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Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov.

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Legume species belonging to the genus *Lespedeza* are annual or perennial herb or shrub plants that grow in the northern hemisphere. They are known for the formation of root nodules, but little information is available about their microsymbionts. In this study, 58 root-nodule isolates from *Lespedeza* spp., obtained from China and the USA, were characterized using numerical taxonomic analysis of phenotypic features, SDS-PAGE analysis of whole-cell proteins, DNA–DNA hybridization, 16S rRNA gene sequence analysis and cross-nodulation with selected legume species. From the results generated using these approaches, it was concluded that *Lespedeza* spp. were promiscuous hosts for rhizobia. Four main clusters of bacteria, which included 35 of the strains isolated from *Lespedeza* spp., were defined upon numerical taxonomic analysis; these groups corresponded to those determined from analyses of protein electrophoretic and DNA–DNA hybridization data. The four clusters were found to define strains belonging to one of four species, *Sinorhizobium saheli*, *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii* or a novel species of the genus *Bradyrhizobium*. The strains of *B. japonicum* and *B. elkanii* were all from the USA soil samples, and their representative strains could not nodulate soybean. The seven strains found to represent the novel *Bradyrhizobium* sp. were from China. These were differentiated from recognized species of the genus *Bradyrhizobium* by all of the taxonomic methods used here; hence, it is proposed that the novel strains isolated from *Lespedeza* spp. represent a novel species of the genus *Bradyrhizobium*, *Bradyrhizobium yuanmingense*. The type strain of the novel species, CCBAU 10071^T (= CFNEB 101^T), formed ineffective nodules on *Medicago sativa* and *Melilotus albus* but did not nodulate soybean. The other 23 bacterial strains isolated from *Lespedeza* spp. were found to form single branches or small groups (two to three strains) that were related to *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* spp. on the basis of numerical taxonomic analysis, indicating the possibility that other rhizobial species are also associated with *Lespedeza* spp.

Keywords: *Lespedeza*, *Bradyrhizobium*, *Sinorhizobium*, diversity, phylogeny

INTRODUCTION

Legume species of the genus *Lespedeza* are annual or perennial wild plants, of which there are known to be around 140 species of herbs or shrubs. Most of these species (125) are native to eastern Asia and about 15 are indigenous to the south-eastern United States (Allen & Allen, 1981). Most *Lespedeza* spp. are drought-enduring plants, and are held in high esteem as foliage, green manure crops or honey resources and in preventing soil erosion (Allen & Allen, 1981). On

The GenBank accession number for the 16S rRNA gene sequence of *Bradyrhizobium yuanmingense* CCBAU 10071^T is AF193818.

some de-forested hills in north-eastern China, *Lespedeza* spp. are the main pioneer plants after the trees have been cut; hence, they may be potential plants for re-forestation. Different *Lespedeza* spp. are used in Chinese herbal medicine, e.g. *Lespedeza cyrtobotrya*, *Lespedeza buergeri*, *Lespedeza davidii*, *Lespedeza bicolor* and *Lespedeza cuneata* are used for treating coughs and fevers, and *Lespedeza chinensis* is used for treating diarrhoea (He, 1993). Clinical tests using constituents (flavonoids or extracts) from *Lespedeza* spp. have been performed (Campanacci *et al*., 1965). Research has also been done on the biological degradation of tannins in sericea lespedeza (*L. cuneata*) by white rot fungi, in an attempt to increase the potential value of *L. cuneata* as a forage crop (Gamble *et al*., 1996). Previous studies on cross-nodulation (performed between 1917 and 1974; summarized by Allen & Allen, 1981) indicated that *Lespedeza* plants belonged to the cowpea miscellany. Marked host specificity relative to the effectiveness of nodulation was also revealed in these previous studies. The perennial *Lespedeza* spp. showed effective responses to bacterial strains isolated from perennial species, while the annual species had effective responses to the strains isolated from annual species (Allen & Allen, 1981). The microsymbionts of the leguminous plants of the cowpea miscellany were classified as belonging to the bacterial genus *Bradyrhizobium*, by using a taxonomic approach (Jordan, 1984). However, the specific taxonomic positions for most of the bacteria associated with the cowpea miscellany were unknown at that time. Since its creation in 1982 (Jordan, 1982), three species have been assigned to the genus *Bradyrhizobium*: *Bradyrhizobium japonicum* (Jordan, 1982), *Bradyrhizobium elkanii* (Kuykendall *et al*., 1992) and *Bradyrhizobium liaoningense* (Xu *et al*., 1995).

