UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DAGUTAT, HELGA

IDENTITY AND TAXONOMY OF INDIGENOUS SOUTH AFRICAN RHIZOBIA

PhD **UP** **1995**

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

IDENTITY AND TAXONOMY OF INDIGENOUS SOUTH AFRICAN RHIZOBIA

by

HELGA DAGUTAT

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy
PhD (Microbiology)
in the Department of
Microbiology and Plant Pathology,
Faculty of Biological and Agricultural Sciences,
at the University of Pretoria,
Pretoria, Republic of South Africa

Promoter: Prof. Dr. P L Steyn
1995

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

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

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

finis coronat opus

Aan my dierbare ouers

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

INDEX

Summary viii

Opsomming ix

CHAPTER 1 : Introduction 1

CHAPTER 2 : The isolation of nodulated leguminous species, from the three subfamilies Mimosoideae, Caesalpinioideae and Papilionoideae from South African soil 7

CHAPTER 3 : Characterization and diversity among strains of rhizobia indigenous to South African soils: isolated from a wide variety of Fabaceae spread through different geographical areas 24

CHAPTER 4 : The South African context 69

CHAPTER 5 : Conclusions 72

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

IDENTITY AND TAXONOMY OF INDIGENOUS SOUTH AFRICAN RHIZOBIA

by

HELGA DAGUTAT

PROMOTER: Prof. P.L. Steyn

DEPARTMENT: Microbiology and Plant Pathology

DEGREE: PhD (Microbiology)

SUMMARY

A phenotypic-molecular method (analysis of SDS-PAGE of whole-cell proteins) was used to investigate the taxonomic position of 346 new rhizobia isolates from 147 legumes, mainly indigenous species, from geographically separate localities in South Africa. No comprehensive study of the indigenous South African rhizobia has been done before, despite the richness of indigenous legume species and their possible practical importance in intensive as well as extensive, sustainable agriculture. Our isolates were compared with 45 authentic rhizobial cultures. The resulting protein profiles were analyzed by the GelCompar 3.0 programme and a dendrogram constructed. These organisms aggregated into 24 distinct clusters, 10 of which do not contain any reference strains. The results in this study show that the genus *Rhizobium* and related genera are very heterogeneous. A large group of the isolates clustered with members of the recognised genera *Rhizobium, Bradyrhizobium* and

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

Sinorhizobium. However, we also found differences from published taxonomic data, as well as possibly new taxa. Whereas *R. tropici* and *R. etli* are reportedly nodulating only species of *Phaseolus* and *Leucaena*, and *Phaseolus* respectively, we found that *R. tropici* revealed a strong resemblance to isolates from *Bolusanthus* and *Spartium,* and *R. etli* to strains from *Desmodium*, *Melolobium*, *Indigofera* and two tree legumes *Acacia melanoxylon* and *Chamaecrista stricta.* Members isolated from the tree legumes formed an assemblage of heterogeneous isolates and a network of lines and groups of various relationships. It seems obvious from the results that any comprehensive taxonomic exploration will result in a proliferation of new taxa. Genetic studies are needed to further clarify the taxonomic and phylogenetic position of the new isolates and recognised genera of rhizobia.

Rhizobium were isolated from hitherto unconfirmed nodulated legume genera *Cassia, Bauhinia* and *Schizolobium.* Indigenous counterparts have been found for each of the 9 rhizobia strains used for commercial legume inoculant production in South Africa.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

IDENTIFISERING EN TAKSONOMIE VAN INHEEMSE RHIZOBIUMS IN SUIDER-AFRIKA

deur

HELGA DAGUTAT

PROMOTOR: Prof. P.L. Steyn
DEPARTEMENT: Mikrobiologie en Plantpatologie
GRAAD: PhD (Mikrobiologie)

OPSOMMING

Die doel van die studie was om soveel as moontlik inheemse rhizobiums te isoleer, hul identitieit te bepaal met behulp van SDS-PAGE van heelsel proteïne en indien moontlik, 'n taksonomie te konstrueer. 147 verskillende peulplante van diverse geografiese streke is versamel waaruit 346 verskillende kulture uit die nodules geïsoleer is. Geen omvattende studie van inheemse rhizobiums is tot dusver gedoen nie ten spyte van die rykdom inheemse peulplantspesies in Suider-Afrika en hul moontlike praktiese belang in intensiewe en ekstensiewe landbou. Die nuwe isolate is vergelyk met 45 outentieke rhizobium stamme. Vir die ontleding van proteïen profiele is gebruik gemaak van die Gelcompar 3.0 program waardeur 'n dendrogram saamgestel is. Die diversiteit van die rhizobiums is duidelik te sien in die groeperings in 24 verskillende bondels waarvan 10 geen verwysingstamme bevat nie. Die resultate toon dat die genus *Rhizobiums* en verwante genusse baie uiteenlopend is. 'n Groot groep van die isolate het saam met lede van die erkende genusse *Rhizobium, Bradyrhizobium* en *Sinorhizobium* gebondel. Verskille is ook gevind van gepubliseerde

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

taksonomiese data asook moontlike nuwe taksons. Volgens gepubliseerde bevindings noduleer *R. tropici* en *R. etli* slegs spesies van *Phaseolus* en *Leucaena,* en *Phaseolus* onderskeidelik. Daarenteen is gevind dat *R. tropici* sterk ooreenkoms toon met isolate uit *Bolusanthus* en *Spartium,* en *R. etli* met die uit *Desmodium, Melolobium, Indigofera* en twee boompeulplante *Acacia melanoxylon* en *Chamaecrista stricta.* Die boompeulplant-isolate het 'n heterogene versameling gevorm met 'n netwerk van lyne en groepe met verskeie verwantskappe. Dit is duidelik dat enige omvattende taksonomiese ondersoek sal uitloop op 'n toename in nuwe taksons. Genetiese studies is nodig vir die verdere opheldering van die taksonomiese en filogenetiese posisie van die nuwe isolate en erkende rhizobium-genusse.

Rhizobiums is geïsoleer uit die genusse *Cassia, Schizolobium* en *Bauhinia* wat voorheen as nie-nodulerend gereken is. Inheemse ekwivalente is gevind vir elk van die 9 rhizobium rasse wat vir kommersiële peulplantentstofproduksie in Suid Afrika gebruik word.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 1

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

INTRODUCTION

Greek and Roman writers described an agricultural system which involved leguminous plants to improve the condition of the soil (1). However, an important synapomorphy (common character) of rhizobia was first documented in the 1880s, namely that nitrogen assimilating bacteria grow in nodules on roots of legumes. This has subsequently led to the most salient symbiotic nitrogen fixation system in agriculture. Considerable nitrogen can be added to soils through fixation brought about by rhizobia. The influence of inoculation upon crop yield and the amount of nitrogen fixed by rhizobia in South Africa is particularly striking. Normally about 100 kg nitrogen (N) is fixed for each 3 000 kg dry mass plant material produced. A fixation magnitude in South Africa of 95 600 t of N per year was reported by Wasserman and Strijdom (22). Most of the plants infected by rhizobia belong to the family Fabaceae, although one non-legume species, *Parasponia andersonii*, is known to develop nodules and the bacteria existing in it (*Rhizobium* strain ANU 289) are proven nitrogen fixers (19). Some legume species belonging to the genera *Neptunia, Aeschynomene* and *Sesbania* form stem as well as root nodules, both resulting in nitrogen fixation. In the case of *Aeschynomene* the bacteria are able to produce the photosynthetic pigment bacteriochlorophyl *a* (Bchl *a*) and appear to harvest light energy resulting in a unique energy economising physiological pathway of nitrogen fixation (7). *Azorhizobium caulinodans*, the stem and root-nodulating organism isolated from *Sesbania rostrata*, represents a single species that can fix atmospheric nitrogen both symbiotically and *ex planta* (16). The rhizobia that nodulate the roots of *S. rostrata* only, do not fix nitrogen in culture (6). Stem nodules are of great significance because few environmental factors play a role to discourage nodulation, whereas soil conditions (aeration, drainage, moisture, pH, the amount of active calcium and available nitrogen in the soil) largely contribute to the failure of root nodule bacteria and thereby reduce fixation. The potential of using stem nodules is approaching a renaissance which has attracted much interest in agriculturally important legumes as a possible alternative method of inoculation, offering exciting improvements to symbiotic nitrogen fixation.

The importance of the role of rhizobia in ecology as well as in bacterial taxonomy has been met with increased interest all over the world, and is being further disseminated by large numbers of publications. Since the beginning of 1994, 523 articles on various aspect of

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

rhizobia have been published. There is unanimity that the taxonomy of rhizobia is being hampered by inadequate exploration of a wide variety of legumes because of an understandable emphasis on agriculturally important plants, and, since only 15% of the 16 000 to 19 000 species of the family Fabaceae has been examined for the nodulation and characterization of rhizobia, the taxonomy is still incomplete. Any study of a large assemblage of strains isolated from a wide variety of legumes will eventually result in the emergence of several new groups. Even though in recent years more interest has been elicited in expanding the examination of legume hosts and characterising the nodulating rhizobia, but much more encouragement is needed for advancing this concept. The taxonomy of rhizobia has previously been based solely on the type of host plant from which the strains were isolated, whereas the potential of a given strain to effectuate nodules on certain plants and not others, tended to be specific. With the incorporation of phylogenetic classification, an increased number of new species and genera as well as the revision of several groups have since been uncovered. Within the genus *Rhizobium* the following three species were described by Jordan (1984): *Rhizobium meliloti* (*Medicago, Melilotus, Trigonella*), *Rhizobium loti* (*Lotus* spp.) and *Rhizobium leguminosarum* containing three biovars namely, biovar *viciae* (*Pisum, Vicia*), biovar *trifolii* (*Trifolium*) and biovar *phaseoli* (*Phaseolus*). *R. meliloti* has been reassigned to the genus *Sinorhizobium* as *S. meliloti* (1994): (5). In addition, the following six species have been created: *Rhizobium galegae* (1989, *Galega officinalis*, *Galega orientalis* (17)); *Rhizobium huakuii* (1991, *Astragalus sinicus* (2)); *Rhizobium tropici* llA and llB (1991, biovar phaseoli type ll strains, *Leucaena* spp. (18)); *Rhizobium etli* (1993, biovar phaseoli type 1 strains (21)); *Rhizobium tianshanense* (1995, soil in Xinjiang region of People's Republic of China (3)), and *Rhizobium fredii* (1984, from fast growing soybean-nodulating strains (20)). *R. fredii* has been reassigned to the genus *Sinorhizobium* (1988, (4)). The genus *Sinorhizobium* has been expanded to five species: *S. meliloti* and *S. fredii*, as already mentioned, *Sinorhizobium xinjiangensis* (1988, fast-growing soybean strains obtained from Xinjiang region in the People's Republic of China (4)); *Sinorhizobium teranga* (1994, *Sesbania* spp., *Acacia* spp., *Leucaena leucocephala*, and *Neptunia oleracea*), and *Sinorhizobium saheli* (1994, *Sesbania* spp., *Acacia seyal*, *Leucaena leucocephala*, and *N. oleracea*) (5). The genus *Bradyrhizobium* consists of two species, *Bradyrhizobium japonicum* (1984, *Glycine max* (14)), and *Bradyrhizobium elkanii* (1992 *Glycine max* (15)) and includes

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

all the slow-growing rhizobia. The genus *Azorhizobium* consists of one species: *Azorhizobium caulinodans* (1988, *Sesbania rostrata* (6)).

With its different geographical areas South Africa is undeniably a rich source of a wide variety of legumes. Grobbelaar (8, 9, 10, 11, 12, 13), published descriptions of approximately 960 nodulated legumes in South Africa but he neither isolated nor characteriz ed the bacteria. Consequently, there has been a paucity of information concerning the identification and characterization of rhizobia in South Africa.

The intention of this study was to develop the rhizobia taxonomy with special emphasis on indigenous South African rhizobia. The basis needed for a future comprehensive taxonomic classification prompted efforts to explore as many nodulating rhizobia from different legumes as possible. This is imperative for the vindication of stability and coherency in rhizobia taxonomy. For the identification and characterization of the large collection of rhizobia we made use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total soluble whole-cell proteins to construct a database that is essential for the future rational development of rhizobia taxonomy in South Africa. The resulting observations not only provide substantial evidence of the diversity that exists in the relationships between rhizobia but also broaden the knowledge of the legume host range that is nodulated by specific rhizobia species. The information also calls into question a rhizobia taxonomy that has been dependent explicitly on legume host plants and phenotypic data, and strengthens the impact of a polyphasic approach. The search to expand the scope of rhizobia taxonomy by embracing a combination of legumes such as trees, annuals and perennials, shrubs and vines while not concentrating on specific hosts only, has resulted in a network of relationships. New members isolated from the same legume species have been found to belong to different phenotypic-molecular lineages and reflect no systematical grouping according to their legume host, indicating that the root nodules of the same legume species are occupied by emulous, different rhizobia. However, attention should be paid to the fact that up to four different rhizobia were isolated from the same root nodule. This needs further research with respect to the ability to fix nitrogen effectively. Rhizobia isolated from the tree legumes were intermingled with the rest of the isolates and could not be differentiated as a separate entity.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Many of the new isolates remain dubious and further genetic research is necessary to clarify their taxonomic position.

Chapters 2 and 3 are presented in the form of articles ready for submission to Plant and Soil and Applied and Environmental Microbiology respectively. The style of each chapter has been adapted accordingly.

REFERENCES

1. **Brady, N. C.** 1974. The nature and properties of soil *8th Edition.* p. 1-6. Macmillan publishing Co., Inc. New York.
2. **Chen, W. X., G. S. Li, Y. L. Qi, E. T. Wang, H. L. Yuan, and J. L. Li.** 1991. *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus.* Int. J Syst. Bacteriol. **41:**275-280.
3. **Chen, W., E. Wang, S. Wang, Y. Li, X. Chen, and Y. Li.** 1995. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from and arid saline environment in Xinjiang, People's Republic of China. Int. J. Syst. Bacteriol. **45:**153-159.
4. **Chen, W. X., G. H. Yan, and J. L. Li.** 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. **38:**392-397.
5. **De Lajudie, P., A. Willems, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M. D. Collins, B. Dreyfus, K. Kersters, and M. Gillis.** 1994. Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Bacteriol. **44:**715-733.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

6. **Dreyfus, B., J. L. Garcia, and M. Gillis.** 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bactriol. **38:**89-98.

7. **Eaglesham, A. R. J., J. M. Ellis, W. R. Evans, D. E. Fleischman, M. Hungria, and R. W. F. Hardy.** 1990. The first photosynthetic N_2-fixing *Rhizobium:* characteristics, p. 805-811. *In* P. M. Gresshoff et al. (ed.). Nitrogen fixation: achievements and objectives. Chapman and Hall, Ltd., London.

8. **Grobbelaar, N., and B. Clarke.** 1972. A qualitative study of the nodulating ability of legume species: List 2. Jl S. Afr. Bot. **38:**241-247.

9. **Grobbelaar, N., and B. Clarke.** 1974. A qualitative study of the nodulating ability of legume species: List 4. Agroplantae **6:**59-64.

10. **Grobbelaar, N., and B. Clarke.** 1975. A qualitative study of the nodulating ability of legume species: List 3. Jl S. Afr. Bot. **41:**29-36.

11. **Grobbelaar, N., M. C. Beyma, and C. M. Todd.** 1967. A qualitative study of the nodulating ability of legume species: List 1. Publs. of the Univ. of Pretoria, New Series No. **38:**1-9.

12. **Grobbelaar, N., and M. W. van Rooyen.** 1979. A qualitative study of the nodulating ability of legume species: List 5. Jl S. Afr. Bot. **45:**267-272.

13. **Grobbelaar, N., M. W. van Rooyen, and N. van Rooyen.** 1983. A qualitative study of the nodulating ability of legume species. List 6. S. Afr. J. Bot. **2:**329-332.

14. **Jordan, D. C.** 1984. Family lll: *Rhizobiaceae* Conn 1938, p. 234-244. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's mannual of systematic bacteriology. vol. 1. Williams & Wilkins, Baltimore.

15. **Kuykendall, L. D., B. Saxena, T. E. Devine, and S. E. Udell.** 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal of *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. **38:**501-505.

16. **Ladha, J. K., R. P. Pareek, R. So, and M. Becker.** 1990. Stem-nodule symbiosis and its unusual properties, p. 633-640. *In* P. M. Gresshoff et al. (ed.), Nitrogen fixation: achievements and objectives. Chapman and Hall, Ltd., London.

17. **Lindström K.** 1989. *Rhizobium galegae,* a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. **39:**365-367.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

18. **Martinez-Romero, E., L. Segovia, F. Martins, A. A. Franco, P. Graham, and M. A. Pardo.** 1991. *Rhizobium tropici,* a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41:**417-426.

19. **Price, G.D., S. Mohapatra, and P. M. Gresshoff.** 1984. Structure of nodules formed by rhizobium strain ANU298 in the nonlegume *parasponia* and the legume siratro (*Macroptilium atropurpureum*). Bot. Gaz. **4:**444-451.

20. **Scholla, M. H., and G. H. Elkan.** 1984. *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. Int. J. Syst. Bacteriol. **34:**484-486.

21. **Segovia L., J. P. W. Young, and E. Martinez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type l strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. **43:**374-377.

22. **Wassermann, V. D., B. W. Strijdom.** 1987. Potential nitrogen contribution of legumes to agriculture. Plant Production: 175-177.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 2

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

THE ISOLATION OF NODULATED LEGUMINOUS SPECIES, FROM THE THREE SUBFAMILIES MIMOSOIDEAE, CAESALPINIOIDEAE AND PAPILIONOIDEAE FROM SOUTH AFRICAN SOIL.

Key words: climatic regions, nodulation, Rhizobia, symbiosis, variety legumes

ABSTRACT

Nodulated legumes were collected from various geographic and climatic regions in the Republic of South Africa. Three hundred and forty six isolates were obtained from the nodules and purified. The legumes confirmed for rhizobial infection comprised 14 spp. of the Mimosoideae, mostly *Acacia* spp., 9 spp. of the Caesalpinioideae and 71 spp. of the Papilionoideae. Rhizobia were isolated from *Cassia* spp., *Schizolobium parahybum* and *Bauhinia variegata* (subfamily Caesalpinioidaea) which according to previous reports, are not known to be nodulated. The collection of indigenous rhizobia will be further investigated for their identity, taxonomy and possible application in agriculture.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

INTRODUCTION

The Fabaceae is the third largest flowering plant family and comprises 750 genera and some 16 000 to 19 000 species. Many species are economically important and pulse is, second to the *Poaceae* (grasses, *Zea mays*), considered one of the most important foodstuffs for man and livestock (Allen and Allen, 1981). The Fabaceae is widely distributed and a large number of species occur in South Africa. This is primarily the result of great variation in climate, vegetation and different kinds of soil. A unique complex, symbiotic association between the plant roots and nitrogen-fixing rhizobia species in the soil is characteristic of certain members of the family (Caetano-Anollés and Gresshoff, 1991).

