CHAPTER 4

Taxonomy of the rhizobia

1. Introduction

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

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

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

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

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

2. The genera of the *Rhizobiaceae*

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

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

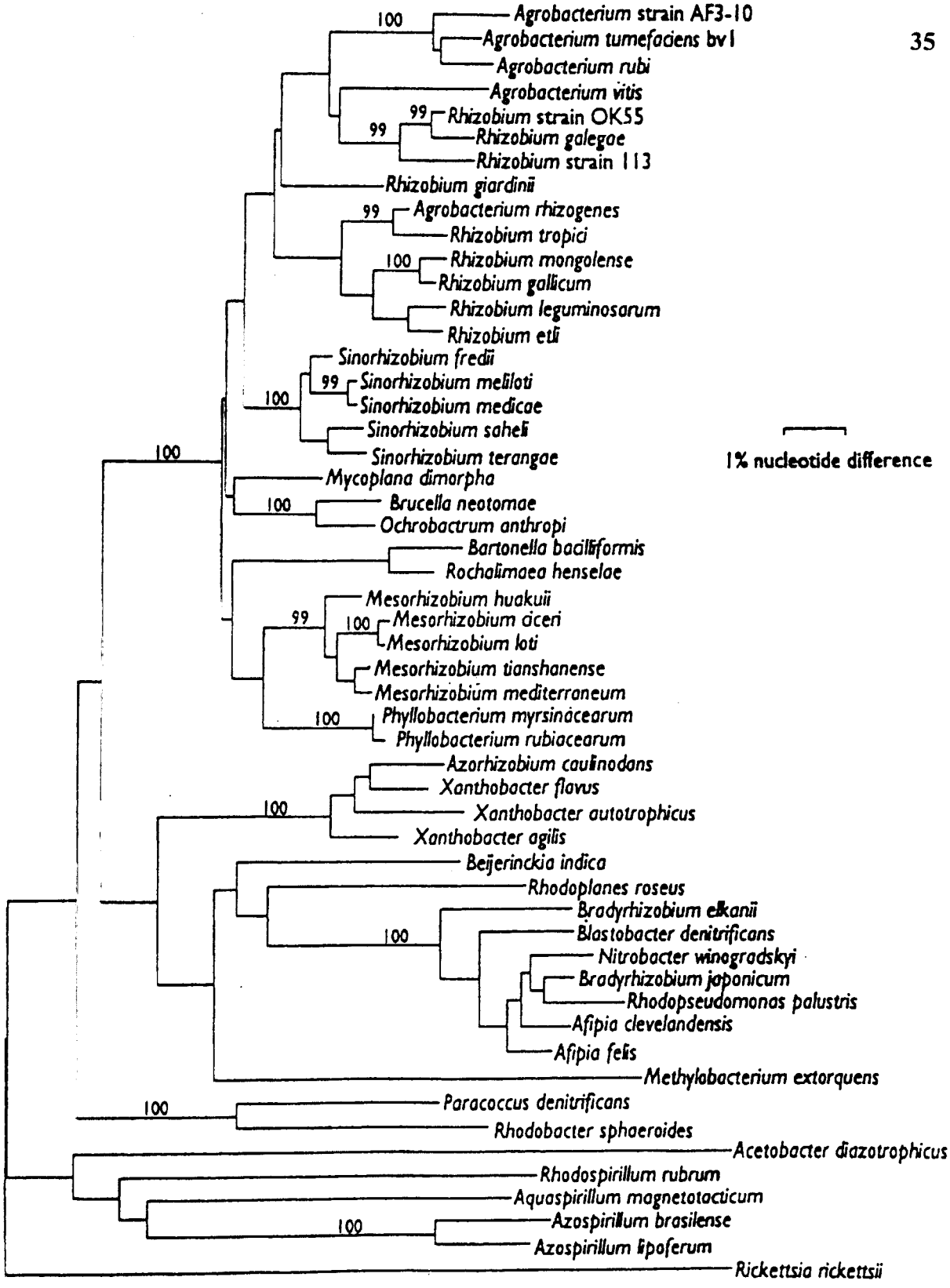

Figure 4.1. Phylogenetic relationships of the *Rhizobiaceae* within the α-subdivision of the Purple bacteria based on the SSU rRNA gene. Jukes-Cantor distances were derived from the aligned sequences to construct the unrooted tree using the unrooted Neigbour-joining method. Five hundred replicates were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