So far, about 70 species of *Lespedeza* have been recorded in China (He, 1993). However, the nodulation of these species and their microsymbionts have not been documented. During a survey of rhizobial resources in the northern parts of China, we obtained a number of rhizobial isolates from a variety of different plants and geographical regions. The characterization of some populations of these isolates according to their geographical origin or host has been done already (Chen *et al*., 1995; Tan *et al*., 1999; Wang *et al*., 1999). Because of the potential values of *Lespedeza* spp. in agriculture and re-forestation and the uncertain taxonomic position of the rhizobial species associated with them, we decided to characterize the rhizobial strains isolated from these plants using a polyphasic approach. The aims of this research were to check the diversity of these strains and to classify them.

METHODS

Isolates and strains. Fifty-eight nodule strains isolated from 15 *Lespedeza* spp. that grow in the USA and in Chinese soils were used in this study (Table 1). The following reference strains were used: *B. liaoningense* FSI 2062^T (Xu *et al*., 1995); *B. japonicum* USDA 6^T (Jordan, 1982); *B. japonicum* USDA 110 (Gao *et al*., 1994); *B. japonicum* B15 (Gao *et al*., 1994); *B. elkanii* USDA 76^T (Kuykendall *et al*., 1992); *Mesorhizobium loti* NZP 2213^T (Jarvis *et al*., 1997); *Mesorhizobium huakuii* CCBAU 2609^T (Chen *et al*., 1991); *Mesorhizobium ciceri* UPM-Ca7^T (Nour *et al*., 1994); *Mesorhizobium tianshanense* A-1BS^T (Chen *et al*., 1995); *Mesorhizobium amorphae* ACCC 19665^T (Wang *et al*., 1999); *Mesorhizobium plurifarium* USDA 4413 (de Lajudie *et al*., 1998b); *Rhizobium leguminosarum* USDA 2370^T (Jordan, 1984); *Rhizobium tropici* type B CIAT 899^T (Martínez-Romero *et al*., 1991); *Rhizobium tropici* type A CFN 299 (Martínez-Romero *et al*., 1991); *Rhizobium etli* CFN 42^T (Segovia *et al*., 1993); *Rhizobium galegae* HAMBI 540^T (Lindström, 1989); *Sinorhizobium meliloti* USDA 1002^T (Jordan, 1984); *Sinorhizobium fredii* USDA 205^T (Scholla & Elkan, 1984); *Sinorhizobium saheli* USDA 4102^T (de Lajudie *et al*., 1994); *Sinorhizobium terangae* USDA 4101^T (= ORS 1037^T) (de Lajudie *et al*., 1994); *Escherichia coli* DH5α (Sambrook *et al*., 1989). Plasmid pUC18 (Sambrook *et al*., 1989) was also used as a vector for cloning. The Chinese strains were isolated in six provinces or cities in northern China, all within an area of about 2000 km east to west by 1000 km north to south. The provinces of Gansu, Shanxi and Shaanxi are located in the Loess Plateau in north-western China; they have a semi-arid climate and soils that are poor in organic matter. The climate and soils of Beijing and Inner Mongolia are similar to those of Shanxi, but these regions have more rain in the summer. Jilin is a province in north-eastern China; the soils in this region contain more organic matter and water, but the region has very cold winters (below −20 °C). The strains isolated in the USA were obtained from the USDA-ARS Rhizobium Germplasm Resource Collection (United States Department of Agriculture); some of these strains were isolated over half a century ago (Table 1). The symbiotic ability of each novel strain was confirmed by nodulating its original host using the method of Vincent (1970). Of the 15 host species tested, *Lespedeza stipulacea* and *Lespedeza striata* are annual herbs; the others are perennial plants.

Phenotypic characterization and numerical taxonomy. Phenotypic features, i.e. the utilization of sole carbon sources and sole nitrogen sources, resistance to antibiotics, tolerance to dyes and chemicals, tolerance to NaCl, temperature and pH ranges for growth, and some physiological and biochemical reactions, were determined using protocols described previously (Gao *et al*., 1994). The results of the phenotypic characterization were converted into a binary dataset which was used to estimate the simple matching similarity coefficient (Ssm) of each strain pair and to generate a similarity matrix (Sneath & Sokal, 1973). The similarity matrix was used for cluster analysis to construct a dendrogram using the unweighted pair group method with averages (UPGMA) (Sneath & Sokal, 1973). The mean similarity for each strain within a cluster was estimated to present the phenotypic variation in the cluster, as described previously, and the central strain as the representative for the phenotypic cluster was calculated (Sneath & Sokal, 1973). Generation times were determined in PY medium (5 g peptone of casein, 3 g yeast extract, 0·6 g $CaCl_2$; all vales given are per litre of distilled water) by using a spectrophotometric method (Vincent, 1970).