According to Graham et al. (1991), as few as 15% of the species of Fabaceae have been examined for nitrogen-fixing bacteria. We agree with Dupuy et al. (1994) and Vauterin et al. (1990), that for the comprehensiveness of the two genera *Rhizobium* and *Bradyrhizobium* (Jordan, 1982; Jordan, 1984), more isolates of independent strains from a wide variety of legumes plants are needed. The subfamily Papilionoideae with its 505 genera predominates over the members of Mimosoideae (66 genera) and Caesalpinioideae (177 genera). From an agricultural point of view the former is much more important in providing protein food crops, an essential nutritional source for man and animal (Allen and Allen, 1981; Stacey and Upchurch, 1984). The identity and taxonomy of rhizobia in South African soils have not been investigated in depth. Grobbelaar et al. (1964, 1967, 1972, 1974, 1975, 1979, 1983), surveyed and reported on nodulated legumes in South Africa, but did not isolate any bacteria from nodules.

South Africa, with its growing population, depends on plants for food and is becoming a Third World country where fertilizers are quite costly. The nitrogen-fixing system between bacteria and legumes represents an important part of agriculture. In general, the use of legumes as green manures adds nitrogen to the soil. In this paper we report on nodulated legumes collected from various diverse environments from which rhizobia were isolated.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MATERIALS AND METHODS

Environment and Soil Site Description

The field-work extended over 2 500 km of South Africa with a phytogeography of tropical forest, woodland with temperate grassveld, mountain mixed grassveld and trees, mixed savanna with woodland and grass, and true forest (Figure 1). Samples were taken during the summer rainfall period over three consecutive seasons. The main soil types were sandy, stony sandy, clay loam, sandy loam, sandy humic and humic soils.

Plant samples and identification

Plant samples were identified by the Botanical Research Institute, Pretoria.

Isolation of bacteria from veld plants

Excised nodules were surface sterilized with 5% H_2O_2 for 1 to 4 min depending on their sizes and washed four times with sterile water. Duplicate samples of nodules were squashed individually in 200 to 500μl sterile distilled water. The squashed nodule suspensions were poured onto Yeast extract mannitol (YM) agar plates, supplemented with Congo Red (Allen, 1959). Plates were incubated at 25 to 28°C. Development of colonies depended on the individual growth rate of the organisms. As soon as colonies became visible, they were purified by further streaking on the same medium. This process was repeated at least three times or more until pure cultures were obtained. Purified cultures were transferred to YM agar slants (Vincent, 1970). Where sufficient growth was obtained, the cells were washed from the agar surface with a sterile 25% (vol/vol) solution of glycerol in water. 1,5 ml of this was transferred to a sterile cryotube and stored at -70°C.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

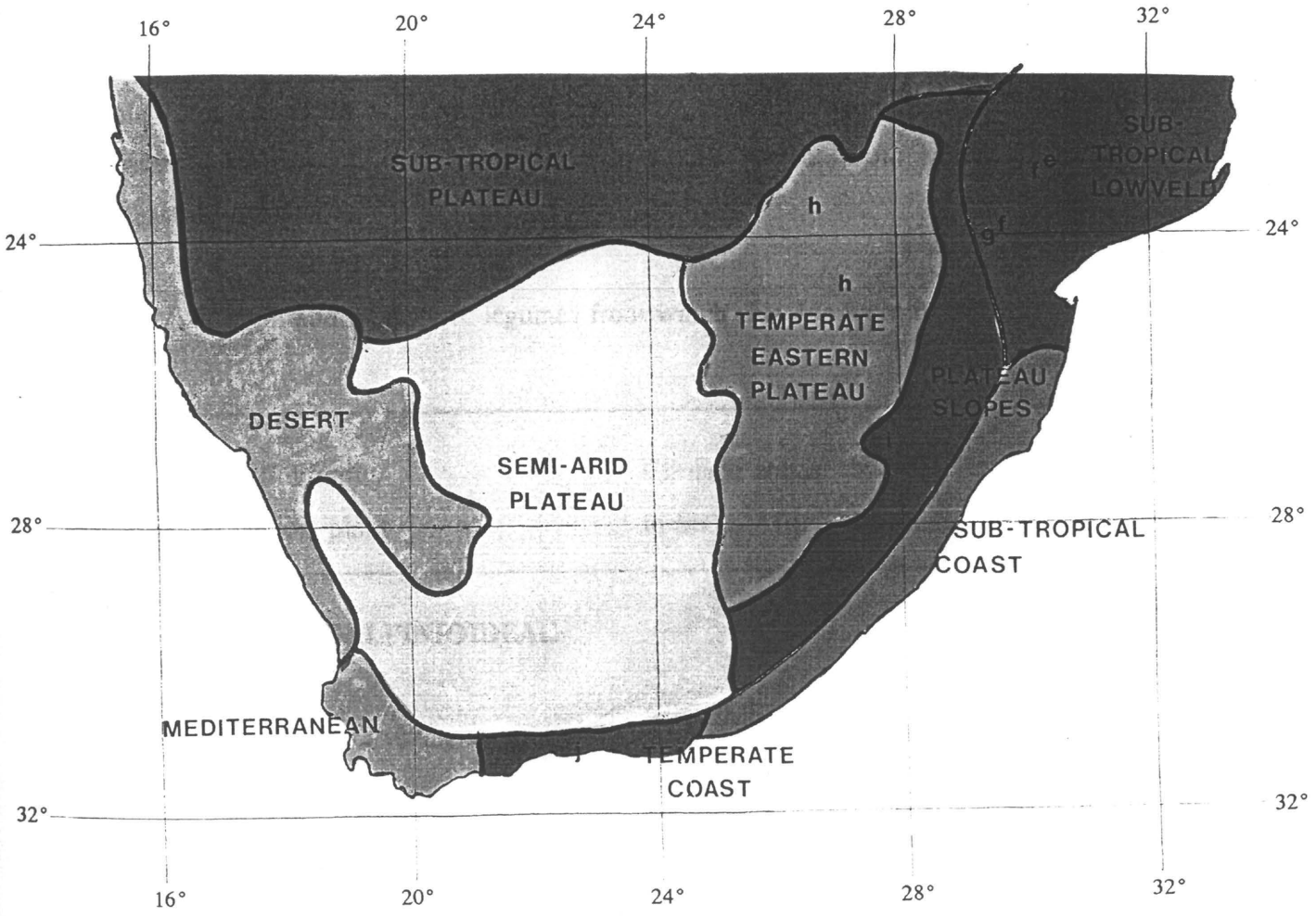


FIG. 1. Climatic regions and natural vegetation of South Africa.
e, Woodland and temperate grassland; **f,** Tropical forest;
g, Sour grassveld; **h,** Mixed savanna, woodlands and grass;
i, Mountain mixed grassveld and trees; **j,** True forest.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

RESULTS

One hundred and forty seven nodulated legumes were collected country wide. Some species occurred in more than one locality, including untouched, virginal environments.

Table 1 represents the wide variety of nodulated legumes obtained from diverse soils and different climatic zones and vegetation in South Africa, from which rhizobia were successfully isolated.

Table 1. Origin and identity of legumes from which rhizobia were isolated.

Plant no of isolation	Subfamily Host plant	Climatic zones in South Africa[a]	Natural Vegetation[b]	Type of soil
	CAESALPINIOIDEAE			
86	*Bauhinia variegata*	A	e	humic
97	*Cassia didymobotrya*	A	e	humic
47	*Cassia floribunda*	A	e	sandy loam
83	*Cassia* sp.	A	e	humic
39	*Chamaecrista abrus*	A	e	sandy
51	*Chamaecrista biensis*	B	h	stony sandy
*102	*Chamaecrista biensis*	A	g	stony sandy
50	*Chamaecrista comosa*	B	h	stony sandy
61	*Chamaecrista comosa*	B	h	stony sandy
73	*Chamaecrista mimosoides*	A	f	clay loam
90	*Chamaecrista mimosoides*	A	e	humic
105	*Chamaecrista stricta*	A	g	stony sandy

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Plant no of isolation	Subfamily Host plant	Climatic zones in South Africa[a]	Natural Vegetation[b]	Type of soil
	MIMOSOIDEAE			
104	*Chamaecrista sp.*	A	g	stony sandy
79	*Acacia caffra*	A	e	sandy
146	*Acacia cyclops*	D	j	sandy loam
54	*Acacia dealbata*	B	h	sandy humic
109	*Acacia dealbata*	A	f	humic
147	*Acacia longifolia*	D	j	sandy loam
145	*Acacia mearnsii*	D	j	sandy loam
119	*Acacia mearnsii*	C	i	stony sandy
138	*Acacia melanoxylon*	D	j	sandy loam
158	*Acacia nigrescens*	A	e	humic
151	*Acacia podalyriaefolia*	D	j	sandy loam
66	*Acacia robusta*	B	h	sandy humic
152	*Acacia saligna*	D	j	sandy loam
46	*A. sieberana* var. *woodii*	A	e	humic
92	*A. sieberana* var. *woodii*	A	e	sandy loam
85	*Acacia xanthophloea*	A	e	humic
130	*Acacia xanthoploea*	A	e	humic
157	*Albizia adianthifolia*	A	e	humic
99	*Albizia adianthifolia*	A	e	humic
53	*Elephantorrhiza obliqua*	B	h	sandy humic
84	*Schizolobium parahybum*	A	e	humic

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Plant no of isolation	Subfamily Host plant	Climatic zones in South Africa[a]	Natural Vegetation[b]	Type of soil
	PAPILIONOIDEAE			
7	*Alysicarpus rugosus*	B	h	stony sandy
29	*Alysicarpus rugosus*	A	e	sandy loam
118	*Argyrolobium pauciflorum*	C	i	stony sandy
21	*Argyrolobium tomentosum*	A	e	sandy loam
98	*Bolusanthus speciosus*	A	e	humic
70	*Crotalaria brachycarpa*	A	f	clay loam
5	*Crotalaria distans*	B	h	stony sandy
100	*Crotalaria doidgeae*	A	g	stony sandy
32	*Crotalaria pallida*	A	e	sandy loam
37	*Crotalaria pallida*	A	e	sandy
38	*Crotalaria pallida*	A	e	sandy
80	*Crotalaria pallida*	A	e	sandy
95	*Crotalaria vasculosa*	A	e	sandy loam
33	*Crotalaria* sp.	A	e	sandy loam
44	*Crotalaria* sp.	A	e	stony
23	*Desmodium repandum*	A	f	clay loam
123	*Desmodium repandum*	C	i	stony sandy
69	*Desmodium setigerum*	A	f	clay loam
107	*Desmodium setigerum*	A	g	sandy loam
15	*Desmodium tortuosum*	A	e	sandy loam
28	*Desmodium tortuosum*	A	e	sandy loam
77	*Desmodium velutinum*	A	e	sandy
131	*Dipogon lignosus*	C	i	sandy loam

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Plant no of isolation	Subfamily Host plant	Climatic zones in South Africa[a]	Natural Vegetation[b]	Type of soil
31	*Eriosema psoraleoides*	A	e	loam humic
67	*Eriosema psoraleoides*	A	f	clay loam
76	*Eriosema psoraleoides*	A	e	sandy
45	*Erythrina lysistemon*	A	e	humic
22	*Flemingia grahamiana*	A	f	clay loam
71	*Flemingia grahamiana*	A	f	clay loam
41	*Indigofera arrecta*	A	e	sandy
74	*Indigofera arrecta*	A	f	clay loam
93	*Indigofera arrecta*	A	e	sandy loam
52	*Indigofera daleoides*	B	h	sandy humic
103	*Indigofera hilaris*	A	g	stony sandy
83	*Indigofera incarnata*	A	e	humic
131t	*Indigofera jucunda*	A	e	humic
49	*Indigofera melanadenia*	B	h	stony sandy
116	*Indigofera oxalidea*	A	g	sandy loam
63	*Indigofera oxytropis*	B	h	stony sandy
12	*Indigofera rhytidocarpa*	B	h	stony sandy
108	*Indigofera swaziensis*	A	g	sandy loam
115	*Indigofera swaziensis*	A	g	sandy loam
125	*Indigofera woodii*	C	i	stony sandy
117	*Indigofera woodii*	C	i	stony sandy
112	*Indigofera* sp.	A	g	sandy loam
129	*Indigofera* sp.	C	i	stony sandy
126	*Lessertia perennans*	C	i	stony sandy
13	*Lotononis bainesii*	B	h	stony sandy
121	*Lotononis* sp.	C	i	stony sandy

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Plant no of isolation	Subfamily Host plant	Climatic zones in South Africa[a]	Natural Vegetation[b]	Type of soil
136	*Lotus hispidus*	D	j	sandy loam
122	*Macrotyloma axillare*	C	i	stony sandy
8	*Medicago lupulina*	B	h	stony sandy
132	*Medicago lupulina*	D	j	sandy loam
149	*Medicago sativa*	D	j	sandy loam
1	*Melilotus alba*	B	h	stony sandy
148	*Melilotus indica*	D	j	sandy loam
128	*Melolobium obcordatum*	D	j	sandy stony
156	*Millettia caffra*	A	e	humic
68	*Mucuna coriacea*	A	f	clay loam
81	*Mucuna coriacea*	A	e	sandy
19	*Neonotonia wightii*	A	e	sandy loam
25	*Neonotonia wightii*	A	f	clay loam
34	*Neonotonia wightii*	A	e	sandy loam
36	*Neonotonia wightii*	A	e	sandy loam
42	*Neonotonia wightii*	A	e	sandy
154	*Otholobium bracteolatum*	D	j	sandy loam
120	*Pisum sativum*	C	i	loam
155	*Podalyria myrtillifolia*	D	j	sandy loam
35	*Pseudarthria hookeri*	A	e	sandy loam
114	*Pseudarthria hookeri*	A	g	sandy loam
143	*Psoralea asarina*	D	j	sandy loam
144	*Psoralea pinnata*	D	j	sandy loam
18	*Pueraria lobata*	A	e	sandy loam
106	*Rhynchosia caribaea*	A	g	stony sandy
87	*Rhynchosia hirta*	A	e	sandy loam

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Plant no of isolation	Subfamily Host plant	Climatic zones in South Africa[a]	Natural Vegetation[b]	Type of soil
96	*Rhynchosia hirta*	A	e	sandy loam
110	*Rhynchosia hirta*	A	f	humic
124	*Rhynchosia minima*	C	i	stony sandy
55	*Rhynchosia monophylla*	B	h	sandy humic
60	*Rhynchosia monophylla*	B	h	stony sandy
2	*Rhynchosia nervosa*	B	h	stony sandy
40	*Sesbania bispinosa*	A	e	sandy
89	*Sesbania punicea*	A	e	sandy loam
113	*Sesbania punicea*	A	g	sandy loam
150	*Spartium junceum*	D	j	sandy loam
59	*Sphenostylis angustifolia*	B	g	stony sandy
26	*Strongylodon macrobotrys*	A	f	till
127	*Sutherlandia frutescens*	C	i	stony sandy
134	*Sutherlandia frutescens*	D	j	sandy loam
142	*Tephrosia capensis*	D	j	sandy loam
75	*Tephrosia glomeruliflora*	A	f	clay loam
62	*Tephrosia multijuga*	B	h	sandy stony
30	*Tephrosia polystachya*	A	e	loam humic
43	*Tephrosia purpurea*	A	e	sandy
64	*Tephrosia purpurea*	B	h	stony sandy
72	*Tephrosia purpurea*	A	e	clay loam
82	*Tephrosia purpurea*	A	e	sandy
48	*Tephrosia purpurea*	B	h	stony sandy
57	*Tephrosia semiglabra*	B	h	sandy humic
58	*Tephrosia semiglabra*	B	h	sandy humic
91	*Tephrosia* sp.	A	e	sandy loam

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Plant no of isolation	Subfamily Host plant	Climatic zones in South Africa[a]	Natural Vegetation[b]	Type of soil
4	*Teramnus labialis*	B	h	sandy stony
27	*Teramnus labialis*	A	e	sandy loam
153	*Trifolium angustifolium*	D	j	sandy loam
141	*Trifolium pratense*	D	j	sandy loam
3	*Trifolium* sp.	B	h	stony sandy
111	*Vicia sativa*	A	f	sandy loam
133	*Vicia* sp	D	j	sandy loam
137	*Vicia* sp	D	j	sandy loam
94	*Vigna subterranea*	A	e	sandy loam
88	*Vigna unguiculata*	A	e	clay loam
6	*Vigna vexillata*	B	h	stony sandy
140	*Virgilia divaricata*	D	j	sandy loam
101	*Zornia capensis*	A	g	stony sandy

[a]**A**, Sub-tropical Lowveld; **B**, Temperate Eastern Plateau; **C**, Plateau Slopes; **D**, Temperate coast.