(a) The *Bradyrhizobium* genus

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

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

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

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

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

(ii) *B. liaoningense*

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

(b) The *Azorhizobium* genus

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

(c) The genus *Allorhizobium*

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

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

(d) The genus *Rhizobium*

(i) *R. leguminosarum*

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

(ii) *R. tropici*

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

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

(iii) *R. etli*

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

(iv) *R. galegae*

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

(v) *R. huautlense*

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

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

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

(vii) *R. hainanense*

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

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

(viii) *Rhizobium mongolense*

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

(ix) *R. yanglingense*

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

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

(e) The genus *Sinorhizobium*

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

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

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

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

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

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

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

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

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

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

(f) The *Mesorhizobium* genus

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

(i) *M. loti*

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

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

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

(ii) *M. ciceri*

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

(iii) *M. mediterraneum*

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

(iv) *M. huakuii*

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

(v) *M. tianshanense*

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

(vi) *M. plurifarium*

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

(vii) *M. amorphae*

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

(viii) *M. chacoense*

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

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

(g) The genus *Agrobacterium*

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

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

(i) *Agrobacterium tumefaciens*

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

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

(ii) *Agrobacterium rhizogenes*

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

(iii) *Agrobacterium rubi*

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

(iv) *Agrobacterium larrymoorei*

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

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

(v) *Agrobacterium vitis*

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

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

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

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

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

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

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

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

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

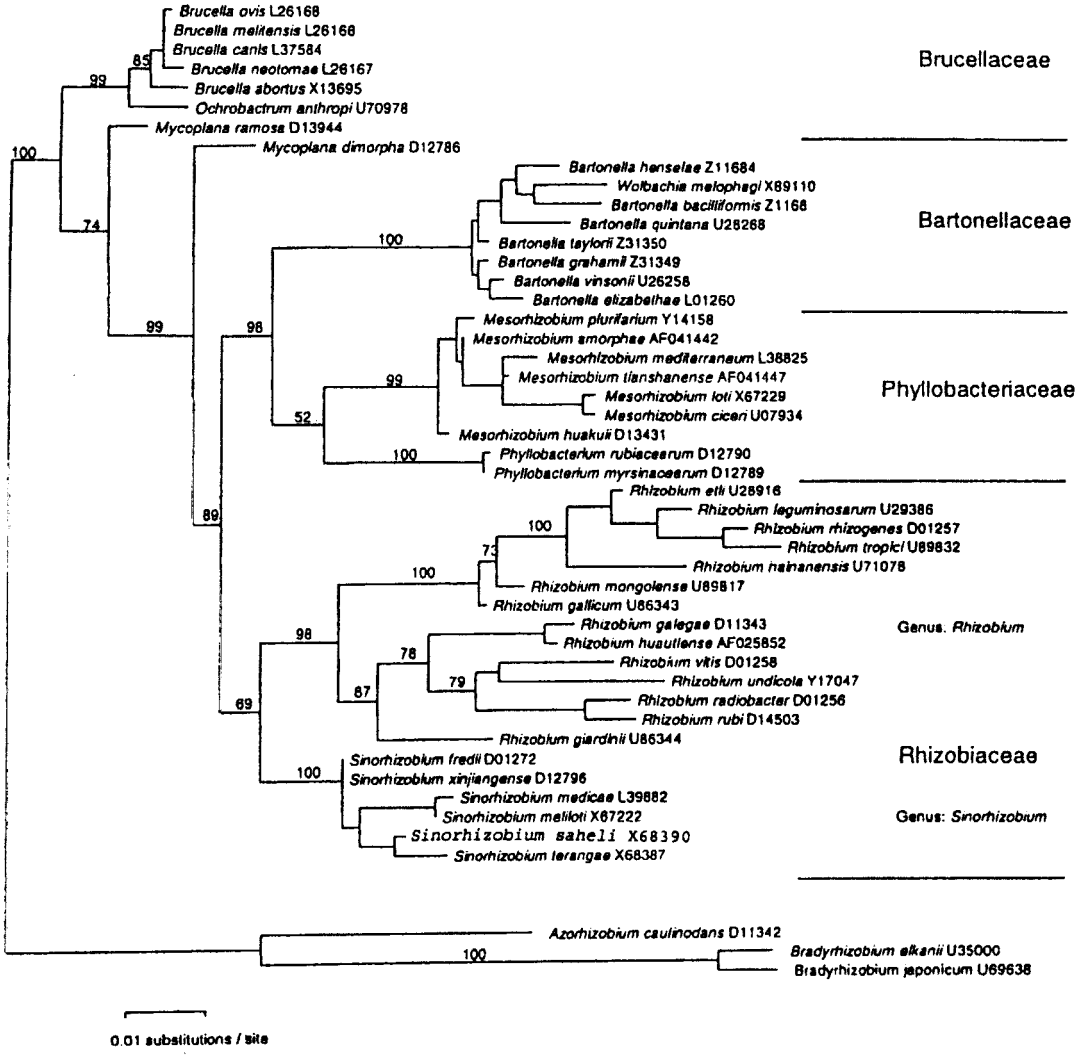

Figure 4.2. Maximum-likelihood tree expressing the relationships between the *Rhizobiaceae* and their relatives based on 16S rDNA sequences. Horizontal branch lengths are proportional to the estimated number of nucleotide substitutions and local bootstrap probabilities (as percentages) were determined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