SDS-PAGE of whole-cell proteins. Soluble-protein extraction, SDS-PAGE and scanning of the protein patterns using a Densitometer Extra-Scanner (LKB) were done as described

Table 1. Bacterial strains used in this study

Strain	*Lespedeza* host species	Geographical origin (year of isolation)
Cluster 1 (*Sinorhizobium saheli*)		
CCBAU 10012, CCBAU 10032, CCBAU 10043, CCBAU 10063, CCBAU 10171, CCBAU 10091, CCBAU 10113, CCBAU 10143, CCBAU 10152, CCBAU 10155, CCBAU 10163	*L. inschanica*	Beijing, China (1995)
CCBAU 13072, CCBAU 13074	*L. daurica*	Jilin, China (1995)
CCBAU 13145	*L. tomentosa*	Jilin, China (1995)
CCBAU 13146	*L. cyrtobotrya*	Jilin, China (1995)
Cluster 2 (*Bradyrhizobium yuanmingense*)		
CCBAU 10011, CCBAU 10030, CCBAU 10031, CCBAU 10038, CCBAU 10040, CCBAU 10071^T, CCBAU 10073	*L. cuneata*	Beijing, China (1995)
Cluster 3 (*Bradyrhizobium japonicum*)		
USDA 3399	*L. juncea* var. *sericea*	USA (unknown)
USDA 3638	*L. juncea*	OH, USA (1960)
USDA 3651	*L. daurica*	USA (1960)
USDA 3652	*L. daurica*	MO, USA (1960)
USDA 3654	*L. juncea*	USA (1960)
Cluster 4 (*Bradyrhizobium elkanii*)		
USDA 3199	*L. striata*	MD, USA (1933)
USDA 3203	*L. capitata*	OH, USA (1940)
USDA 3204	*L. capitata*	USA (1932)
USDA 3205	*L. stipulacea*	NJ, USA (1933)
USDA 3211	*L. juncea* var. *sericea*	VA, USA (1914)
USDA 3212	*L. juncea* var. *sericea*	TN, USA (1933)
USDA 3220	*L. procumbens*	SC, USA (1941)
USDA 3222	*L. bicolor*	SC, USA (1940)
Other *Bradyrhizobium* strains		
CCBAU 10033	*L. cuneata*	Beijing, China (1995)
USDA 3198	*L. striata*	USA (unknown)
USDA 3202	*L. capitata*	OH, USA (1940)
USDA 3208	*L. stipulacea*	IN, USA (1938)
USDA 3219	*L. juncea* var. *serpens*	SC, USA (1941)
Other fast or moderately growing rhizobial strains		
USDA 3197	*L. hirta*	OH, USA (1940)
USDA 3215	*L. striata*	MD, USA (1937)
CCBAU 10201, CCBAU 10241, CCBAU 10301	*L. daurica*	Beijing, China (1995)
CCBAU 73041	*L. formosa*	Gansu, China (1995)
CCBAU 13008, CCBAU 13132	*L. bicolor*	Jilin, China (1995)
CCBAU 13143	*L. hydysaroides*	Jilin, China (1995)
CCBAU 10112, CCBAU 10134	*L. inschanica*	Beijing, China (1995)
CCBAU 01104	*L. bicolor*	Inner Mongolia, China (1995)
CCBAU 71055	*L. daurica*	Shaanxi, China (1995)
CCBAU 71082	*L. bicolor*	Shaanxi, China (1995)
CCBAU 03208, CCBAU 03216	*L. floribunda*	Shanxi, China (1995)
CCBAU 03220, CCBAU 03248	*L. daurica*	Shanxi, China (1995)

previously (Tan *et al.*, 1997). Clustering analysis of the protein patterns was performed as described previously (Tan *et al.*, 1997).