[b]**e**, Woodland and temperate grassland; **f**, Tropical forest; **g**, Sour grassveld; **h**, Mixed savanna, woodlands and grass; **i**, Mountain mixed grassveld and trees; **j**, True forest.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Normally, only two types of rhizobia can be found within a nodule (Johnston and Beringer, 1975). However, in this study up to four different rhizobia were found colonising the same nodule. Rhizobia were isolated not only from agriculturally important plants such as peas, lupins and clover, but also from leguminous trees as well as a variety of other legumes, some of which are valuable in improving soil and preventing erosion. 346 rhizobium isolates were isolated. Fig. 2 shows transmission electron micrographs of nodules from the indigenous *Millettia caffra,* revealing densely packed intracellular bacteriods.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

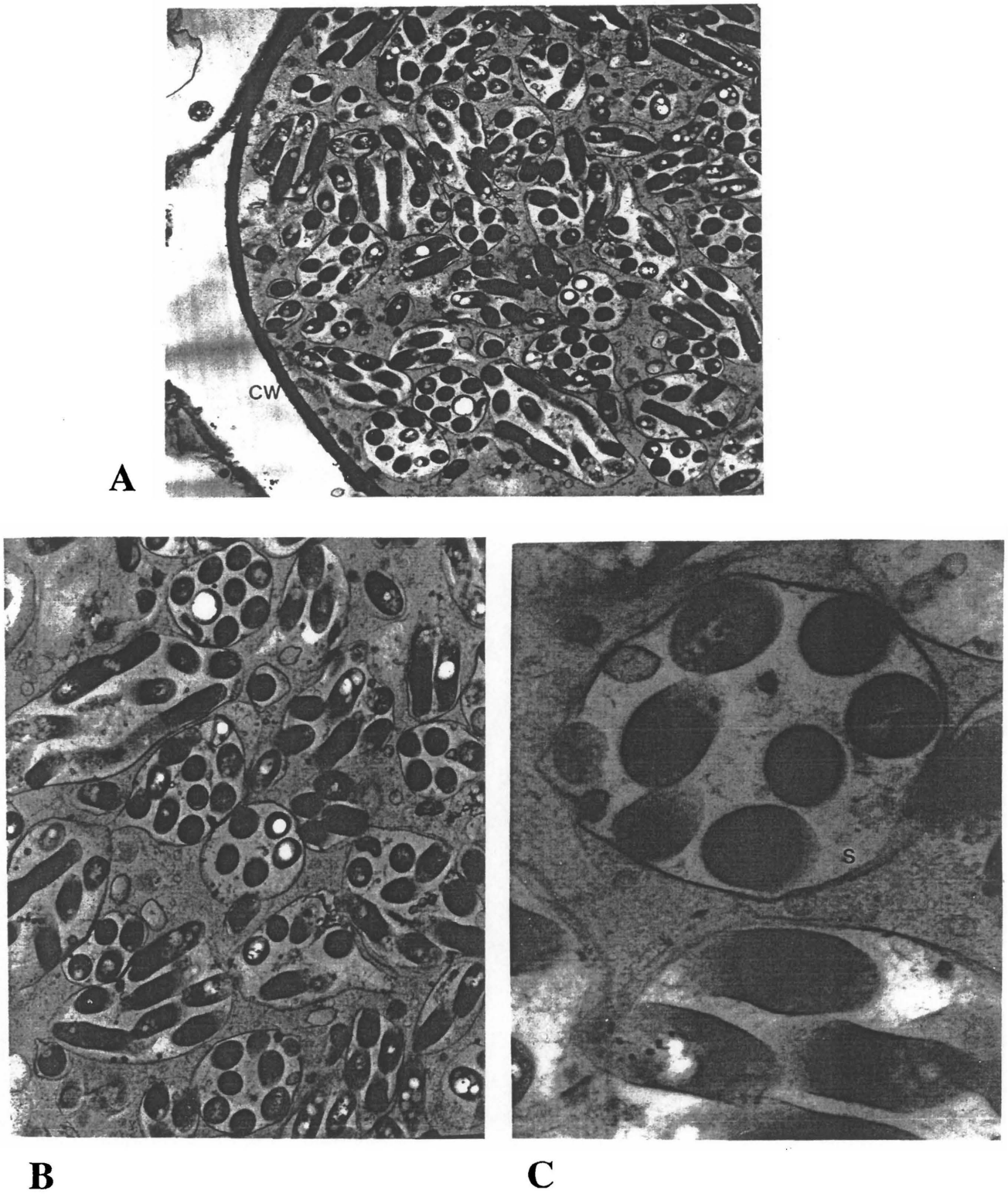


FIG. 2. Appearance of rhizobia in root nodules of the tree legume *Millettia caffra*, indigenous in South Africa.
(A) Electron micrograph of root nodule cross section showing plant cells densely packed with rhizobia. **CW** = cell wall
(B) and **(C)** High magnification of bacteriods. **S** = saccule

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DISCUSSION

Numerous indigenous Fabaceae occur in South Africa, many of which are nodulated, which suggest that the soil maintains populations of indigenous rhizobia. Competition between indigenous rhizobia and effective strains affect the capacity of inoculation, which may lead to the elimination of selected rhizobial strains and thus decreasing crop yield in cultivated legumes (Bromfield et. al, 1986; Dowdle and Bohlool, 1987). For optimal nitrogen fixation, one should attempt to find stronger competitive strains for supernodulation, and from the large variety of rhizobia in this study the relative effectiveness of selected strains and isolated indigenous rhizobia can be compared.

As many as four different rhizobia occupying the same nodule were isolated from some legumes. Information of this nature is valuable from an ecological point of view. New opinions might arise concerning the competition between rhizobia strains during infection of legumes. None of the nodules from which rhizobia were isolated showed any sign of greenness, a sign of ineffectivity, though effectivity remains to be determined.

There is the view that no association between rhizobia and *Cassia* sp. exists (Allen and Allen, 1981; Sprent, 1994), but, on the contrary the results of this study show the existence of nodules on the roots of *Cassia* sp.. Rhizobia, together with the plant infection process, are in a certain sense enigmatic. It should be emphasized that we are dealing with an elaborate system, and that the importance of suitable ecological rhizosphere conditions along with the presence of effective rhizobia for infecting the roots of the host plant, (a fragment of the whole network), can only be mentioned here. The same applies to the hypotheses that exist regarding the role of lipopolysaccharides in establishment of an effective symbiosis (Russell et al., 1987(a); Russell et al., 1987(b); De Maagd et al., 1989) and a variety of other factors (Wall and Favelukes, 1991; Thies et al., 1991). Furthermore, nodules were observed on *Schizolobium parahybum* and *Bauhinia* sp.. This has never before been reported. However, there is one report of nodulation of senescent roots of only one *Bauhinia* plant, but this is viewed with scepticism (Allen and Allen, 1981).

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

To gain a better understanding of rhizobia associated with legumes, attention must be given to a large variety of legumes under various climatic and vegetation conditions. Indigenous and improved legumes which affect a successful agricultural system can and should be used. Our culture collection of rhizobial isolates will form a valuable part of such an investigation.

Further progress towards exploitation of rhizobia as inoculants by selecting good strains that are effective and competitive for application in South Africa can be gained from this large collection. It has come to be regarded as axiomatic that effective nitrogen fixation implies fertility.

REFERENCES

Allen O N 1959 Experiments in soil bacteriology. Third edition. Burgess publishing co. Minneapolis, Minnesota.

Allen O N and Allen E K 1981 The Leguminosae. A Source Book of Characteristics Uses and Nodulation. The University of Wisconsin Press, USA.

Bromfield E S P, Sinha I B and Wolynetz M S 1986 Influence of location, host cultivar, and inoculation on the composition of naturalized populations of *Rhizobium meliloti* in *Medicago sativa* nodules. Appl. Environ. Microbiol. 51, 1077-1084.

Caetano-Anollés G and Gresshoff P M 1991 Plant genetic control of nodulation. Annu. Rev. Microbiol. 45, 345-382.

De Maagd R A, Rao A S, Mulders I H M, Goosen-de Roo L, van Loosdrecht M C M, Wijffelman C A and Lugtenberg B J J 1989 Isolation and characterization of mutants of *Rhizobium leguminosarum* bv. *viciae* 248 with altered lipopolysaccharides: Possible role of surface charge or hydrophobicity in bacterial release from the infection thread. J. Bacteriol. 171, 1143-1150.

Dowdle S F and Bohlool B B 1987 Intra-and inter-specific competition in *Rhizobium fredii* and *Bradyrhizobium japonicum* as indigenous and introduced organisms. Can. J. Microbiol. 33, 990-995.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Dupuy N, Willems A, Pot B, Dewettinck D, Vandenbruaene I, Maestrojuan G, Dreyfus B, Kersters K, Collins M D and Gillis M 1994 Phenotypic and genotypic characterization of Bradyrhizobia nodulating the leguminous tree *Acacia albida*. Int. J. Syst. Bacteriol. 44, 461-473.

Graham P H, Sadowsky M J, Keyser H H, Barnet Y M, Bradley R S, Cooper J E, De Ley D J, Jarvis B D W, Roslycky E B, Strijdom B W and Young J P W 1991 Proposed Minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. Int. J. Syst. Bacteriol. 41, 582-587.

Grobbelaar N, Beijma M C and Saubert S 1964 Additions to the list of nodule-bearing legume species. S.Afr.J.Agric.Sci. 7, 265-270.

Grobbelaar N and Clarke B 1972 A qualitative study of the nodulating ability of legume species: List 2. Jl S. Afr. Bot. 38, 241-247.

Grobbelaar N and Clarke B 1974 A qualitative study of the nodulating ability of legume species: List 4. Agroplantae 6, 59-64.

Grobbelaar N and Clarke B 1975 A qualitative study of the nodulating ability of legume species: List 3. Jl S Afr. Bot. 41, 29-36.

Grobbelaar N, Beyma M C and Todd C M 1967 A qualitative study of the nodulating ability of legume species: List 1. Publs. of the Univ. of Pretoria, New Series No. 38, 1-9.

Grobbelaar N and van Rooyen M W 1979 A qualitative study of the nodulating ability of legume species: List 5. Jl S. Afr. bot. 45, 267-272.

Grobbelaar N, van Rooyen M W and van Rooyen N 1983 A qualitative study of the nodulating ability of legume species. List 6. S. Afr. J. Bot. 2, 329-332.

Johnston A W B and Beringer J E 1975 Identification of the rhizobium strains in pea root nodules using genetic markers. J. Gen. Microbiol. 87, 343-350.

Jordan D C 1982 Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol 32, 136-139.

Jordan D C 1984 Family III. Rhizobiaceae *In* Bergey's Manual of Determinative Bacteriology, Vol.I. Ed. Krieg N R and Holt J G. pp 234-242. Williams and Wilkins, Baltimore.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Russell W C, Kalembasa S, Turowski D, Pachori P and Noel K D 1987(a) Characterization of the lipopolysaccharide from a *Rhizobium phaseoli* mutant that is defective in infection thread development. J. Bacteriol. 169, 4923-4928.

Russell W C, Shatters R, Duh J, Turnbull E, Hanley B, Rolfe B G and Djordjevic M A 1987(b) The isolation and partial characterization of the lipopolysaccharides from several *Rhizobium trifolii* mutants affected in root hair infection. Plant Physiol. 84, 421-427.

Stacey G and Upchurch R G 1984 Rhizobium inoculation of legumes. Trends in Biotech. 2, 65-70.

Sprent J I 1994 Evolution and diversity in the legume-rhizobium symbiosis: chaos theory? Plant and Soil 161, 1-10.

Thies J E, Singleton P W and Bohlool B B 1991 Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol. 57, 19-28.

Vauterin L, Swings J, Kersters K, Gillis M, Mew T W, Schroth M N, Palleroni N J, Hildebrand D C, Stead D E, Civerolo E L, Hayward A C, Maraite M, Stall R E, Vidaver A K and Bradbury J F 1990 Towards improved taxonomy of *Xanthomonas*. Int. J. Syst. Bacteriol. 40, 312-316.

Vincent J M 1970 A manual for the practical study of the root-nodule bacteria. IBP Handbook no. 15. Blackwell Scientific Publishers, Oxford.

Wall L G and Favelukes G 1991 Early recognition in the *Rhizobium meliloti*-alfalfa symbiosis: Root exudate factor stimulates root adsorption of homologous rhizobia. J. Bacteriol. 173, 3492-3499.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 3

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DIVERSITY OF RHIZOBIA INDIGENOUS TO SOUTH AFRICAN SOILS, ISOLATED FROM A WIDE VARIETY OF FABACEAE DISTRIBUTED ALONG DIFFERENT GEOGRAPHICAL AREAS

The purpose of this study was to determine the degree of phenotypic-molecular relatedness, with particular emphasis on indigenous rhizobia in South Africa to 45 selected representative reference strains of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Agrobacterium* and *Azorhizobium*. An extensive study which included a total of 346 strains isolated from a wide variety of 147 legumes obtained from different geographical areas is presented. Up to four different rhizobia were found to be colonising the same nodule. Rhizobia were isolated from hitherto unconfirmed nodulated legumes genera *Cassia*, *Bauhinia* and *Schizolobium*. A dendrogram was constructed by using average-linkage (UPGMA) cluster analysis based on sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole-cell proteins. This dendrogram produced 24 clusters demonstrating the diversity that exists between members of rhizobia.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

A wide diversity of leguminous species occurs in South Africa with its different geographical areas. Characteristic of many members of the family Fabaceae is a symbiotic relationship between the roots and certain nitrogen-fixing rhizobia in the soil (2). There has been a dearth of information concerning the identity and taxonomy of rhizobia in South Africa. Precise identification of these taxa is often difficult because apart from the rhizobia included in this study, numerous legumes remain to be tested for nodulation in order to complete a resultant large database. Extensive research is necessary to provide stability and coherency in the taxonomy of rhizobia (32, 35). In the past few years, there has been increased interest in searching for new rhizobia isolates from legumes that do not represent agriculturally important plants, and several groups of rhizobia have been revised (13, 23, 27, 40). The present study includes 147 nodulated legumes which were collected from different geographical areas. This study embraces leguminous trees, annuals and perennials, shrubs and vines. Their origins are discussed in a previous paper. Most of the legume species that were collected are indigenous to South Africa. The areas where legumes were collected include virgin soils, with the focus on the occurrence of indigenous rhizobia, which will hopefully result in a more comprehensive study.

It was the aim of this study to include all isolates and conceivable relatives and to gauge the relationships of these organisms as criteria for further study. Representative reference strains were selected from the five genera *Rhizobium*, *Sinorhizobium, Bradyrhizobium*, *Azorhizobium* and *Agrobacterium*. In order to study and compare the diversity of this large number of organisms and their phenotypic - molecular relationships, with representative strains of rhizobia, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins was used, since it is relatively rapid and can be used with confidence and also provides for the establishment of a stable database to detect as wide a rhizobia diversity as possible. The information obtained from the analysis of complex protein patterns has shown that the reference strains used in this study reflect a remarkable agreement with the data obtained with phenotypic and phylogenetic analysis used in recent literature (13, 23, 26, 39, 40). Some of the isolates were clearly related to previously described species in the genera *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium*. However, because of loose associations it is presumed that some of these new isolates could constitute separate taxonomic entities.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Although relationships among some of the tree rhizobia clearly identify them as members of the genera *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium*, the status of a large group of tree rhizobia remains equivocal. This study provides an indication of the huge numbers of new rhizobia that is still to be detected from the wide range of remaining nodulated legume plants.

MATERIAL AND METHODS

Bacterial strains. A total of 346 strains were isolated from 147 different legumes. Isolation of these microorganisms was carried out as described previously. The purity of each strain was checked by repeated streaking on Yeast Extract Mannitol (YEM) (37) agar plates supplemented with Congo Red and by microscopic examination of living and Gram-stained cells. Different strains isolated from the same nodules were designated with different symbols, for example 134 a, 134 b, 134 c. To compare the new isolates with previously described rhizobia species, reference strains of the five genera, *Azorhizobium*, *Agrobacterium*, *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* were obtained (Table 1) (Fig.1).

Growth and culture conditions. Strains were maintained on YEM agar, which contained 0,4 g Yeast extract, 10 g of Mannitol, 0,5 g of K_2HPO_4, 0,2 g of $MgSO_4.7H_2O$, 1 litre distilled water, and 15 g of agar. The pH was adjusted to 6,8-7,0 before sterilization. Cultures were incubated for 3 to 5 days at 28°C.

Test for *Agrobacterium*. For the production of 3-Ketolactose, the method of Holding and Collee (14) was used.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

PAGE of total bacterial proteins. Strains were grown on sterile YEB medium (10) containing 5 g of peptone, 1 g of Yeast extract, 5 g of Beef extract, 5 g of sucrose, 1 litre distilled water, and 20 g of agar, in petri dishes at 28°C, for 5 days. This was repeated three times before preparation started for SDS-PAGE. Whole-cell protein extract was prepared and SDS-PAGE was performed as described by D. Dewettinck, B. Pot, and K. Kersters (May 1991, unpublished data, corresponding authors). To eliminate the excessive amount of ever-present slime which interfered with electrophoresis the extract was first frozen at -12°C, then heated to 94°C for 30 min, cooled to room temperature and centrifuged for 10 min at 15000 rpm in a Sigma (2 MK) centrifuge. The supernatant was decanted into clean Eppendorf centrifuge tubes and stored at -12°C. The protein concentration of each sample was assayed with Pierce-Coomassie Plus Protein Assay Reagent (Boehringer) as well as according to Stegemann et al. (33). In the event that there was still some slime present after the first run, the heated protein extracts were diluted to a concentration permitting "clean" protein profiles. The protein electrophoretic patterns were scanned with a Hoefer densitometer (Hoefer Scientific Instruments; San Francisco; GS300 Transmittance/Reflectance). Normalized densitometric traces were grouped and similarities were calculated between all organisms by using the Pearson product moment correlation coefficient (*r*), which was converted to a percentage (29), while cluster analysis was performed by the GelCompar 3.0 software package (Applied Maths, Kortrijk, Belgium) (36).

The protein profiles of *Psychrobacter immobilis* LMG 1125 were used as references patterns in each gel (five tracks/gel). Reproducibility of electrophoresis was determined by comparing these tracks with the *P. immobilis* protein profile selected in the GelCompar 3.0 as standard. Correlation of 94% was presumed as acceptable.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

FIG. 1. Computer print-out of the normalized profiles obtained for the 45 reference strains.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

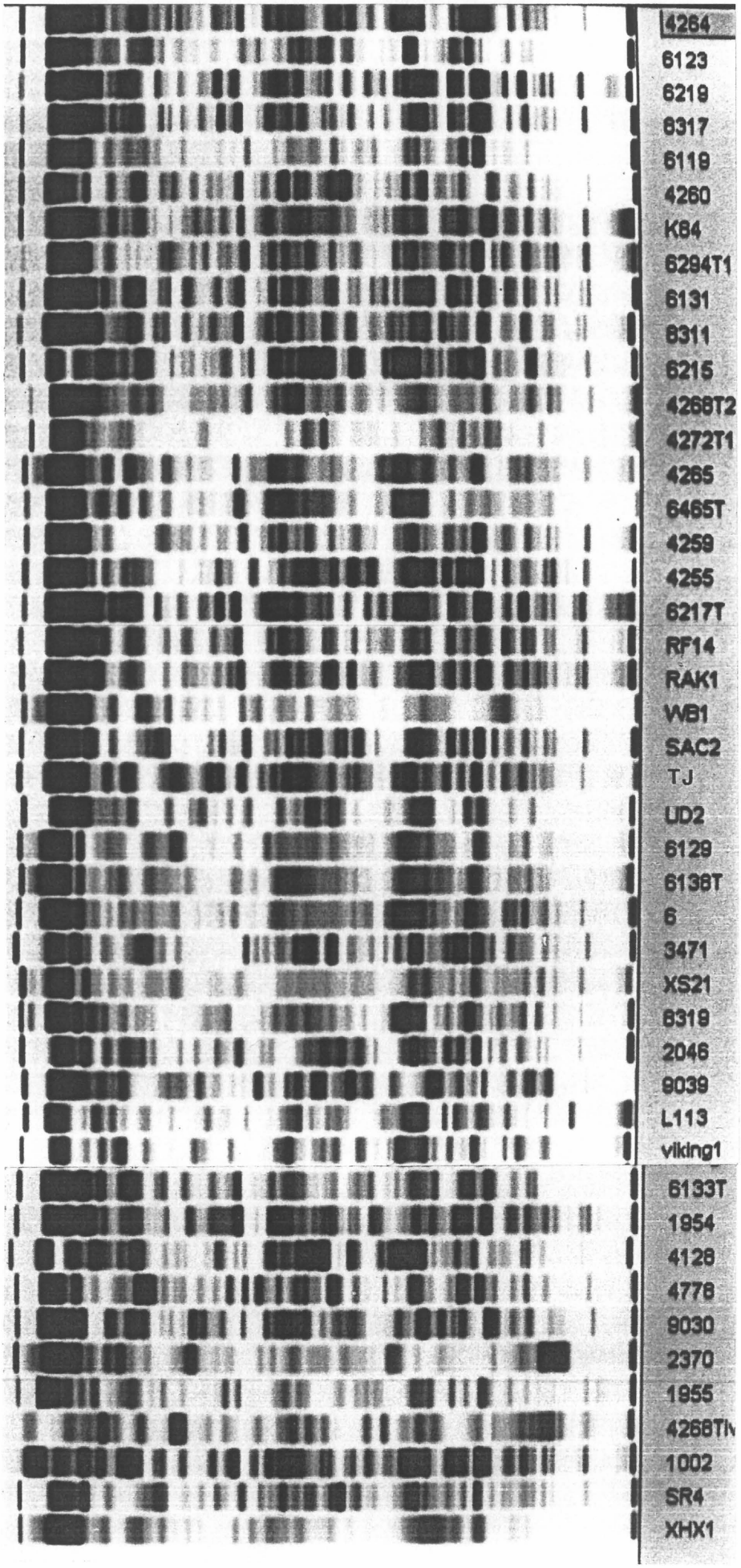


FIG. 1

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

RESULTS AND DISCUSSION

Medium. The use of YEB medium instead of the conventional TY medium, which contained (per liter) 5 g of tryptone, 0.75 g of yeast extract, 0.454 g of KH_2PO_4, 2.388 g of $Na_2HPO_4 .12H_2O$, 1 g of $CaCl_2$, and 20 g of agar (pH 6,8 to 7), to culture strains for SDS-PAGE of whole-cell proteins, yielded a higher protein production and thus better resolution of a greater number of individual protein bands used for comparison. Diminution of polysaccharide production was also achieved by repeated streaking on the YEB medium, since the excessive slime production by most isolates on TY medium interfered with obtaining suitable protein bands thus leading to a decrease in reproducibility. The exposure of the protein extracts to extra heating decreased residual slime.