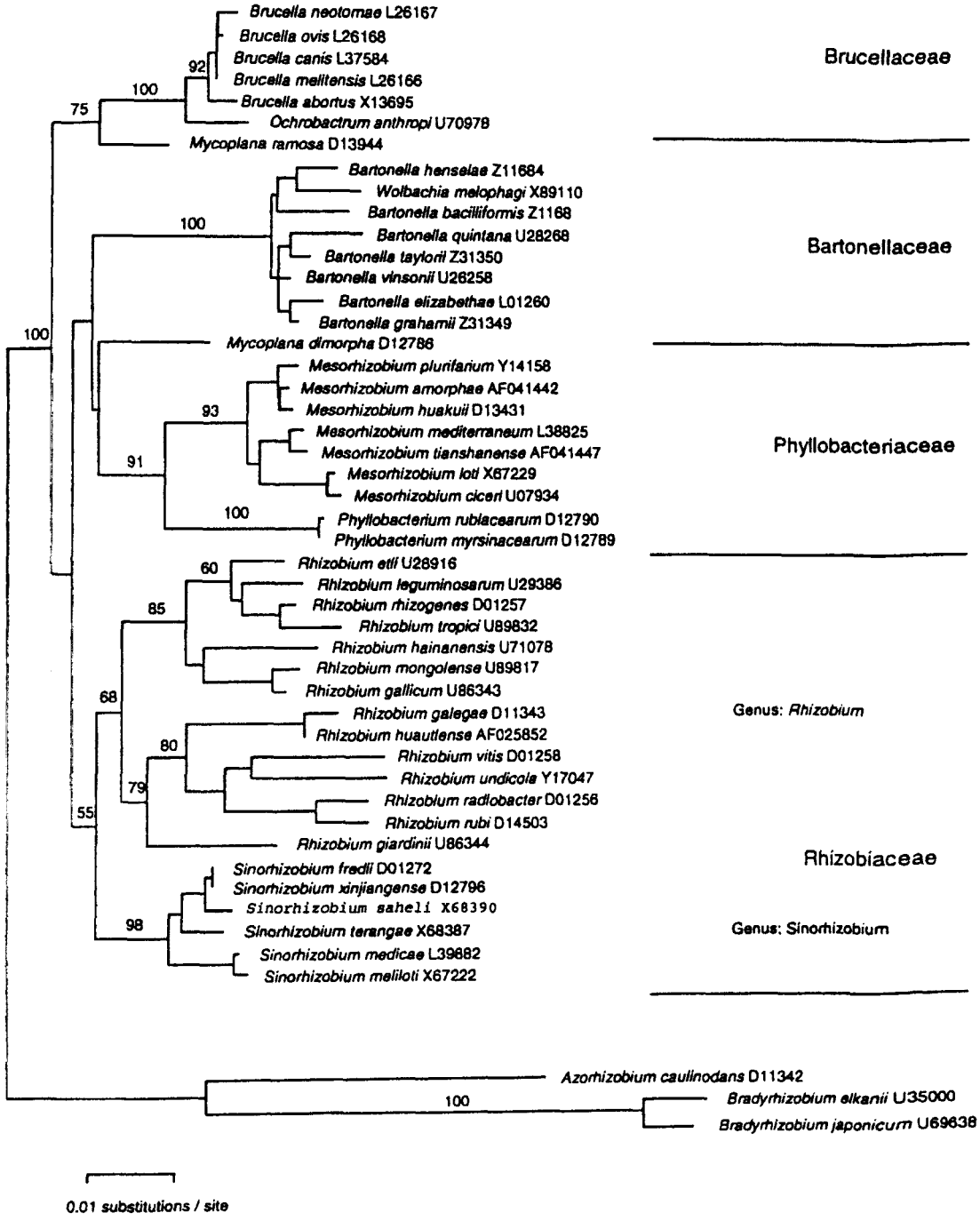

Figure 4.3. Neighbour-joining tree expressing the relationships between the *Rhizobiaceae* and their relatives, based on 16S rDNA sequences. Sites that include gaps in more than one sequence were excluded. Horizontal branch lengths are proportional to the estimated number of nucleotide substitutions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

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

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

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

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

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

4. Conclusion

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