Analyses of 16S rRNA gene sequence data and phylogeny. These were performed only for the representative strain of the novel species, strain CCBAU 10071^T (Cluster 2). The complete 16S rRNA gene ($\sim$ 1500 bp) was amplified by PCR using the universal forward primer P1 (5′-CGgatcc-AGFGTTTGATCCTGGTCAGAACGAACGCT-3′) and the universal reverse primer P6 (5′-CGggatccTACGGCTA-CCTTGTTACGACTTCACCCC-3′) and a standard protocol. The PCR product was cloned into pUC18, which was then transformed into *E. coli* DH5α (Sambrook *et al.*, 1989), as described by Tan *et al.* (1997). An automated DNA sequencer and primers P1, P2, P3, P4, P5 and P6 (Tan *et al.*, 1997) were used for single-strand sequencing of the cloned 16S rRNA gene fragments, after purification of the plasmids.

The 16S rRNA gene sequence of CCBAU 10071^T and the 16S rRNA gene sequences of its related bacterial species (retrieved from GenBank) were aligned by using the program PILEUP in the GCG package (Genetics Computer Group, 1995). Sequence similarities and a phylogenetic tree were obtained using CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic tree constructed was visualized by using TREE VIEW (Page, 1996).

Determination of DNA G+C content and DNA–DNA homology. DNA was prepared by a standard phenol/chloroform extraction (Marmur, 1961). The DNA G+C content of strains was determined using the thermal melting protocol of De Ley (1970), using *E. coli* K-12 DNA as the standard. DNA–DNA homologies were estimated spectrophotometrically (De Ley *et al.*, 1970). DNA was sheared by passage through a syringe, to generate fragments of $2–5 \times 10^5$ Da in size. Renaturation was performed in 2 × SSC at the optimum temperature (De Ley *et al.*, 1970).

PCR-based RFLP analysis of 16S rDNA. Primers fD1 and rD1 (Weisburg *et al.*, 1991) and procedures described previously (Wang *et al.*, 1999) were used for amplification of the complete 16S rRNA genes, for digestion of the PCR products with *Msp*I, *Hin*fI, *Hha*I or *Sau*3AI, and for separation of the digested fragments in 3·0 % (w/v) agarose gels.

Cellular plasmid contents. The Eckhardt (1978) procedure modified by Hynes & McGregor (1990) was used to visualize any plasmids present in the strains.

Cross-inoculation tests. Standard protocols were used (Vincent, 1970). Plants were grown in pots filled with vermiculite moistened with a nitrogen-free plant nutrition solution (Vincent, 1970). Nodulation and nitrogen-fixing abilities of the plants were observed after 1 month. The recommended legume species of Graham *et al.* (1991), *Medicago sativa*, *Melilotus albus*, *Vigna unguiculata*, *Glycyrrhiza uralensis*, *Glycine max*, *Phaseolus vulgaris*, *Pisum sativum*, *Galega officinalis*, *Trifolium repens* and *Leucaena leucocephala*, were used as hosts for strain CCBAU 10071^T, which was isolated from *Lespedeza cuneata* and was the representative strain of the novel *Bradyrhizobium* sp. described here. *L. cuneata* was inoculated with *S. saheli* USDA 4102^T, *B. japonicum* USDA 6^T and *B. elkanii* USDA 76^T. *Glycine max* was inoculated with USDA 3204 and USDA 3654, representatives of the *B. japonicum* and *B. elkanii* strains, respectively, isolated from *Lespedeza* spp.

RESULTS

Isolation

The growth rate of and acid/alkali production by each strain were determined on YMA medium (Vincent, 1970) plates during the isolation procedure. Of the 58 strains isolated, 30 from China and two from the USA were fast-growing, acid-producing rhizobia that produced colonies > 2 mm in diameter after 3–5 days incubation. Eight strains from China and 17 from the USA were slow-growing, alkali-producing bacteria that belonged to the genus *Bradyrhizobium*, with colonies $\leqslant 1$ mm in diameter after 7–10 days incubation (Table 1).