Agrobacterium test. All isolates were 3-ketolactose negative. Therefore, the probability that agrobacteria were included in this study, is small.

SDS-PAGE of whole-cell proteins. The diversity that exists among indigenous rhizobia strains is revealed in the 24 distinct clusters formed within the dendrogram (Fig. 2) and the variation in protein profiles from which the groupings were derived. The new isolates clustered with members of the three genera *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium*, but also formed groups that are well separated from these rhizobia clusters.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

TABLE 1. Geographic origin and host plants of Rhizobial reference strains

Strain	Host plant	Geographical origin or Authors	Cluster
Azorhizobium caulinodans			
LMG 6465T	*Sesbania rostrata*	Senegal	4
Agrobacterium tumefaciens			
SARCC K84			7
Bradyrhizobium sp.			
LMG 6129	*Lotus pedunculatus*		4a
LMG 8319	*Macrotyloma africanicus*	Zimbabwe	4b1
SARCC XS21			4b1
SARCC XHX1			4b2
Bradyrhizobium japonicum			
LMG 6138T	*Glycine max*	Japan	4a
LMG 4265	*Ulex europaeus*		4b2
LMG 4272T1	*Pueraria lobata*		4e
USDA 6			4b1
SARCC WB1	*Glycine max*		4d1
Rhizobium sp.			
LMG 8311	*Acacia farnesiana*	Senegal	7
LMG 6463	*Sesbania rostrata*	Senegal	18

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Strain	Host plant	Geographical origin or Authors	Cluster
Rhizobium leguminosarum			
LMG 4260	*Vigna unguiculata*		8a
LMG 4259	*Vicia sativa*		2b
LMG 6294T1	*Lathyrus* sp.	St. Petersburg	2b
SARCC TJ9	*Pisum, Viciae*		2b
SARCC UD2	*Phaseolus vularis*		2b
Rhizobium leguminosarum biovar *trifolii*			
USDA 2046		van Berkum (34)	6b1
LMG 4255	*Trifolium pratense*		9
LMG 6119	*Trifolium repens*	New Zealand	2b
SARCC SAC2			9
SARCC SR4			9
Rizobium leguminosarum biovar *viciae*			
L113		G. Laguerre (21)	13a1
USDA 2370	*Pisum sativum*	van Berkum (34)	16a2

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Strain	Host plant	Geographical origin or Authors	Cluster
Rhizobium etli			
biovar *phaseoli* (type I)			
Viking 1	*Phaseolus vulgaris* L.		13b
Rhizobium tropici			
group IIA			
USDA 9039(=CFN 299)	*Phaseolus vulgaris*	Martinez-Romero (26)	11b
group IIB			
USDA 9030(=CIAT 899)	*Phaseolus vulgaris*	Martinez-Romero (26)	8a
Rhizobium loti			
LMG 4268T1	*Lotus americanus*		8a, 23
LMG 4268T2	*Lotus americanus*		2a
LMG 4264	*Lupinus densiflorus*		2a
LMG 6123	*Lotus divaricatus*	New Zealand	2a
USDA 3471	*Lotus corniculatus*	New Zealand	2a
Rhizobium huakuii			
USDA 4778	*Astragalus sinicus*	People's Republic of China	2a
Rhizobium galegae			
LMG 6215	*Galega orientalis*	Finland	20
USDA 4128	*Galega orientalis*	USSR	20

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

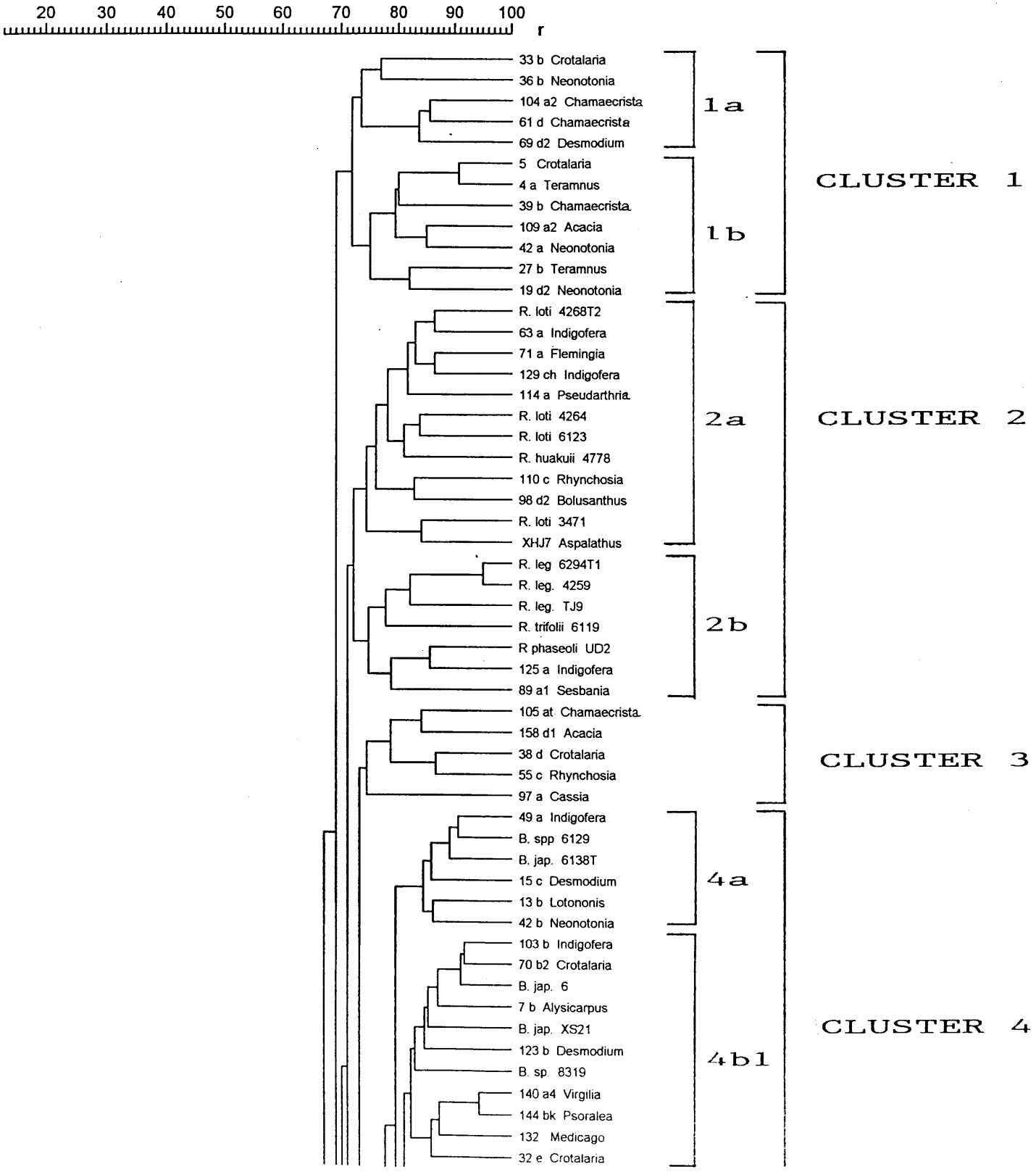
UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Table 1. Continued

Strain	Host plant	Geographical origan or Authors	Cluster
Sinorhizobium meliloti			
LMG 6131	*Medicago sativa*		7
LMG 6133T	*Medicago sativa*		7
SARCC RAK1	*Medicago sativa*	Australia	7
USDA 1954	*Trigonella suavissima*	Australia	7
USDA 1955 (M1)	*Medicago* sp.	Mediterranean basin	19
USDA 1002 (ATCC 9930)	*Medicago sativa*	United States	19
Sinorhizobium fredii			
LMG 6219	*Glycine max*	Honan China	7
LMG 8317	soil	Shanghai China	7
LMG 6217T	*Glycine max*	Honan China	7

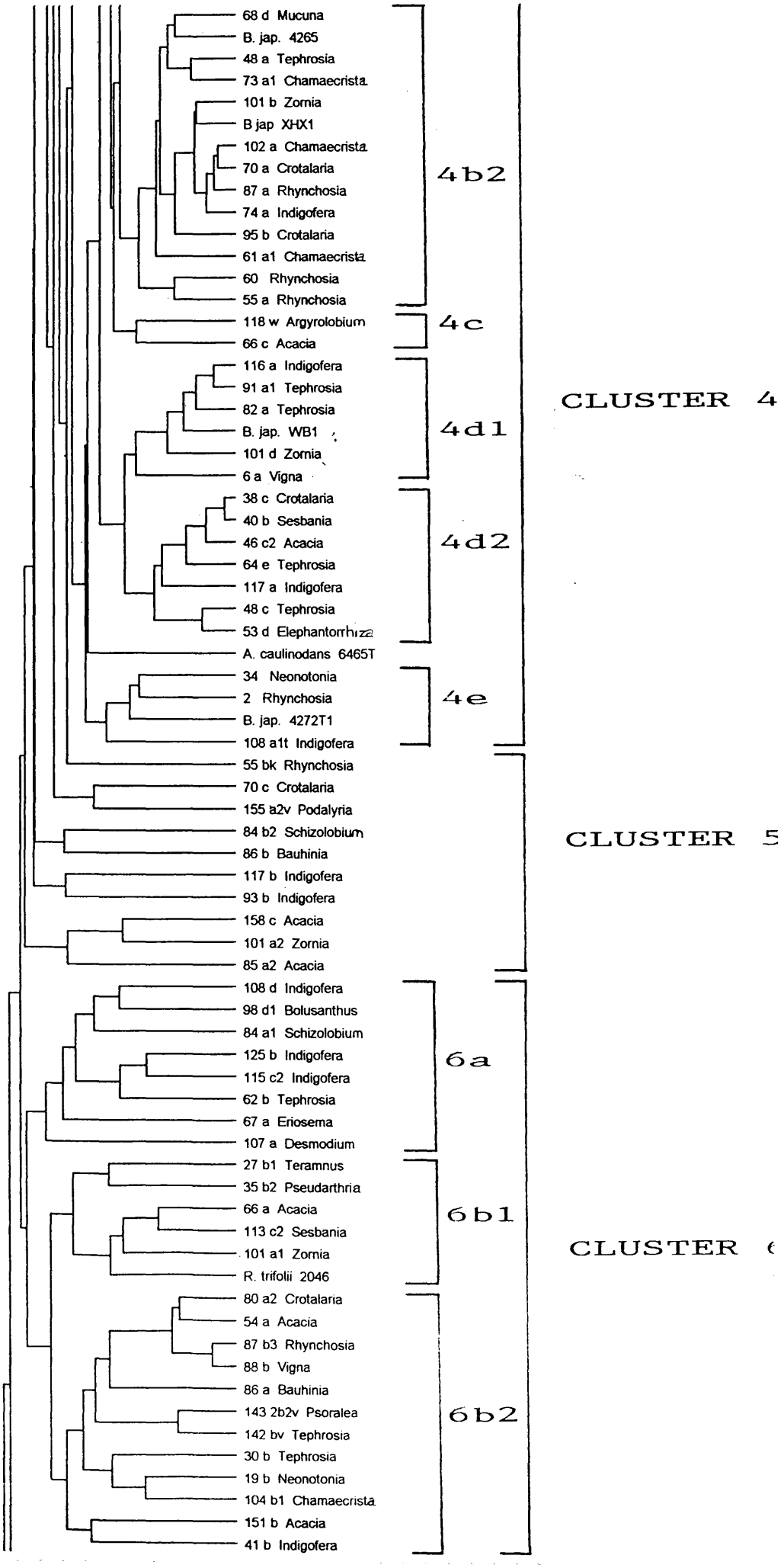
Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



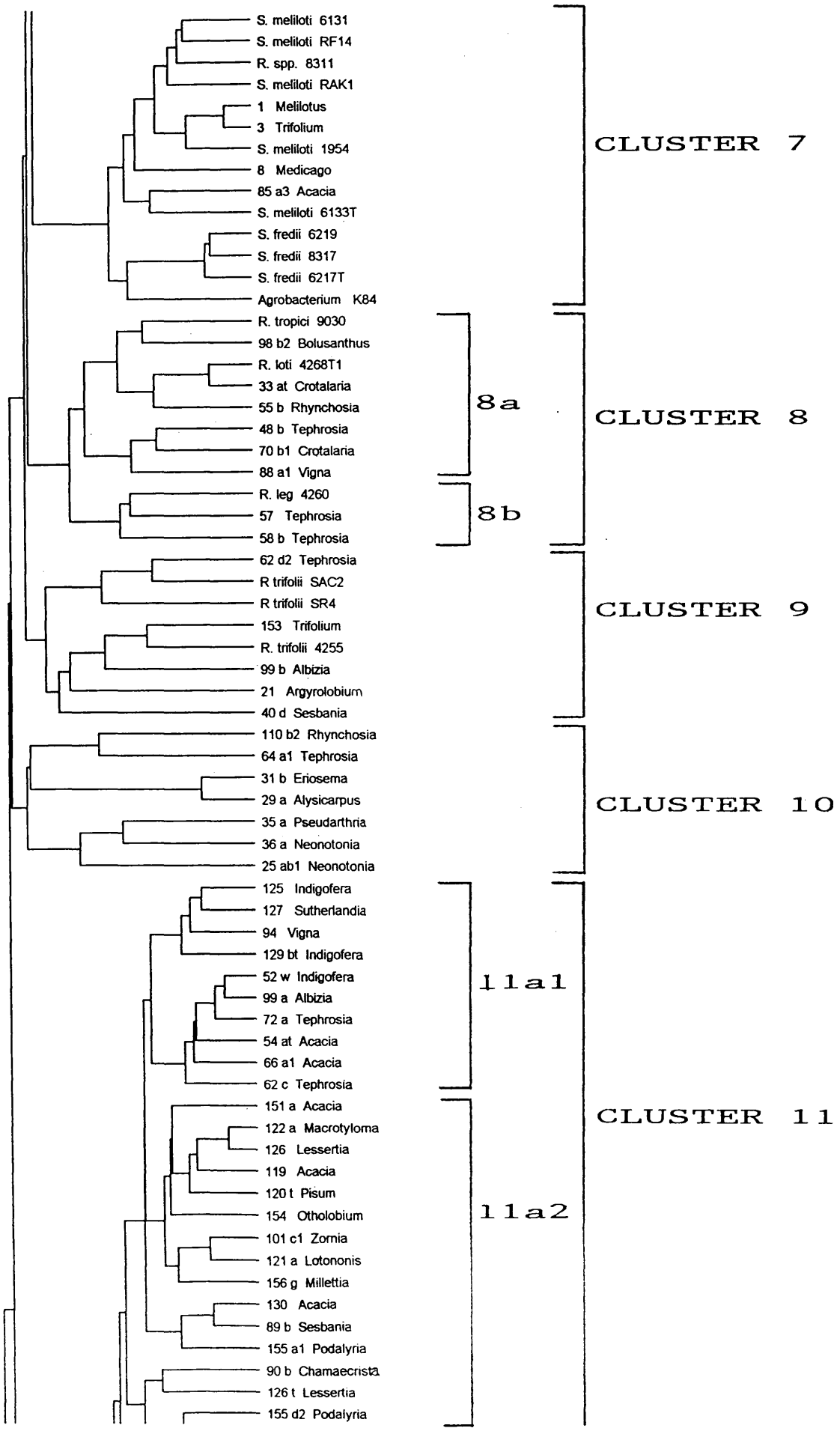
Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



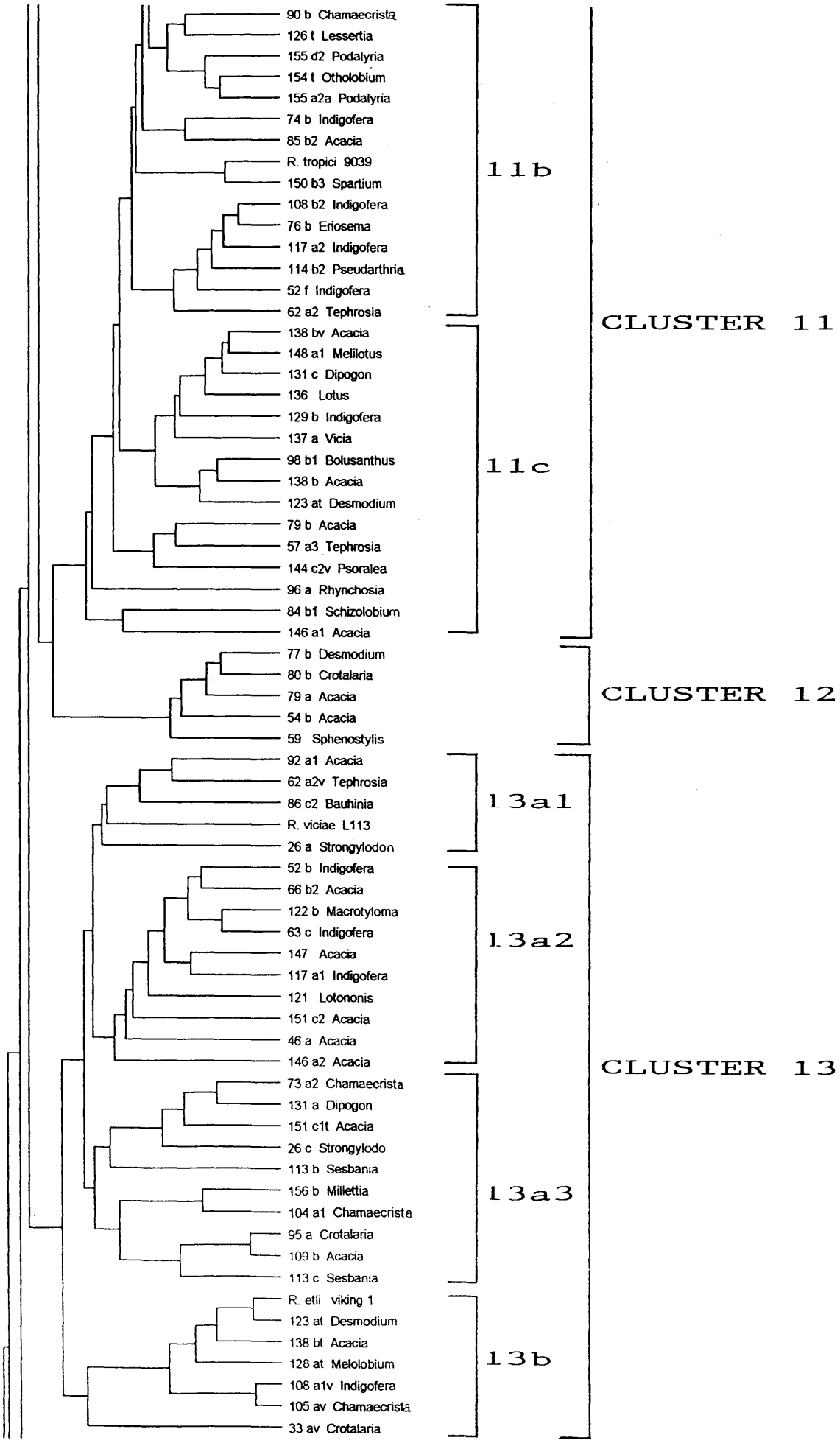
Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



