Isolates from nodules of supposedly non-nodulating legume genera are members of the genus *Bradyrhizobium*.
- Supposedly non-nodulating legume genera should be systematically investigated to determine their nodulation status.
- The partial sequencing approach supported the separation of the stem- and root-nodulating bacteria into six genera.
- No conclusions about the evolution of the rhizobia included in this study could be drawn from the trees obtained using the partial sequencing approach.
- The two different partial approach methods are equally suited for the identification and characterisation of the isolates.
- Most of the indigenous isolates examined are slow-growers belonging to the genus *Bradyrhizobium*.
- Some of the indigenous isolates belong to the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*.
- The sequencing results corroborate results obtained by other workers as part of the polyphasic approach to identify and determine the diversity of indigenous rhizobia.

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

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

CHAPTER 7

CHAPTER 7
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