Phenotypic characterization and numerical taxonomy

For all of the strains (including the reference strains), 125 phenotypic features were tested. None of the strains tested used malonate, oxalate, sorbose or D-methionine as a sole carbon source, and none used DL-alanine, L-glutamine or L-threonine as a sole nitrogen source. All of the strains were resistant to 5 μg ampicillin ml^{-1}, 5 μg chloramphenicol ml^{-1}, 5 μg erythromycin ml^{-1} and 0·1 % Congo red; they were sensitive to 100 μg kanamycin ml^{-1}, 300 μg erythromycin ml^{-1}, 300 μg neomycin ml^{-1} and 0·1 % crystal violet and methylene blue. They were all sensitive to heat treatment at 60 °C for 10 min, and were catalase-positive. The 105 features found to be variable among the strains were used for the numerical taxonomic analysis. Based on the results of cluster analysis (Fig. 1), the levels of similarity between most of the type strains of recognized species were less than 80 %. The exceptions were *Mesorhizobium ciceri* and *Mesorhizobium huakuii*, which were separated at the 83 % similarity level. Thirty-five of the 58 strains isolated from *Lespedeza* spp. belonged to one of four clusters, with around 80 % similarity; other strains formed single branches or small groups (of two to three strains) that consisted of the strains isolated from *Lespedeza* spp. only or which were related to recognized species (Fig. 1). Some important features of the four clusters are described below.

Cluster 1 consisted of 15 fast-growing strains isolated from four species of *Lespedeza* (*L. inschanica*, *L. daurica*, *L. tomentosa* and *L. cyrtobotrya*) that grow in the Beijing and Jilin Provinces of China; *S. saheli* ORS 609^T was also found in this cluster. The mean similarities for the strains ranged from 82·0 %, in the case of strain CCBAU 13145, to 88·2 %, in the case of strain CCBAU 10091 – this latter strain was chosen as the central strain for the cluster. Cluster 1 strains produced colonies that were > 2 mm in diameter after 2–3 days incubation at 28 °C. All of the strains in this cluster produced acid when grown on YMA plates. They were resistant to 100 μg erythromycin ml^{-1}, but sensitive to 50 μg kanamycin ml^{-1}, 300 μg erythromycin ml^{-1} and 300 μg polymyxin ml^{-1}. Growth of Cluster 1 strains was obtained on YMA supplemented with 0·1 % Congo red, deoxycholate or nitrite, but not with crystal violet or methylene blue. The strains grew on YMA supplemented with 1 % (w/v) NaCl and at pH 5–10. No nitrate reduction or growth in Luria–Bertani broth was observed for Cluster 1 strains.

The seven strains of Cluster 2 were slow-growing rhizobia that had been isolated from *L. cuneata* found in Beijing. Generation times for strains CCBAU 10011, CCBAU 10038, CCBAU 10040, CCBAU 10071^T and CCBAU 10073 were 12·5, 16·0, 9·5, 10·2 and 10·2 h, respectively. The mean similarities for the seven strains ranged from 83·3 to 89·2 %. CCBAU 10071^T was designated the representative strain of the cluster. Cluster 2 strains produced single colonies of 1–2 mm in