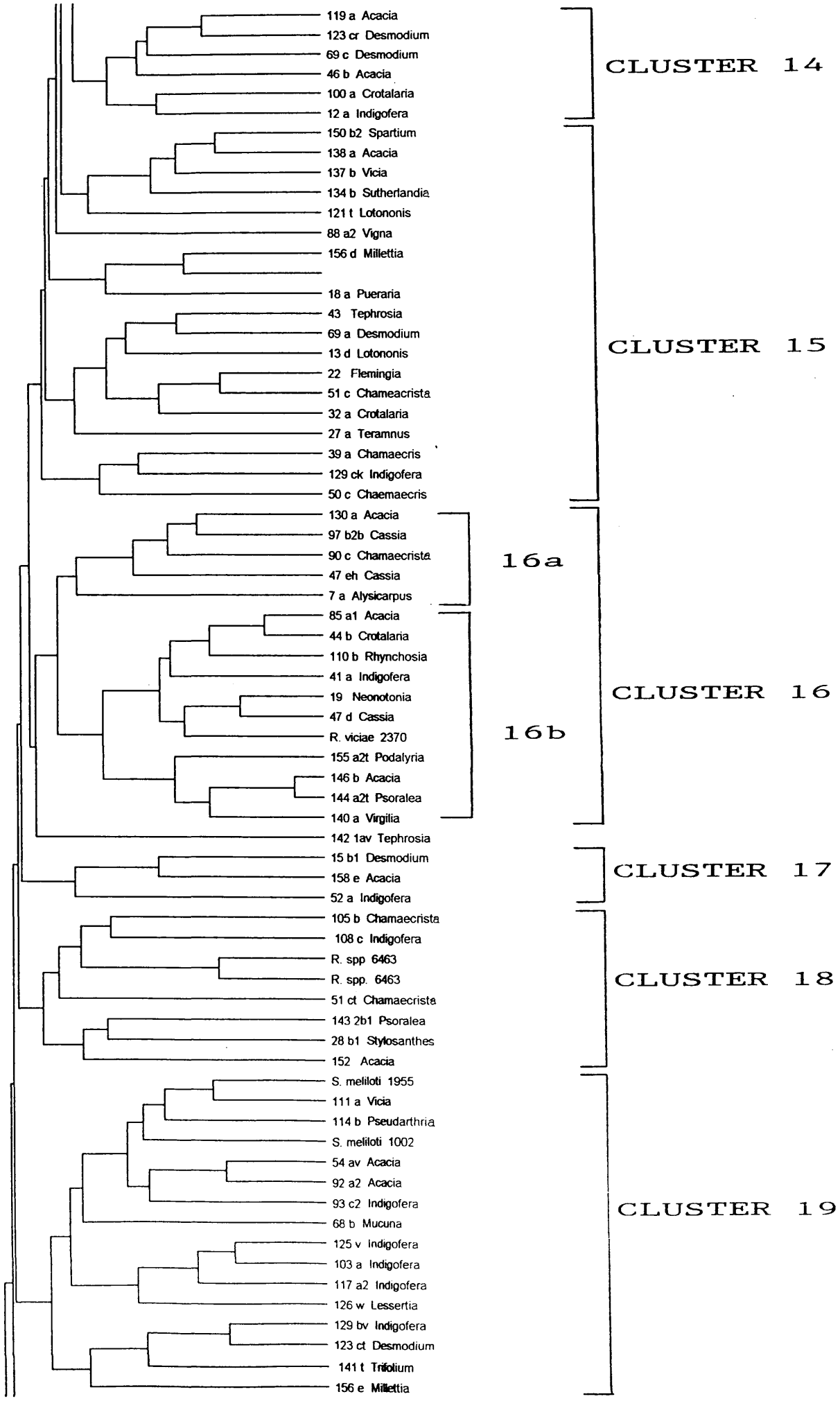
Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



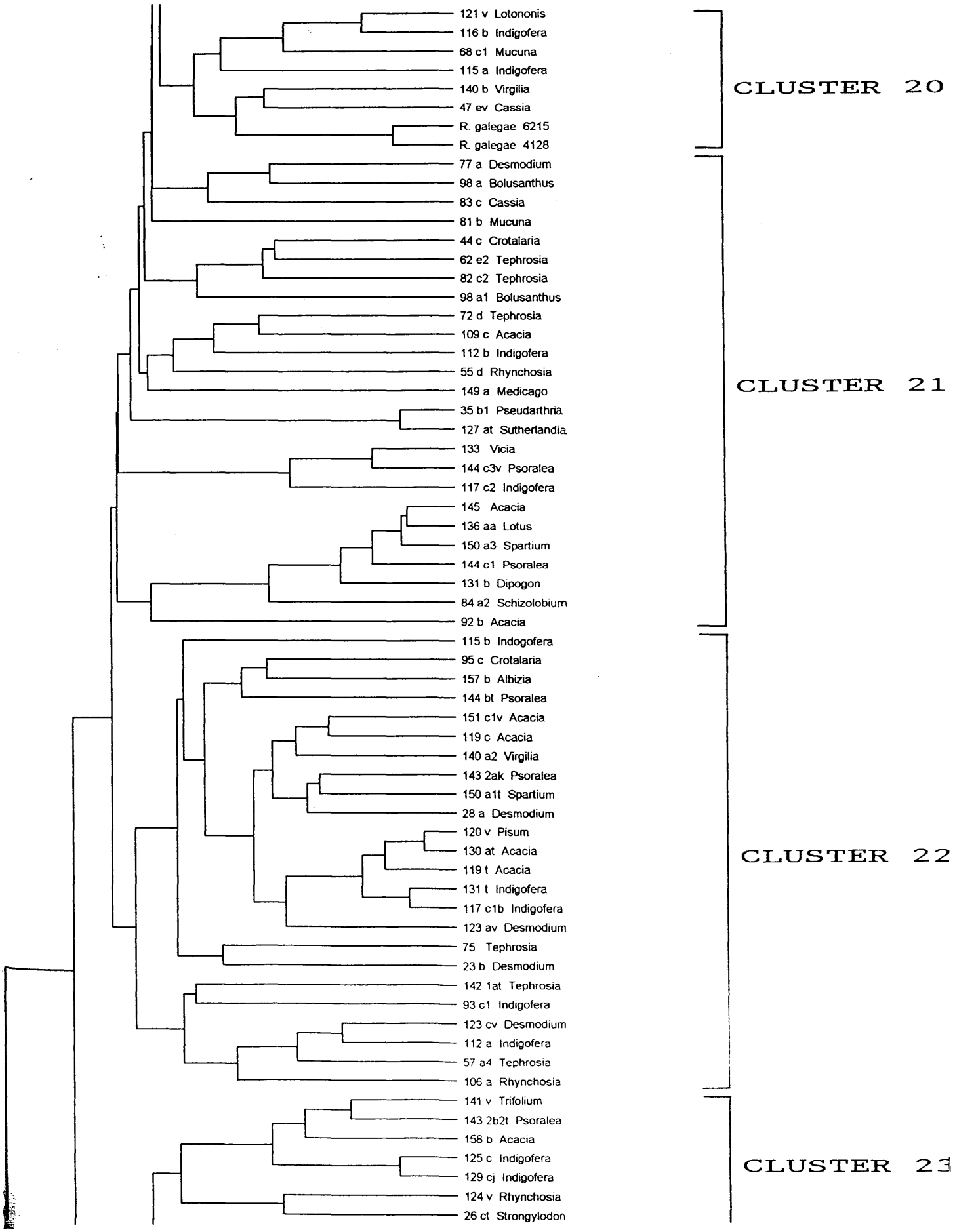
Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

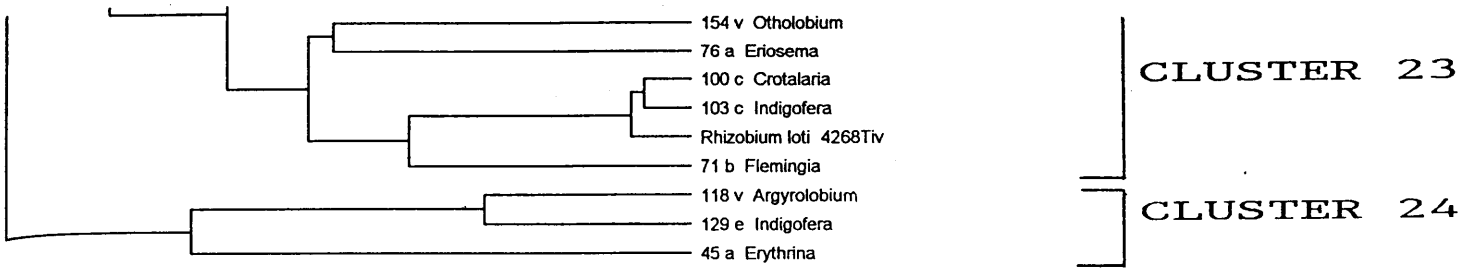


FIG. 2. Dendrogram defined 24 clusters showing the relationships based on protein profiles among the indigenous strains isolated from different legumes, and reference stains of *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Sinorhizobium* species. The relationships are determined by using the mean correlation coefficient (*r*) values, which were grouped by the unweighted average pair group method, using Gelcompar 3.0.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

FIG. 3. **(A)** The diversity based on protein profiles that exists between the divergent groups (A1, A2 and B) of *S. meliloti* strains and the close relationship of group A2 with *S. fredii* strains. **(B) Similarity** matrix of *S. meliloti* and *S. fredii* strains showing the relationships based on *r*-values.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

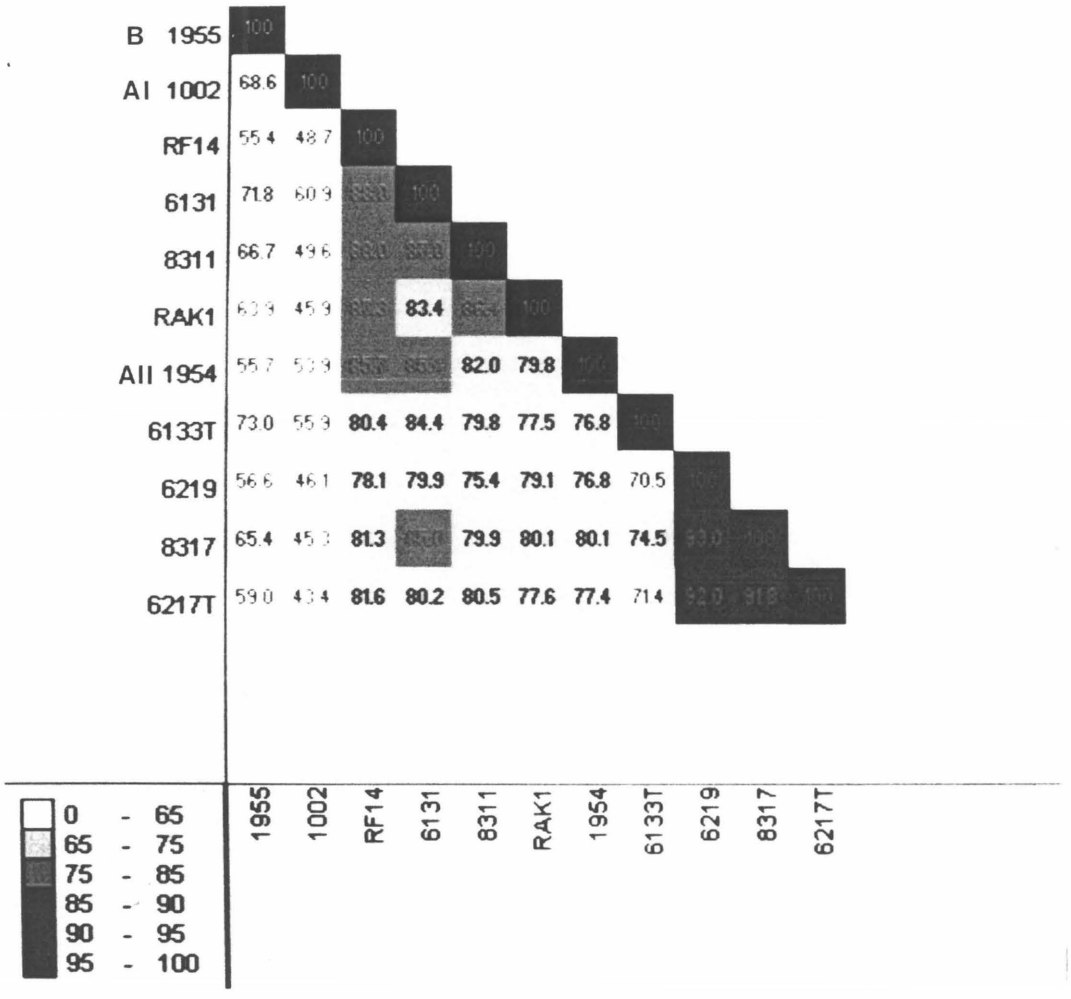


FIG. 3

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Cluster 1. Cluster 1 contains 12 slow-growing new isolates, four from tree legumes. Cluster 1 is segregated into two sub-groups that were related at a similarity value of $r = 0{,}71$.

Subgroup 1a. The profiles of the strains belonging to cluster 1a showed little variation. Strains include (33b) from *Crotalaria* sp., (36b) from *Neonotonia wightii*, (61d) from the tree legume *Chamaecrista comosa*, (104a2) from the tree legume *Chamaecrista* sp. and (69d2) from *Desmodium setigerum.*

Subgroup 1b. The profiles of the organisms in this subgroup were very similar and they clearly differed from those of the strains belonging in subgroup 1a. Strains include (5) from *Crotalaria distans*, (4a) from *Teramnus labialis*, (39b) from the tree legume *Chamaecrista absus*, (109a2) from the tree legume *Acacia dealbata*, (42a) and (19d2) from *Neonotonia wightii* and (27b) from *Teramnus labialis*.

Cluster 2. Cluster 2 was separated into two subgroups (2a and 2b). The intersubgroup similarity value of $r = 0{,}72$ indicates that the two subgroups share some similar protein bands but diversity is also detectable.

Subgroup 2a. Subgroup 2a forms a tight cluster between reference strains *R. loti* LMG 4268T2, *R. loti* LMG 4264, *R. loti* LMG 6123, *R. loti* USDA 3471 and *R. huakuii* USDA 4778 (=CCBAU 2609), together with a number of new isolates, one (71a) from *Flemingia grahamiana*, two isolates (129ch, 63a) from *Indigofera* sp., one (114a) from *Pseudarthria hookeri*, one (110c) from *Rhynchosia hirta*, one (98d2) from the tree legume *Bolusanthus speciosus*, and one (XHJ7) from *Aspalathus linearis* (Supplied by Ms. J. F. Bloem, Unit for Rhizobiology, Institute for Plant protection. ARC, Pretoria). Differences can be detected in their protein profiles.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Subgroup 2b. Subgroup 2b contains five reference strains. *R. leguminosarum* LMG 6294T1 and *R. leguminosarum* LMG 4259 exhibit very high levels of similarity (r = 0,95), expressed in their identical protein profiles. However, homogeneous protein patterns were also detected in their close association with *R. leguminosarum* SARCC TJ9, *R. leguminosarum* bv. *trifolii* LMG 6119 and *R. leguminosarum* bv. *phaseoli* SARCC UD2. The two new isolates include (125a) from *Indigofera woodii* and (89a1) from *Sesbania punicea*.

Cluster 3. Cluster 3 consists of five slow growing isolates differing considerably in their protein profiles and forming a distinct subline exhibiting affinity to the slow growing isolates in cluster 4. Strains isolated from three different tree legumes belong to this cluster. The intracluster similarity value is r = 0,74 and contains the following isolates: (105at) from the tree legume *Chamaecrista stricta*, (158d1) from the tree legume *Acacia nigrescens*, (55c) from *Rhynchosia monophylla*, (38d) from *Crotalaria pallida* and (97a) from the tree legume *Cassia didymobotrya*.

Cluster 4. Cluster 4 contains slow-growing new isolates together with representative strains of *Bradyrhizobium japonicum* and *Bradyrhizobium* sp.. This is one of the largest groups of rhizobia within the dendrogram and forms rather tight subgroups except for the loose association of subgroup 4e. Within this cluster, *A. caulinodans* LMG 6465T forms a loose association with the slow growers. The effect of growth rate on members in this cluster is significant in terms of the absence of distinct sharp protein bands, clearly contrasting with the protein profiles of fast growers.

Subgroup 4a. Subgroup 4a forms a tight cluster composed of six isolates, which includes two references strains. The protein profiles of the two reference strains, *Bradyrhizobium* sp. LMG 6129 and *B. japonicum* LMG 6138T were found to be identical to the new isolates, (49a) from *Indigofera melanadenia*, (15c) from *Desmodium tortuosum*, (42b) from *Neonotonia wightii* and (13b) from *Lotononis bainesii*, thereby confirming their placement in subgroup 4a.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Subgroup 4b. Subgroup 4b is separated into two groups (4b1 and 4b2) with an intersubgroup similarity value of $r = 0{,}82$, indicating that the close relationship among these strains is conspicuous. Protein profiles between members within subgroup 4b are very homogeneous.

Subgroup 4b1 comprises three reference strains. *B. japonicum* USDA 6 was found to exhibit protein patterns identical to that of (103b) from *Indigofera hilaris*, (70b2) from *Crotalaria brachycarpa* and (7b) from *Alysicarpus rugosus*. *B. japonicum* SARCC XS21 clearly possesses very similar protein bands to (123b) from *Desmodium repandum*. The remaining isolates include *Bradyrhizobium* sp. LMG 8319, (140a4) from *Vigna divaricata*, (144bk) from *Psoralea pinnata*, (132) from *Medicago lupulina* and (32e) from *Crotalaria pallida*.

Members of subgroup 4b2 exhibited protein patterns almost identical to one another with an intragroup similarity value of $r = 0{,}84$. The three isolates, (55a) and (60) isolated from *Rhynchosia monophylla* and (61a1) from the tree legume *Chamaecrista comosa*, displayed small differences in their protein profiles. When they were not taken into account, the similarity level value of this group was $r = 0{,}89$. The rest of the isolates, (68d) from *Mucuna coriacea*, (73a1) from the tree legume *Chamaecrista mimosoides*, (48a) from *Tephrosia purpurea*, (101b) from *Zornia capensis*, (102a) from the tree legume *Chamaecrista biensis*, (70a) from *Crotalaria brachycarpa*, (87a) from *Rhynchosia hirta*, (74a) from *Indigofera arrecta*, (95b) from *Crotalaria vasculosa*, (61a1) from the tree legume *Chamaecrista comosa*, (55a) and (60) from *Rhynchosia monophylla*, including the two reference strains *B. japonicum* LMG 4265 and *B. japonicum* SARCC XHX1, clearly exhibited very high levels of protein pattern relatedness to each other. It is interesting to note that three of the strains isolated from the tree legume species *Chamaecrista* clustered within this subgroup.

Subgroup 4c. Subgroup 4c contains two new isolates, (118w) from *Argyrolobium pauciflorum* and (66c) from the tree legume *Acacia robusta* with a similarity value of $r = 0{,}85$.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Subgroup 4d. Subgroup 4d is separated into two groups (4d1 and 4d2) with an intersubgroup similarity value of $r = 0{,}82$. Diversity between groups 4d1 and 4d2 exist within the protein profiles.

Subgroup 4d1 contains five new isolates, (116a) from *Indigofera oxalidea*, (91a1) from *Tephrosia* sp., (82a) from *Tephrosia purpurea*, (101d) from *Zornia capensis* and (6a) from *Vigna vexillata*, exhibiting protein patterns identical to the reference strain *B. japonicum* SARCC WB1, thereby confirming their classification within subgroup 4d1.

Subgroup 4d2 consists of seven new isolates. It is clear that fewer protein bands are present in the profiles of members within group 4d2, which distinguishes them from group 4d1. The isolates in this group are (40b) from *Sesbania bispinosa*, (38c) from *Crotalaria pallida*, (46c2) from the tree legume *Acacia sieberana* var. *woodii*, (64e) from *Tephrosia purpurea*, (117a) from *Indigofera woodii*, (53d) from *Elephantorrhiza obliqua* and (48c) from *Tephrosia purpurea*. The intrasubgroup similarity value of $r = 0{,}88$ is an indication of the very close relationship that exists among these isolates.

The reference strain *Azorhizobium caulinodans* LMG 6465T formed a long individual line separating sub-group 4e from the rest of the forming sub-groups in cluster 4.

Subgroup 4e. Subgroup 4e is composed of three isolates: (2) from *Rhynchosia nervosa*, (34) from *Neonotonia wightii* and (108a1t) from *Indigofera swaziensis* and one reference strain *Bradyrhizobium japonicum* LMG 4272T1, exhibiting a loose association with the rest of the subgroups within cluster 4.

Cluster 5. Cluster 5 contains ten weakly growing isolates which produce light yellow colonies on YEB medium. Four of the ten isolates were isolated from tree legumes. Members of this cluster form distinct sublines that are linked separately at different correlation values with no specific association to previously mentioned clusters. Protein patterns among these

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

isolates are very diverse. Isolates that clustered within this group are (55bk) from *Rhynchosia monophylla*, (70c) from *Crotalaria brachycarpa*, (155a2v) from *Podalyria myrtillifolia*, (84b2) from the tree legume *Schizolobium parahybum*, (86b) from the tree legume *Bauhinia variegata*, (93b) from *Indigofera arrecta*, (117b) from *Indigofera woodii*, (158c) from the tree legume *Acacia nigrescens*, (101a2) from *Zornia capensis* and (85a2) from the tree legume *Acacia xanthophloea*.

Cluster 6. Cluster 6 is divided into two subgroups.

Subgroup 6a. Subgroup 6a contains eight slow-growing isolates, two (108d, 115c2) from *Indigofera swaziensis*, one (98d1) from the tree legume *Bolusanthus speciosus*, one (84a1) from the tree legume *Schizolobium parahybum*, one (125b) from *Indigofera woodii*, one (62b) from *Tephrosia multijuga*, one (67a) from *Eriosema psoraleoides* and one (107a) from *Desmodium setigerum*. The protein patterns of members within this group are rather diverse.

Subgroup 6b is separated into two groups (6b1 and 6b2), with an intrasubgroup similarity value of $r = 0{,}70$, indicating the level of protein pattern divergence that exists among them.

Subgroup 6b1 contains five new isolates and one reference strain *R. leguminosarum* bv. *trifolii* USDA 2046. The strains include (27b1) from *Teramnus labialis*, (35b2) from *Pseudarthria hookeri*, (66a) from the tree legume *Acacia robusta*, (113c2) from *Sesbania punicea* and (101a1) from *Zornia capensis*. Similarities can be detected in their protein profiles.

Subgroup 6b2. Within subgroup 6b2 the following isolates exhibit marked similarities in respect of their protein profiles: (80a2) from *Crotalaria pallida*, (54a) from the tree

legume *Acacia dealbata*, (88b) from *Vigna unguiculata*, (87b3) from *Rhynchosia hirta*, (86a) from the tree legume *Bauhinia variegata*, (143 2b2v) from *Psoralea asarina*, (142bv) from *Tephrosia capensis*, (30b) from *Tephrosia polystachya*, (104b1) from the tree legume *Chamaecrista* sp. and (19b) from *Neonotonia wightii*. The two isolates (151b) from the

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

tree legume *Acacia podalyriaefolia* and (41b) *Indigofera arrecta* reflect some differences in their protein patterns.

Overall, the similarity value of $r < 0,70$ and visual analysis of protein patterns confirmed the diversity that exists between the subgroups distinguished within cluster 6.

Cluster 7. Cluster 7 contains, besides the *S. meliloti* and *S. fredii* reference strains, the intermingling of the two reference strains *Agrobaterium radiobacter* SARCC K84 which was found to exhibit specific affinity for *S. fredii* strains, and *Rhizobium* sp. LMG 8311, isolated from *Acacia farnesiana* which was found to be closely related to *S. meliloti* strains. Four new isolates were also included in cluster 7 and were specifically closely related to *S. meliloti* strains. Cluster 7 comprises *S. fredii* LMG 6219, *S. fredii* LMG 8317, *S. fredii* LMG 6217T, *Agrobacterium radiobacter* SARCC K84, *S. meliloti* LMG 6131, *S. meliloti* SARCC RF14, *S. meliloti* SARCC RAK1, *Rhizobium* sp LMG 8311, *S. meliloti* USDA 1954, *S. meliloti* LMG 6133T and strains (1) from *Meliloti alba*, (3) from *Trifolium* sp., (8) from *Medicago lupulina* and (85a3) from the tree legume *Acacia xanthophloea*. The whole-cell protein patterns of each member were found to be almost identical within cluster 7. The only marked difference that distinguishes *S. fredii* strains and *A. radiobacter* strains from *S. meliloti* strains and the new isolates is one protein band that was found to be more prominent (thicker) in the case of the *S. fredii* and *A. radiobacter* association. Within this cluster type strain *S. meliloti* 6133T was found to be closely related to strain (85a2), isolated from the tree legume *A. xanthophloea*, the only close association involving the new tree legume isolate in this *S. meliloti-fredii* relationship.

Cluster 8. Cluster 8 is separated into two subgroups (8a and 8b) which differ in their protein profiles.

Subgroup 8a comprises two reference strains, *R. tropici* IIB USDA 9030 (CIAT899) and

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

type strain *R. loti* LMG 4268T1(t). These two strains revealed almost identical protein patterns to each other (the t in brackets will be explained later). Six new isolates that cluster within subgroup 8a, exhibited protein patterns closely related to the reference strains, thereby confirming their identity. The new isolates consist of one strain (98b2) from the tree legume *Bolusanthus speciosus*, (33at) from *Crotalaria* sp., (55b) from *Rhynchosia monophylla*, (48b) from *Tephrosia purpurea*, (70b1) from *Crotalaria brachycarpa* and (88a1) from *Vigna unguiculata*.

Subgroup 8b consists of *R. leguminosarum* LMG 4260, and two strains (57, 58b) from *Tephrosia semiglabra*. Differences appear in their protein profiles.

Cluster 9. Three reference strains of *R. leguminosarum* bv. *trifolii* group in cluster 9. *R. leguminosarum* bv. *trifolii* SARCC SAC2 and *R. leguminosarum* bv. *trifolii* SARCC SR4 were found to possess identical protein bands to that of strain (62d2) isolated from *Tephrosia multijuga*. *R. leguminosarum* bv. *trifolii* LMG 4255 and isolate (153) from *Trifolium angustifolium* exhibited very similar protein patterns. The remaining three isolates (99b) from the tree legume *Albizia adianthifolia*, (21) from *Argyrolobium tomentosum* and (40d) from *Sesbania bispinosa* display a close protein profile relationship with members within cluster 9. Overall, the analysis of protein profiles indicates that the relationship among these strains is of a relatively homogeneous nature.

Cluster 10. Cluster 10 consists of seven isolates. The isolates (29a) from *Alysicarpus rugosus* and (31b) from *Eriosema psoraleoides* were found to display identical protein profiles (similarity value of $r = 0{,}91$), but to differ completely from the other strains, by no means possessing protein bands identical to members within this cluster. Isolates (36a) from *Neonotonia wightii* and (35a) from *Pseudarthria hookeri* revealed almost identical protein patterns to each other. The remaining isolates (64a1) from *Tephrosia purpurea*, (110b2) from *Rhynchosia hirta* and (25ab1) from *Neonotonia wightii* have no significant relationship with one another or with any members belonging to cluster 10 and the protein profiles of isolates

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

within this group are heterogeneous.

Cluster 11. Cluster 11, comprising 46 isolates and one reference strain, *R. tropici* IIA USDA 9039 (CFN 299), is the largest of the rhizobia groups. Strains isolated from seven different *Acacia* spp. are present in this large set of strains. The low linkage of cluster 11 with the previous groups in the dendogram, indicates that these organisms form a relatively distinct groupline.

Subgroup 11a consists of two tight groups (11a1 and 11a2) within which the protein profiles of members are remarkably identical, with an intersubgroup similarity value of $r = 0{,}81$.

Subgroup 11a1. The isolates belonging to this group clearly display protein profiles identical to each other with a correlation value of $r = 0{,}83$, indicating that the relationship among these isolates is significant. Subgroup 11a1 consists of isolates (125) from *Indigofera woodii*, (127) from *Sutherlandia frutescens*, (94) from *Vigna subterranea*, (129bt) from *Indigofera* sp., (52w) from *Indigofera daleoides*, (99a) from the tree legume *Albizia adianthifolia*, (72a) from *Tephrosia purpurea*, (54at) from the tree legume *Acacia dealbata*, (661a) from the tree legume *Acacia robusta* and (62c) from *Tephrosia multijuga*. Three of the strains isolated from tree legumes clustered within this group.

Subgroup 11a2. This tight association contains twelve isolates of which four were isolated from tree legumes. Protein profiles indicate that members of this group are closely related to one another. Subgroup 11a2 consists of (151a) from the tree legume *Acacia podalyriaefolia* (122a) from *Macrotyloma axillare*, (126) from *Lessertia perennans*, (119) from the tree legume *Acacia mearnsii*, (120t) from *Pisum sativum*, (154) from *Otholobium bracteolatum*, (121a) from *Lotononis* sp., (101c1) from *Zornia capensis*, (156g) from the tree legume *Millettia caffra*, (89b) from *Sesbania punicea*, (130) from the tree legume *Acacia xanthoploea* and (155a1) from *Podalyria myrtillifolia*.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Subgroup 11b. Subgroup 11b forms several distinct sublines that are specifically linked to subgroup 11a. However, visual analysis of the protein patterns indicates that these organisms exhibit a highly significant affinity for one another as well as for members in subgroup 11a. The reference strain *R. tropici* llA USDA 9030 (CFN 299) belongs to this cluster and possesses exactly the same protein profile as strain (150b3) isolated from *Spartium junceum*. The rest of the isolates includes (126t) from *Lessertia perennans*, (90b) from the tree legume *Chamaecrista mimosoides*, (155d2 and 155a2a) from *Podalyria myrtillifolia*, (154t) from *Otholobium bracteolatum*, (85b2) from the tree legume *Acacia xanthophloea*, (74b) from *Indigofera arrecta*, (76b) from *Eriosema psoraleoides*, (108b2) from *Indigofera swaziensis*, (117a2) from *Indigofera woodii*, (114b2) from *Pseudarthria hookeri*, (52f) from *Indigofera daleoides* and (62a2) from *Tephrosia multijuga*.

Subgroup 11c. Despite some distinct sublines the linkages are well within the boundaries of association with cluster 11. Some differences appear in the protein bands of members within sub-group 11c, but overall the similarities that exist in the protein profiles of these organisms for each other as well as for members in cluster 11, clearly place them as a sub-group within cluster 11.

Subgroup 11c consists of 15 isolates, six of which are from tree legumes: (138bv) from the tree legume *Acacia melanoxylon*, (148a1) from *Melilotus indica*, (131c) from *Dipogon lignosus*, (136) from *Lotus hispidus*, (129b) from *Indigofera* sp., (137a) from *Vicia* sp., (98b1) from the tree legume *Bolusanthus speciosus*, (138b) from the tree legume *Acacia melanoxylon*, (123at) from *Desmodium repandum*, (79b) from the tree legume *Acacia caffra*, (57a3) from *Tephrosia semiglabra*, (144c2v) from *Psoralea pinnata*, (96a) from *Rhynchosia hirta*, (84b1) from the tree legume *Schizolobium parahybum* and (146a1) from the tree legume *Acacia cyclops*. Overall, the members of cluster 11 exhibit very similar protein profiles and the two prominent protein bands that exist within each pattern are significant and clearly reveal a very close association.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Cluster 12. Cluster 12 forms a tight cluster composed of five strains. The results of our analysis of protein patterns indicate clearly that these five strains possess profiles almost identical to each other, with an intracluster similarity value of $r = 0{,}80$. Cluster 12 includes strains from (80b) *Crotalaria pallida*, (77b) from *Desmodium velutinum*, (79b) from *Acacia caffra*, (54b) from *Acacia dealbata* and (59) from *Spenostylis angustifolia*. The subline forms a loose association with cluster 11.

Cluster 13. Cluster 13 is separated into two heterogeneous subgroups (13a and 13b), with an intersubgroup similarity value of $r = 0{,}63$.

Subgroup 13a. A diversity of protein profiles was present within members of subgroup 13a (subdivided into 13a1, 13a2 and 13a3).

Subgroup 13a1 forms a loose association between four new isolates and the reference strain *R. leguminosarum* bv. *viciae* L113. The new isolates consist of (62a2v) from *Tephrosia multijuga*, (92a1) from the tree legume *Acacia sieberana* var. *woodii*, (86c2) from the tree legume *Bauhinia variegata* and (26a) from *Strongylodon macrobotrys*.

Subgroup 13a2. The protein profiles of members within this group are rather homogeneous and this group includes the isolates (52b) from *Indigofera daleoides*, (66b2) from *Acacia robusta*, (122b) from *Macrotyloma axillare*, (63c) from *Indigofera oxytropis*, (117a1) from *Indigofera woodii*, (147) from *Acacia longifolia*, (121) from *Lotononis* sp., (151c2) from *Acacia podalyriaefolia*, (46a) from *Acacia sieberana* var. *woodii*. and from (146a2) *Acacia cyclops*. Five strains isolated from different *Acacia* sp. appear in this group.

Subgroup 13a3. Five of the isolates within subgroup 13a3 exhibit similar protein profiles namely, (131a) from *Dipogon lignosus*, (73a2) from the tree legume *Chamaecrista mimosoides*, (151c1t) from the tree legume *Acacia podalyriaefolia*, (26c) from *Strongylodon macrobotrys*, (113b) from *Sesbania punicea*. These five isolates possess a

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

few prominent protein bands that differ significantly from those of the remaining isolates in subgroup 13a3, and lessen the relationship between them. The remaining isolates include (104a1) from the tree legume *Chamaecrista* sp., (156b) from the tree legume *Millettia caffra*, (95a) from *Crotolaria vasculosa*, (109b) from the tree legume *Acacia dealbata* and (113c) from *Sesbania punicea*. The protein profiles of these isolates are very similar to one another. Overall, members of subgroup 13a1 and 13a2 were found to possess one prominent protein band, distinguishing them from subgroup 13a3.

Subgroup 13b. It is remarkable that the protein profiles which exist in six new isolates of subgroup 13b are identical to the reference strain *R. etli* Viking 1. This highly significant association was unexpected but surely confirms the identity of the new isolates. The intrasubgroup similarity value ($r = 0{,}86$) is not a representative reflection of the very high level of relationship that exists among these isolates and *R. etli* Viking 1. The isolates consist of (123at) from *Desmodium repandum*, (138bt) from the tree legume *Acacia melanoxylon*, (128at) from *Melolobium obcordatum*, (108a1v) from *Indigofera swaziensis*, (105av) from the tree legume *Chamaecrista stricta* and (33av) from *Crotalaria* sp..

Cluster 14. Cluster 14 contains six isolates exhibiting rather diverse protein patterns. The isolates consist of (123cr) from *Desmodium repandum*, (119a) from the tree legume *Acacia mearnsii*, (69c) from *Desmodium setigerum*, (46b) from the tree legume *Acacia sieberana* var. *woodii*, (12a) from *Indigofera rhytidocarpa* and (100a) from *Crotalaria doidgeae*.

Cluster 15. Cluster 15 consists of rather detached isolates forming distinct sublines with no specific relationship. The following isolates display similarities in their protein profiles: (150b2) from *Spartium junceum*, (138a) from the tree legume *Acacia melanoxylon*, (137b) from *Vicia* sp., (134b) from *Sutherlanda frutescens* and (121t) from *Lotononis* sp.. Isolate (88a2) from *Vigna unguiculata* forms a long individual line. Isolate (156d) from the tree legume *Millettia caffra* displays some similarity to (18a) isolated from *Pueraria lobata*. Isolates (69a) from *Desmodium setigerum* and (43) from *Tephrosia purpurea* possess similarities in their protein bands and to a lesser extent to (13d) from *Lotononis bainesii*. The

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

three isolates (22) from *Flemingia grahamiana*, (51c) from the tree legume *Chamaecrista biensis* and (32a) from *Crotalaria pallida* exhibit significant affinity for one another based on their protein profiles in having a corresponding, prominent protein band. Isolate (27a) from *Teramnus labialis* is similar in its protein profile to the latter three isolates but needs some compensation for suitable congruency. The remaining three isolates (129ck) from *Indigofera* sp., (39a) from the tree legume *Chamaecrista absus* and (50c) from the tree legume *Chamaecrista comosa* display rather heterogeneous protein patterns and exhibited no specific association.

Cluster 16. Cluster 16 is separated into two subgroups (16a and 16b). Members of this cluster produced yellow colonies on YEB medium and appeared as rather long thin rods with the typical club-Y shaped form of the rhizobia. Protein bands between these two subgroups are rather diverse.