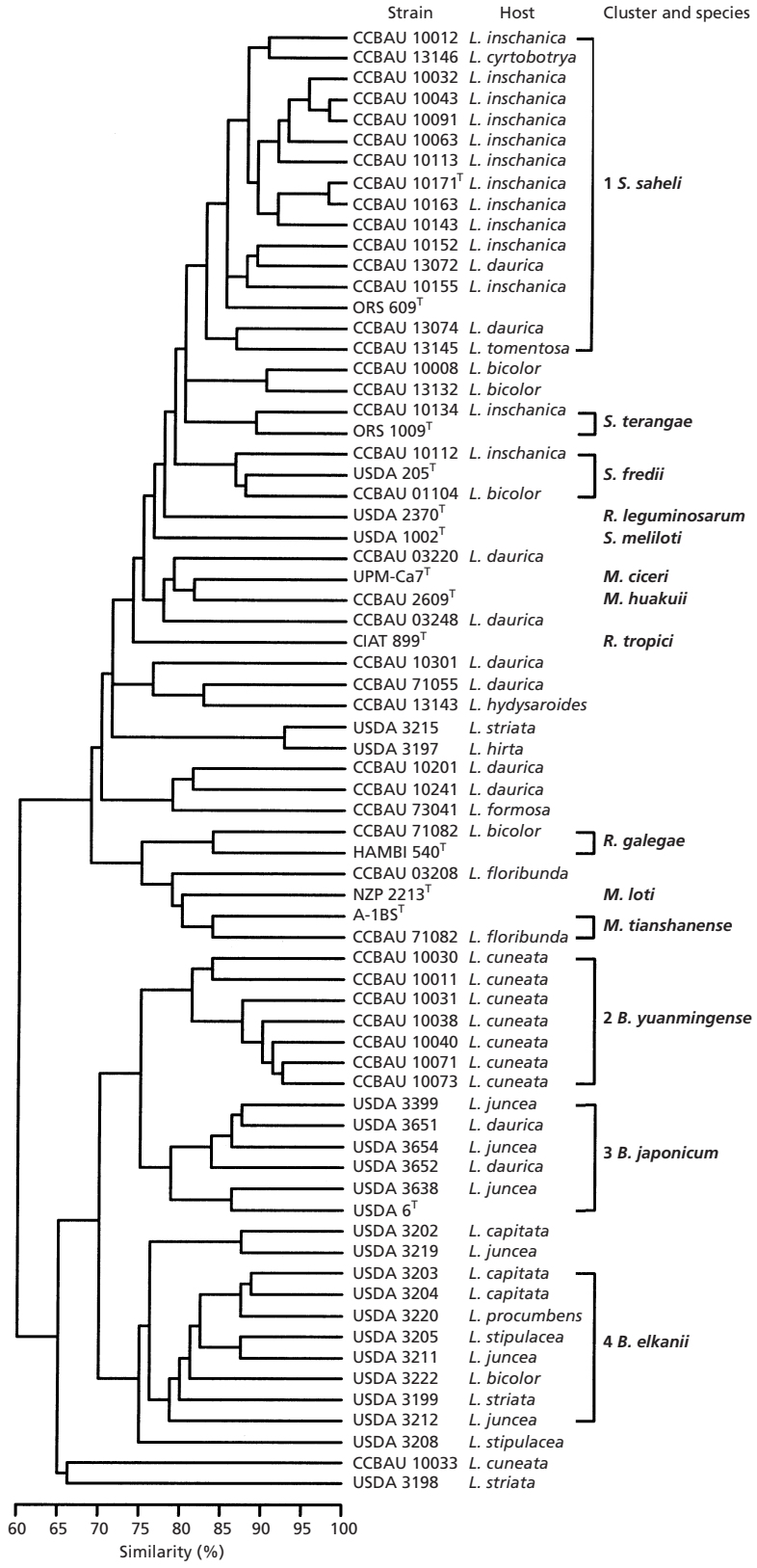

Fig. 1. Dendrogram constructed based on numerical taxonomic analysis using the simple matching similarity coefficient (Ssm) and the UPGMA (Sneath & Sokal, 1973). Four clusters were recovered among the strains isolated from *Lespedeza* spp. at the 80 % similarity level.

diameter after 7–10 days incubation, and alkali when grown on YMA or in litmus milk. They were resistant to 300 μg polymyxin ml^{-1}, but sensitive to 100 μg erythromycin ml^{-1}, 1 % NaCl (w/v) and pH 5 and 10. Nitrate was reduced by all seven strains. None of the Cluster 2 strains grew in Luria–Bertani broth.

Cluster 3 contained five slow-growing strains isolated from *L. daurica* and *Lespedeza juncea* in the USA; *B. japonicum* USDA 6^T was also found in this cluster. The strains within this cluster had mean similarities of 80·0–83·6 %. Strain USDA 3654 was designated the representative strain of the cluster. Cluster 3 strains produced alkali when grown on YMA, and formed single colonies of < 1 mm in diameter after 7 days incubation on YMA plates. Similar to Cluster 2 strains, Cluster 3 strains were resistant to 300 μg polymyxin ml^{-1} and sensitive to 100 μg erythromycin ml^{-1}. No growth was observed in Luria–Bertani broth or on YMA supplemented with 1 % (w/v) NaCl or at pH 5 and 10. All five of the strains reduced nitrate.

Eight slow-growing strains isolated from *Lespedeza bicolor*, *Lespedeza capitata*, *L. juncea* var. *sericea*, *Lespedeza procumbens* and *L. striata* in the USA were grouped in Cluster 4. They had mean similarities of 80·0–84·1 %. Strain USDA 3204 was designated the representative strain of the cluster. Cluster 4 strains produced single colonies of < 1 mm in diameter after 1 week incubation on solid medium, and produced alkali when grown on YMA plates. They were resistant to 100 μg erythromycin ml^{-1} and 300 μg polymyxin ml^{-1}, and tolerant to 1 % NaCl and pH 5. Some of the strains could grow at pH 10. No nitrate reduction or growth in Luria–Bertani broth was observed for the Cluster 4 strains.