Subgroup 16a. Subgroup 16a consists of five isolates, four of which were isolated from tree legumes. Isolate (97b2b) is from the tree legume *Cassia didymobotrya*, (130a) from the tree legume *Acacia xanthoploea*, (90c) from the tree legume *Chamaecrista mimosoides*, (47eh) from the tree legume *Cassia floribunda* and (7a) from *Alysicarpus rugosus*. Analysis of protein patterns confirms that the strains isolated from the tree legumes belong to the same group, while differences appeared in the profile of strain (7a).

Subgroup 16b. Subgroup 16b includes the reference strain *R. leguminosarum* bv. *viceae* USDA 2370. The rest of the strains consists of (85a1) from the tree legume *Acacia xanthoploea*, (44b) from *Crotalaria* sp., (110b) from *Rhynchosia hirta*, (41a) from *Indigofera arrecta*, (19) from *Neonotonia wightii*, (47d) from the tree legume *Cassia floribunda*, (155a2t) from *Podalyria myrtillifolia*, (146b) from the tree legume *Acacia cyclops*, (144a2t) from *Psoralea pinnata* and (140a) from *Virgilia divaricata*. Isolate (1421av) from *Tephrosia capensis* forms a long individual line. The protein patterns of this subgroup are rather diverse (similarity value $r = 0{,}61$) but some distinct corresponding bands are shared between them, indicating the affinity which exists between them.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Cluster 17. Cluster 17 contains three isolates with no specific association. The two isolates (158e) from the tree legume *Acacia nigrescens* and (15b1) from *Desmodium tortuosum* possess comparable similar protein profiles but display some differences from isolate (52a) from *Indigofera daleoides*.

Cluster 18. Cluster 18 is a loose association with isolates exhibiting no specific association. The subline clearly indicates that no specific relationship exists with other clusters. Cluster 18 contains the reference strain *Rhizobium* sp. LMG 6463, indicating no specific association. The remainder of the members within cluster 18 exhibits very diverse protein patterns, indicating an uncertain relationship consisting of isolates (105b) from *Chamaecrista stricta*, (108c) from *Indigofera swaziensis*, (51ct) from *Chamaecrista biensis*, (142 2b1) from *Psoralea asarina* (28b1) from *Desmodium tortuosum*, and (152) from *Acacia saligna*.

Cluster 19. The protein patterns of members within cluster 19 are very heterogeneous and exhibit a low intracluster similarity value of $r = 0{,}50$. The two reference strains *S. meliloti* USDA 1955 (M1) and *S. meliloti* USDA 1002 (ATCC 9930) are included in this group and display some resemblance in the upper half of their protein patterns with each other, although differences are clearly noticeable. The following isolates form a loose association exhibiting no close affinity to the two reference strains, and consist of (111a) from *Vicia sativa* which possesses a rather similar protein pattern to that of *S. meliloti* USDA 1955; (114b) from *Pseudarthria hookeri* which displays differences, while the next two isolates; (54av) from *Acacia dealbata* and (92a2) from *Acacia sieberana* var. *woodii* share some similarities in their protein bands with that of *S. meliloti* USDA 1002. (93c2) from *Indigofera arrecta* and (68b) from *Mucuna coriacea* differ from each other and previously mentioned isolates. Thus, similarities in protein bands are detectable within this association. These isolates produced cream colonies on YEB medium, and differ from the following four isolates: (125v) from *Indigofera woodii*, (103a) from *Indigofera hilaris*, (117a2) from *Indigofera woodii* and (126w) from *Lessertia perennans*, which, in constrast, produced yellow colonies on the same medium. The remaining isolates (129bv) from *Indigofera* sp., (123ct) from *Desmodium*

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

repandum, (141t) from *Trifolium pratense* and (156e) from *Millettia caffra*, produced very similar protein profiles but differ from the rest of the members within this cluster.

Cluster 20. Cluster 20 exhibits a loose association forming a subline with no specific affinity. The two reference strains *R. galegae* LMG 6215 and *R. galegae* USDA 4128 are closely related to each other (level of similarity, $r = 0{,}88$), whereas the remaining isolates, (121v) from *Lotononis* sp., (116b) from *Indigofera oxalidea*, (68c1) from *Mucuna coriacea*, (115a) from *Indigofera swaziensis*, (140b) from *Virgilia divaricata* and (47ev) from the tree legume *Cassia floribunda* are well separated, possessing markedly different protein profiles.

Cluster 21. Most of the 25 isolates included in cluster 21 are slow growers. Small groups are formed within this cluster with sublines exhibiting no specific relationship. The two isolates (77a) from *Desmodium velutinum*, and (98a) from the tree legume *Bolusanthus speciosus* possess some similar protein bands. Isolate (83c) from *Indigofera incarnata* reveals no significant affinity and (81b) from *Mucuna coriacea* forms a long individual line. The protein profiles of the next four isolates share one prominent protein band: (44c) from *Crotalaria* sp., (62e2) from *Tephrosia multijuga*, (82c2) from *Tephrosia purpurea* and (98a1) from the tree legume *Bolusanthus speciosus*. The latter needs some compensation. Marked differences can be detected in the protein bands of the following four isolates: (72d) from *Tephrosia purpurea*, (109c) from the tree legume *Acacia dealbata*, (112b) from *Indigofera* sp. and (55d) from *Rhynchosia monophylla*. However, their association with one another can be seen clearly in the corresponding prominent protein band that exists in their protein profiles. Isolate (149a) from *Medicago sativa* differs significantly in its protein profile. The two isolates (127at) from *Sutherlandia frutescens* and (35b1) from *Pseudarthria hookeri* possess identical protein bands. The tight clustering of isolates (133) from *Vicia* sp., (144c3v) from *Psoralea pinnata* and (117c2) from *Indigofera woodii* is an indication of very similar protein bands. Visual analysis of protein patterns indicates that the relationship (similarity value of $r = 0{,}85$) among isolates (145) from the tree legume *Acacia mearnsii*, (136aa) from *Lotus hispidus*, (150a3) from *Spartium junceum* and (144c1) from *Psoralea pinnata* is

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

significant. Isolate (131b) from *Dipogon lignosus* in its protein pattern displays some similarity in its protein patterns with these four organisms. Isolates (84a2) from the tree legume *Schizolobium parahybum* and (92b) from the tree legume *Acacia sieberana* var. *woodii* differ considerably from the other isolates within cluster 21 with regard to their protein profiles.

Cluster 22. Cluster 22 consists of small groups of isolates exhibiting affinity for one another, but protein profile divergence between these groups is significant. Isolate (115b) from *Indigofera swaziensis* exhibits a loose association with the other organisms. The following three isolates, (157b) from the tree legume *Albizia adianthifolia*, (95c) from *Crotalaria vasculosa* and (144bt) from *Psoralea pinnata* may be grouped together. The next small group of isolates consist of (119c) from the tree legume *Acacia mearnsii*, (151c1v) from the tree legume *Acacia podalyriaefolia*, (140a) from *Virgilia divaricata*, (1432ak) from *Psoralea asarina*, (150a1t) from *Spartium junceum* and (28av) from *Desmodium tortuosum*. This group of organisms belong together on the basis of their protein profiles. The very close association that exists between (120v) from *Pisum sativum*, (130at) from the tree legume *Acacia xanthoploea*, (119t) from the tree legume *Acacia mearnsii*, (117c1b) from *Indigofera woodii* and (131t) from *Indigofera jucunda* is confirmed by the identical protein patterns shared within this grouping. Isolate (123av) from *Desmodium repandum* exhibits affinity with this group. Three of the isolates in cluster 22, namely (123cv) from *Desmodium repandum*, (112a) from *Indigofera* sp. and (57a4) from *Tephrosia semiglabra*, share a very prominent corresponding protein band within their protein patterns. The remaining isolates (75) from *Tephrosia glomeruliflora*, (23b) from *Desmodium repandum*, (1421at) from *Tephrosia capensis*, (93c1) from *Indigofera arrecta* and (106a) from *Rhynchosia caribaea* possess very heterogeneous protein profiles.

Cluster 23. Cluster 23 consists of a rather heterogeneous compilation of isolates and clearly forms a distinct subline with no specific relationship. The reference strain *Rhizobium loti* LMG 4268T1v clusters within this group. The two isolates (141v) from *Trifolium pratense*

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

and (143 2b2t) from *Psoralea asarina* were found to be identical to each other on the basis of their protein bands. Isolate (158b) from the tree legume *Acacia nigrescens* exhibits affinity with the previous two organisms. Isolates (125c) from *Indigofera woodii* and (129cj) from *Indigofera* sp. display similar protein patterns (r = 0,89). Visual analysis of the protein profiles of isolates (26ct) from *Strongylodon macrobotrys* and (124v) from *Rhynchosia minima* indicate that these two organisms are related to each other. Similarities in the protein profiles of the following two isolates, (154v) from *Otholobium bracteolatum* and (76a) from *Eriosema psoraleoides* can be detected. The very high relationship (similarity value of r = 0,93) that exists among the following three isolates: (100c) from *Crotalaria doidgeae*, (103c) from *Indigofera hilaris* and the reference strain *R. loti* LMG 4268T1(v) (the v in brackets will be explained later), is displayed in their identical protein patterns. These strains display typical rhizobia rods under the phase microscope. Isolate (71b) is clearly related to the latter three organisms, although some small differences in its protein profile distinguish it within this association.

Cluster 24. Cluster 24 consists of three isolates which clearly form a distinct subline with no specific association with other clusters. These organisms form club-shaped rods typical of rhizobia strains. Analysis of the protein profiles confirms the relationship that exists between isolate (129e) from *Indigofera* sp. and (118v) from *Argyrolobium pauciflorum*. Strain (45a) from the tree legume *Erythrina lysistemon* displayed no specific relationship.

The results of the present study provide taxonomic information concerning the new isolates and expand previous results where the type of host plant played a role in the classification system (3, 24, 26, 30). On the basis of their whole-cell protein profiles a large number of the new isolates are members of the existing recognized genera *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium*. However, some of the isolates are clearly differentiated and cannot be assigned to any of the previously described recognized species. Further genetic studies are necessary to clarify the taxonomic position of these new isolates that clustered, forming

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

sublines linked to groups of known species, but which cannot be assigned to a particular species, as well as of those that are well separated forming distinct sublines with no specific relationship, since it has not been possible to derive precise boundary conditions based on *r*-values of protein profile relatedness to delineate taxonomic rank. The data did allow for the identification of groups and it is logical to conclude that some of these isolates represent members of new species or new genera within the rhizobia taxonomy.

Progressive improvements in modern bacterial taxonomy have led to the unravelling of new species within the classification of the legume root-nodulating bacteria in recent years (7, 17, 20, 24). These developments have not been without controversy, e.g. the proposal that the placing of the fast-growing soybean-nodulating rhizobium *R. fredii* be reclassified in a new genus, *Sinorhizobium*, as *S. fredii* by Chen et al. (5) using numerical taxonomy. Jarvis et al. (15) demonstrated with 16S rRNA sequence analysis that the sequences of the fast-growing soybean rhizobia are identical to the sequence of *R. meliloti*, thus providing evidence for rejecting the proposed new genus. De Lajudie et al. (7), using a polyphasic approach, opposed this and confirmed that *R. meliloti* and *R. fredii* should be reclassified in a new genus, *Sinorhizobium*, and in addition proposed two new species, *Sinorhizobium saheli* and *Sinorhizobium teranga,* both able to nodulate *Sesbania* and *Acacia* spp., *Leucaena luecocephala,* and *Neptunia oleracea.* The results obtained with the polyphasic approach used by de Lajudie et al. (7) are confusing and showed that there are low levels of relationship between *S. meliloti* and *S. fredii* as far as SDS-PAGE of whole-cell proteins, auxanographic characteristics and DNA-DNA homology are concerned, in contrast with the high level of similarity that was obtained between these two species with 16S rRNA sequence analysis. Previous data published (27) using SDS-PAGE of whole cell proteins, where the same type of reference strains were included for *S. meliloti* and *S. fredii*, as well as results obtained in this study, confirm that *S. meliloti* (LMG 6133T) and *S. fredii* (LMG 6217T) are very closely related and support the conservation of *R. fredii* and *R. meliloti*, while not supporting the interpretation of de Lajudie et al. (7). The two newly proposed species, *Sinorhizobium teranga* and *Sinorhizobium saheli* did not show any signs of a close relationship with *S. fredii* in the interpretation by de Lajudie et al.(7) of SDS-PAGE of whole cell proteins,

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

auxanographic characteristics and DNA-DNA hybridization analysis.

The emergence of new molecular approaches has led to the creation of the following new species: within the genus *Rhizobium*, *R. tropici* (type IIA and IIB) (26), *R. etli* (30), *R. galegae* (23), and *R. huakuii* (3) and within the genus *Bradyrhizobium*, *B. elkanii* (19). Furthermore, *R. tropici* is differentiated into two subgroups, IIA and IIB by means of multilocus enzyme electrophoresis, DNA-DNA hybridization, sequence analysis of 16S rRNA, electrophoretic patterns of PCR products and phenotypic characteristics (21, 26, 34). The two subgroups of the species *R. tropici* were found to be only 36%/39% related, based on DNA-DNA hybridizations studies (21, 26), indicating two different species. Our results confirm the distinctness of each group, and it is of interest that the protein profile of strain (98b2) isolated from the tree legume *Bolusanthus speciosus* that is indigenous to South Africa, showed similarity to that of *R. tropici* USDA 9030 (= CIAT 899) IIB.

The culture of the reference strain *Rhizobium loti* LMG 4268T1 consisted of two different organisms. Pure cultures of the organisms were obtained by isolating single colonies which could be clearly distinguished from each other on YEB medium, but difficult to distinguish on YEM medium supplemented with Congo Red. The one type displayed a cream coloured colony, and consisted of extremely small cells compared with those of the second type, which formed yellow colonies. Examination of cells of both colonies by light microscopy revealed typical rhizobia cells. The cream colony was assigned the font (t) (*R. loti* 4268T1(t)) (cluster 8a). The similarity in protein profiles between *R. loti* 4268T1(t) and *R. tropici* IIB is striking. The yellow colony to which we assigned the font (v) (*R. loti* 4268T1(v)) belongs to a cluster lineage totally different from *R. loti* 4268T1(t), exhibiting an identical protein profile to members within cluster 23. The only conclusion we can draw is that the reference strain originally contained two different rhizobia which were easily detected on YEB medium. It is significant that both strains are misclassified.

In the case of *R. tropici* IIA we found a very close relationship with strain (150 b3) from *Spartium junceum*. From our data it is obvious that the new members within cluster 11, isolated from a wide variety of legumes encompassing *R. tropici* IIA USDA 9039 (=CFN 299), share enough similar prominent protein bands to support the conviction that this new

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

isolate complex should belong with *R. tropici* IIA. *R. tropici* is known to nodulate both *Phaseolus vulgaris*, *Macroptilium atropurpureum* and *Leucaena leucocephala* (26, 28). According to Laguerre et. al (21), members of *R. tropici* and *R. etli* have been found only in the Americas. *R. etli* may be distinguished from other *Rhizobium* species on the basis of 16S rRNA sequence analysis, as well as the fact that strains nodulate and fix nitrogen on *Phaseolus vulgaris* L. exclusively (30). Our results also separate *R. etli* from the other *Rhizobium* species. In addition rhizobia (subgroup 13b) were isolated from legumes such as *Desmodium* sp., *Melolobium* sp., *Indigofera* sp. and from the two tree legumes, *Acacia melanoxylon* and *Chamaecrista stricta*, which surprisingly exhibit identical protein profile relatedness to that of *R. etli* and is similar enough to the latter to warrant assigning identical taxonomic status to them. Our research evidence is in conflict with previous suggestions that *R. etli* isolates are restricted to the Americas and nodulating only beans (25). Contrary to the hitherto accepted opinion, we suggest that the host-plant specificity for *R. etli* be expanded. Of importance are the two strains that were isolated from the tree legumes.

R. loti that nodulates *Lotus* spp. is phylogenetically related to *R. huakuii* that nodulates *Astragalus sinicus* (3, 24). Strains that were isolated from *Flemingia* sp., *Indigofera* sp., *Pseudarthria* sp., *Bolusanthus* sp. and *Rhynchosia* sp. grouped together with *R. loti* and *R. huakuii*. It is interesting to note that strain (98d2) isolated from the tree legume *Bolusanthus speciosus*, clustered together with *R. huakuii*. The role of host plant specificity as a criterion in rhizobia taxonomy becomes more questionable since most of the strains did not group systematically according to their host plant range. The protein profile of the reference strain *R. loti* USDA 3471 differs enough from members within this group (cluster 2a) to warrant placement of this organism in a separate cluster (cluster 15).

R. leguminosarum bv. *viciae* nodulates *Vicia, Pisum, Lens,* and *Lathyrus* spp. (18). It is interesting to note that isolates from five different *Acacia* spp. exhibit affinity with *R. leguminosarum* bv. *viciae* L113 (Cluster 13a1). The two reference strains *R. leguminosarum* bv. *viciae* L113 and *R. leguminosarum* bv. *viciae* USDA 2370 occupy distinct positions within the dendrogram (Fig.2), which eliminates the possibility that these two organisms are equivalent.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

S. meliloti comprises highly divergent groups of strains (A1, A2 and B) based on multilocus enzyme electrophoresis (11) and PCR (6) studies. Our results differentiate between members of these two groups as well as those of subgroup A1 and A2 (Fig. 3). Although strain *S. meliloti* USDA 1955 (M1) from group B and *S. meliloti* USDA 1002 T (ATCC 9930) from group A1 grouped in the same cluster (cluster 19) there is a definite separation (similarity value of $r = 0{,}64$) between these two groups. At the same time, they showed low affinity with the other reference strains of the *S. meliloti* - *S. fredii* cluster (cluster 7). The results obtained by Chen et. al. (4), using numerical analysis, also revealed that *S. meliloti* USDA 1002 T exhibited low levels of similarity with the *S. fredii* strains. In light of these results, the status of *S. meliloti* USDA 1002 T and *S. meliloti* USDA 1955 as representatives of *S. meliloti* strains is questionable. *S. meliloti* USDA 1954 from group A2 is well separated from the previous isolates and exhibits a very close relationship with the rest of the *S. meliloti* strains in cluster 7, which are closely related to each other (16). The reference strain *Rhizobium* sp. LMG 8311, isolated from *Acacia* sp., as well as our own isolate (85a3) from *Acacia xanthophloea*, exhibits a very close relationship to the *S. meliloti* - *S. fredii* group. The similarity in protein profiles is striking, thereby confirms their placement in cluster 7. *Agrobacterium radiobacter* K84 was also linked with the *S. meliloti* - *fredii* group. According to Willems and Collins (38) it is significant that *Rhizobium* and *Agrobacterium* species are phylogenetically interwoven on the basis of 16S rRNA gene sequences.

The reference strains of *R. leguminosarum* bv. *trifolii* form an assemblage of heterogeneous species. They are scattered between subgroup 2b, subgroup 6b and cluster 9, which indicates that these organisms constitute a very diverse group. According to Allen and Allen (1), the host affinities of *R. leguminosarum* bv. *trifolii* strains are limited to *Trifolium* species. Unique host-plant symbiotic affinities exist between *Trifolium* species and strains of *R. leguminosarum* bv. *trifolii* (22). Rhizobial strains that are of European or American origin lack effective symbiobility with most native African legume species (12). It is most interesting that the new isolate (62d2) from *Tephrosia multijuga* revealed a protein pattern identical to that of the reference strain *R. leguminosarum* bv. *trifolii* SARCC SAC2 (cluster 9). Analysis of the protein profiles indicates that the reference strain *R. leguminosarum* bv. *trifolii* LMG 6119 is more closely related to *R. leguminosarum* LMG 6294T1, isolated from

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Lathyrus sp., and *R. leguminosarum* LMG 4295, isolated from *Vicia sativa* (cluster 2b), than to any of the other *R. leguminosarum* bv. *trifolii* species for which the protein profile information is available in this study. According to Allen and Allen (1), there exists a mutual relationship between *Lathyrus*, *Pisum*, *Vicia*, and *Lens* species and their rhizobia. The reference strain *R. leguminosarum* bv. *trifolii* USDA 2046 (subgroup 6b1) displayed an almost identical protein profile to that of isolate (101a1) isolated from *Zornia capensis*. We can only mention that isolate (153) isolated from *Trifolium angustifolium* revealed almost identical protein patterns to that of *R. leguminosarum* bv. *trifolii* LMG 4255. Strains (141t and 141v) isolated from *Trifolium pratense* belong to different clusters (cluster 19 and cluster 23) and strain (3), isolated from a *Trifolium* sp., showed a protein profile identical to *S. meliloti* strains in cluster 7. Attention should be paid to the fact that the *R. leguminosarum* bv. *trifolii* complex is not restricted to strains isolated from *Trifolium* sp. and *vice versa*.

Our SDS-PAGE of whole-cell protein data corroborate the data obtained from DNA homology studies by Lindström (23) that *R. galegae* exhibits no specific affinity for any currently recognized *Rhizobium* species. *R. galegae* was also found not to be closely related to any other isolate within the dendrogram (Fig. 2).

Most of the slow-growing new isolates were found to be members of the genus *Bradyrhizobium* (cluster 4). These organisms were isolated from a variety of legume host-plants. Despite some uncertainty within the taxonomy of the tree legume rhizobia (40) it is evident from our data that the tree rhizobia phenotypically exhibit considerable diversity and that some of the strains isolated from the tree legumes *Chamaecrista mimosoides*, *Chamaecrista biensis*, *Chamaecrista comosa*, *Acacia robusta* and *A. sieberana* var. *woodii* are closely related to members of the genus *Bradyrhizobium*. Recently, Dupuy et al. (8) examined the relationship of rhizobia isolated from *Acacia albida* and found that most of the strains clustered with *Bradyrhizobium* strains. The taxonomic status of the tree legume rhizobia is unsatisfactory since there has been no comprehensive study of relationships among a large collection of different tree rhizobia, although the limited data on the tree rhizobia (9, 40) suggest diversity and the need for research. The present study demonstrates that the new isolates from different tree legumes form a heterogeneous assemblage and a plexus of lines

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

and groups of various relationships. These isolates were scattered throughout the dendrogram (Fig. 2), except for clusters 10 and 23 where none of the tree rhizobia was present. According to Dreyfus and Dommergues (9) the *Acacia* species are nodulated by *Rhizobium* and *Bradyrhizobium* strains. De Lajudie et al. (7) created two new species, *Sinorhizobium saheli* and *Sinorhizobium teranga* from the new Senegalese strains that were isolated *inter alia* from six different *Acacia* species. A remarkable result that has become manifest from our study is that isolates from different *Acacia* and *Chamaecrista* species and to a lesser extent from *Sesbania* sp. mostly fall in the same clusters. Members isolated from the different *Acacia* species dominated most of the clusters (Fig. 2) due to the fact that the *Acacia* species constituted a majority of our collection of the tree legumes. The fact that the new isolates from different *Acacia* species clustered with *Rhizobium, Sinorhizobium* and *Bradyrhizobium* species, as well as their association with distinct clusters, is a demonstration that relationships of members from *Acacia* sp. may be incoherent.

The rest of the tree rhizobia isolated from different spp. of *Albizia*, *Bauhinia*, *Bolusanthus*, *Cassia*, *Erythrina*, *Millettia* and *Schizolobium* represents a phenotypically very broad range of organisms and from these new results it seem obvious that any comprehensive taxonomic exploration of tree rhizobia will result in a proliferation of new species or genera.

Evidence to date points to the conclusion that *Cassia* species lack nodules (1, 31). It is interesting to note that we did isolate rhizobia from two different *Cassia* species showing relationship with members isolated from *Acacia*, *Chamaecrista* and *Bolusanthus*.

Sesbania species are nodulated by *Rhizobium* and/or *Azorhizobium* strains (10). It should be noted that isolate (40b) from *Sesbania bispinosa* belongs to the *Bradyrhizobium* complex. We found that the reference strain *Azorhizobium caulinodans* LMG 6465T showed affinity towards the slow growing isolates clustered within the *Bradyrhizobium* group. According to Dreyfus et. al. (10) *Azorhizobium caulinodans* exhibited no specific relationship with the genera *Rhizobium* and *Bradyrhizobium* on the basis of DNA-rRNA hybridization analysis.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

In conclusion it is obvious that genetic studies are needed to further clarify the taxonomic and phylogenetic position of the new isolates among members of the genus *Rhizobium* and related genera. Along with the ever-changing taxonomic situation of the genus *Rhizobium* and from our data it is evident that the new isolates not only make up phenotypically incoherent groups but also reflect close relationships among existing species. This study emphasizes the vast range of new rhizobia that is still to be detected from the considerable range of remaining nodulated legume plants.

REFERENCES

1. **Allen, O.N., and E.K Allen.** 1981. The Leguminosae. A Source Book of Characteristics Uses and Nodulation. The University of Wisconsin Press, USA.
2. **Caetano-Anollés G., P. M. Gresshoff.** 1991. Plant genetic control of nodulation. Annu. Rev. Microbiol. **45:**345-382.
3. **Chen, W. X., G. S. Li, Y. L. Qi, E. T. Wang, H. L. Yuan, and J. L. Li.** 1991. *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. Int. J. Syst. Bacteriol. **41:**275-280.
4. **Chen, W. X., E. W. S. Wang, Y. Li, X. Chen, and Y. Li.** 1995. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. Int. J. Syst. Bacteriol. **45**:153-159.
5. **Chen, W. X., G. H. Yan, and J. L. Li.** 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. **38:**392-397.
6. **de Bruijn, F. J.** 1992. Use of repetetive (Repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180-2187.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

7. **de Lajudie, P., A. Willems, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M. D. Collins, B. Dreyfus, K. Kersters, and M. Gillis.** 1994. Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov.. Int. J.Syst. Bacteriol. **44:**715-733.

8. **Dupuy, N, A. Willems, B. Pot, D. Dewettinck, I. van den Bruaene, G. Maestrojuan, B. Dreyfus , K. Kersters, M. D. Collins, and M. Gillis.** 1994. Phenotypic and genotypic characterization of Bradyrhizobia nodulating the leguminous tree *Acacia albida*. Int. J. Syst. Bacteriol. **44:**461-473.

9. **Dreyfus, B. L., and Y. Dommergues.** 1981. Nodulation of *Acacia* species by fast- and slow-growing tropical strains of *Rhizobium.* Appl. Environ. Microbiol. **41:**97-99.

10. **Dreyfus, B., J. L. Garcia, and M. Gillis.** 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulation nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bacteriol. **38:**89-98.

11. **Eardly, B. D., L. A. Materon, N. H. Smith, D. A. Johnson, M. D. Rumbaugh, and R. K. Selander.** 1990. Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. Appl. Environ. Microbiol. **56:**187-194.

12. **Friedericks, J. B., C. Hagedorn, and S. W. Vanscoyoc.** 1990. Isolation of *Rhizobium leguminosarum* (biovar *trifolii*) strains from Ethiopian soils and symbiotic effectiveness on African annual clover species. Appl. Environ. Microbiol. **56:**1087-1092.

13. **Gao, J. L., J. G. Sun, Y. LI, E. T. Wang, and W. X. Chen.** 1994. Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, China. Int. J. Syst. Bactriol. **44:**151-158.

14. **Holding, A. J., and J. G. Collee.** 1971. Routine biochemical tests, p. 1-33. *In* Norris and Ribbons (Editors), Methods in Microbiology, 6A. Academic Press Inc., New York.

15. **Jarvis, B. D. W., H. L. Downer, and P. W. Young.** 1992. Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. Int. J. Syst. Bacteriol. **42:**93-96.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

16. **Jarvis, B. D. W., M. Gillis, and J. De Ley.** 1986. Intra- and intergeneric similarities between the ribosomal ribonucleic acid cistrons of *Rhizobium* and *Bradyrhizobium* species and some related bacteria. Int. J. Syst. Bacteriol. **36:**129-138.

17. **Jarvis, B. D. W., and S. W. Tighe.** 1994. Rapid identification of *Rhizobium* species based on cellular fatty acid analysis. Plant and Soil. **161:**31-41.

18. **Jordan, D. C.** 1984. Family lll. *Rhizobiaceae,* P. 234-242. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.

19. **Kuykendall, L. D., B. Saxena, T. E. Devine, and S. E. Udell.** 1992. Genetic diversity in *Bradyrhizobium japonicum* (Jordan, 1982) and a proposal for *Bradyrhizobium elkanii* sp. nov. Can. J. Microbiol. **38:**501-505.

20. **Laguerre, G., M. Allard, F. Revoy, and N. Amarger.** 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. **60:**56-63.

21. **Laguerre, G., M. P. Fernandez, V. Edel, P. Normand, and N. Amarger.** 1993. Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L.. Int. J. Syst. Bacteriol. **43:**761-767.

22. **Leung, K., S. R. Strian, F. J. de Bruijn, and P. J. Bottomley.** 1994. Genotypic and phenotypic comparisons of chromosomal types within an indigenous soil population of *Rhizobium leguminosaurm* bv. *trifolii* Appl. Environ. Microbiol. **60:**416-426.

23. **Lindström, K.** 1989. *Rhizobium galegae*, a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. **39:**356-367.

24. **Martínez-Romero, E.** 1994. Recent developments in *Rhizobium* taxonomy. Plant Soil **161:**11-20.

25. **Martínez-Romero, E., M. Flores, S. Brom, D. Romero, G. Dávilla, and R. Palacios.** 1988. *Rhizobium phaseoli:* a molecular genetics view. Plant Soil **108:**179-184.

26. **Martínez-Romero, E., L. Segovia, F. M. Mercante, A. A. Franco, P. Graham, and M. A. Pardo.** 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41:**417-426.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

27. **Moreira, F. M. S., M. Gillis, B. Pot, K. Kersters, and A. A. Franco.** 1993. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. Syst. Appl. Microbiol. **16:**135-146.

28. **O'Connell, K. P., and J. Handelsman.** 1993. Foliar chlorosis in symbiotic host and nonhost plants induced by *Rhizobium tropici* type B strains. Appl. Environ. Microbiol. **59:**2184-2189

29. **Pot. B., P. Vandamme, and K. Kersters.** 1994. Analysis of electrophoretic whole organism protein fingerprints, p. 493-521. *In* M. Goodfellow and T. O'Donnell (ed.), Chemical methods in prokaryotic systematics. John Wiley & Sons, Chichester, United Kingdom.

30. **Segovia, L., P. W. Young, and E. Martínez-Romero.** 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov.. Int. J. Syst. Bacteriol. **43:**374-377.

31. **Sprent, J. I.** 1994. Evolution and diversity in the legume-rhizobium symbiosis: chaos theory? Plant Soil **161:**1-10.

32. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846-849.

33. **Stegemann, H., W. Burgermeister, H. Francksen, and E. Krögerrecklenfort.** 1987. Panta-manual on gel electrophoresis and isoelectric focusing. Braunschweig, Institut für Biochemie.

34. **van Berkum, P., R. B. Navarro, and A. T. Vargas.** 1994. Classification of the uptake Hydrogenase-Positive (Hup^+) bean rhizobia as *Rhizobium tropici*. Appl. Environ. Microbiol. **60:**554-561.

35. **Vauterin, L., J. Swings, K. Kersters, M. Gillis, T. W. Mew, M. N. Schroth, N. J. Palleroni, D. C. Hildebrand, D. E. Stead, E. L. Civerolo, A. C. Hayward, M. Maraite, R. E. Stall, A. K. Vidaver, and J. F. Bradbury.** 1990. Towards an improved taxonomy of *Xanthomonas*. Int. J. Syst. Bacteriol. **40:**312-316.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

36. **Vauterin, L., and P. Vauterin.** 1992. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur. Microbiol. **1:**37-41.

37. **Vincent, J. M.**1970. A manual for the practical study of root-nodule bacteria, p. 73-97. *In* International biological programme handbook. Blackwell Scientific Publications, Ltd., Oxford.

38. **Willems, A., and M. D. Collins.** 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. **43:**305-313.

39. **Yanagi, M., and K. Yamasato.** 1993. Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107:**115-120.

40. **Zhang, X., R. Harper, M. Karsisto, and K. Lindström.** 1991. Diversity of *Rhizobium* bacteria isolated from root nodules of leguminous trees. Int. J. Syst. Bacteriol. **41:**104-113.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 4

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

THE SOUTH AFRICAN CONTEXT

Nine isolates which are commercially applied in the legume inoculant industry in South Africa as well as an isolate used as an inoculant against crown gall, have been included in this study. Indigenous South African counterparts have been found for most of these isolates.

Isolates SARCC TJ9 and SARCCC UD2 used for *Pisum sativum, Vicia faba, V. sativa, V. villosa* and other *Vicia* spp, and *Phaseolis vulgaris* and *P. coccineus* respectively showed close relationship to the indigenous isolates (125a) from *Indigofera woodii* and (89a1) from *Sesbania punicea*, all clustering in Sub-group 2b.

Bradyrhizobium japonicum strain SARCC XS21 used for *Arachis, Cajanus cajan, Crotalaria spp., Cyamopsis tetragonolobus, Indigofera spp., Lablab purpureus, Microptilium atropurpureum, M. lathyroides, Macrotyloma axillare, Psoralia phaseoloides, Stizolobium deeringianum, Vigna radiata, V. unguiculata, Voandzia subterranea, Neonotonia wightii, Pueraria thunbergiana, Stylosanthes spp.* except *S. guianensis* was closely related to isolate (123b) from *Desmodium repandum* in subgroup 4b1.

Bradyrhizobium japonicum strain SARCC XHX1 used for *Lespedeza cuneata* and *L. striata* was closely related to isolates (68d) from *Mucuna coriacea*, (73a1) from the tree legume *Chamaecrista mimosoides*, (48a) from *Tephrosia purpurea*, (101b) from *Zornia capensis*, (102a) from the tree legume *Chamaecrista biensis*, (70a) from *Crotalaria brachycarpa*, (87a) from *Rhynchosia hirta*, (74a) from *Indigofera arrecta*, (95b) from *Crotalaria vasculosa* and (61a1) from the tree legume *Chamaecrista comosa*, (55a) from *Rynchosia monphylla*, all clustering in sub-group 4b2.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Bradyrhizobium japonicum strain SARCC WB1 used for *Glycine max* was identical to (116a) from *Indigofera oxalidea*, (91a1) from *Tephrosia* sp., (82a) from *T.purpurea*, (101d) from *Zornia capensis* and (6a) from *Vigna vexillata*, clustering in sub-group 4d.

Rhizobium (Sinorhizobium) meliloti strain SARCC RF14 used for *Medicago sativa* and *Melilotus* spp. (annual medics and sweet clover), and RAK1 used for *Medicago murex* and *M. polymorpha*, were found to be closely related to (1) from *Meliloti alba*, (3) from *Trifolium* sp, (8) from *Medicago lupulina* and (85a3) from the tree legume *Acacia xanthophloea*, in cluster 7.

Rhizobium leguminosarum biovar *trifolii* strain SAC2 used for *Trifolium subterraneum*, *T. fragiferum* and *T. vesiculosum* possessed identical protein bands to (62d2) from *Tephrosia multijuga* and (153) from *Trifolium angustifolium*, and close relationships with (99b) from the tree legume *Albizia adianthifolia*, (21) from *Argyrolobium tomentosum* and (40d) from *Sesbania bispinosa*, in cluster 9.

Agrobacterium radiobacter strain SARCC K84 showed high relatedness to *Rhizobium (Sinorhizobium) meliloti* strains SARCC RF14 and RAK1, and the new isolates related to them.

The isolation of new cultures related to commercially applied strains might be of value in agriculture and should be further tested for competetiveness, infectivity and effectivity on the respective legumes.

A culture collection of indigenous rhizobia has been established and a databank of protein profiles of reference and indigenous cultures created. This will facilitate future identification and taxonomy of indigenous isolates. However, a large number of indigenous legumes still has to be investigated.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Further studies should include multilocus enzyme electrophoresis, phenotypic characterization, DNA-studies involving PCR of 16S rRNA genes and restriction fragment length polymorphism analysis, and randomly amplified polymorphic DNA, and host-plant interactions need to be done. It is our firm conviction that once again something new (rhizobial taxa) will come from Africa.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CHAPTER 5

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

CONCLUSIONS

SDS-PAGE of whole-cell proteins was carried out to determine the phenotypic-molecular taxonomic relationships among 346 indigenous South African rhizobia with representative rhizobia strains.

Analysis of protein profiles demonstrated that the new isolates constitute heterogeneous groups of bacteria, but that they are also identical or closely related to previously studied rhizobia strains.

Our data support the differentiation between the slow and fast-growing rhizobia, namely *Bradyrhizobium* and *Rhizobium* as distinct entities.

According to our results, the protein profile relatedness of many of the new isolates is sufficiently different from that of the genera *Rhizobium, Bradyrhizobium* and *Sinorhizobium* and probably merits creation of distinct genera or entities.

Different groups and putative species seem to appear from the large variety of legume hosts. To construct a comprehensive taxonomy of rhizobia, many more isolates from a wide variety of legumes besides agriculturally important plants, are necessary.

The role of host plant specificity as a cirterion in rhizobia taxonomy is questionable. This is emphasized by findings that *R. etli* can infect several legumes and not exclusively *Phaseolus vulgaris,* as was previously reported. The tree rhizobia represent a very broad range of organisms that form groups of various associations.

A polyphasic study is necessary for the delineation of taxa for most of these new isolates.

The isolation of more than two types and sometimes as many as four types of rhizobia from a nodule displays the competition among strains to occupy the same root nodules.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020

From the large collection of indigenous rhizobia there might be some isolates that can play an important role to improve nitrogen fixation in South African agriculture.

The fact that rhizobia were isolated from three genera that were reported as non-nodulating shows that it is necessary to investigate legumes in diverse geographic and climatic environments.

Indigenous counterparts of the rhizobium strains used for commercial legume inoculant production in South Africa should be evaluated for possible practical application.

Digitised by the Department of Library Services in support of open access to information, University of Pretoria, 2020