Five of the slow-growing, alkali-producing strains isolated from *Lespedeza* spp. showed 67–77 % similarity with the *Bradyrhizobium* spp. clusters (Fig. 1), but they could not be included in these clusters. Strains USDA 3202, USDA 3219 and USDA 3208 had similarities of 76–77 % with Cluster 4. Strains CCBAU 10033 and USDA 3198 formed single branches that were divergent from each other and from Clusters 1–4.

The other 17 strains isolated from *Lespedeza* spp. were fast-growing or moderately growing, acid-producing rhizobia that had closer relationships with *Mesorhizobium*, *Rhizobium* or *Sinorhizobium* spp. than the other strains isolated (Fig. 1). Strain CCBAU 10134, isolated from *L. inschanica*, had 87 % similarity with *S. terangae* USDA 4101^T. Strains CCBAU 10112 and CCBAU 01104 clustered with *S. fredii* USDA 205^T at the 83 % similarity level. Strain CCBAU 03220 shared 80 % similarity with *Mesorhizobium ciceri* UPM-Ca7^T and *Mesorhizobium huakuii* CCBAU 2609^T. Strain CCBAU 71082 was 83 % similar to *R. galegae* HAMBI 540^T. The remaining 12 strains either formed strain pairs or single branches, which had similarities of 71–80 % with the recognized species.

SDS-PAGE of soluble proteins

Representative strains and reference strains were selected based on the clustering results of the numerical taxonomic analysis. The grouping of strains based on protein patterns (data not shown) was in general agreement with the groupings seen in the numerical taxonomic analysis. *S. saheli* USDA 4102^T, *B. japonicum* USDA 6^T and *B. elkanii* USDA 76^T clustered with strains of Clusters 1, 3 and 4, respectively. The similarities of the protein patterns ranged from 79 to 94·5 % in Cluster 1 (including *S. saheli* USDA 4102^T), 90·5 to 94 % in Cluster 2, 78 to 90 % in Cluster 3 (including *B. japonicum* USDA 6^T) and 80 to 93·5 % in Cluster 4 (including *B. elkanii* USDA 76^T). The similarities between strains of the different clusters were < 68 %.

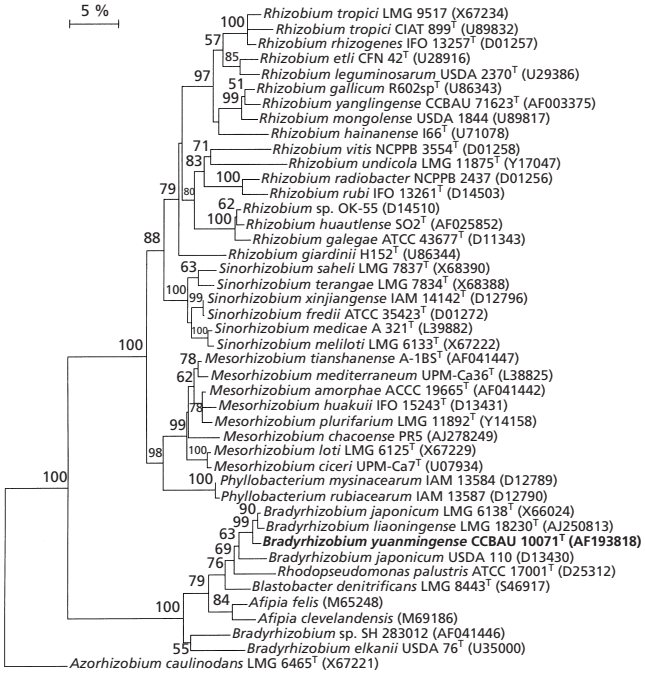

Fig. 2. Dendrogram showing the phylogenetic position of strain CCBAU 10071^T (Cluster 2; Fig. 1) with respect to the genus *Bradyrhizobium* and related genera. 16S rRNA gene sequences were aligned using the PILEUP program in the GCG package (Genetics Computer Group, 1995). CLUSTAL W (Thompson *et al.*, 1994) was used to construct the tree; bootstrap values are shown at the nodes of the tree, and are expressed as a percentage of 1000 replications. Bar, 5 nucleotide substitutions per 100 nucleotides.

16S rRNA gene sequence and phylogenetic analyses

Two independent 16S rRNA gene clones from strain CCBAU 10071^T were sequenced; the sequences from the two clones were identical. The 16S rRNA gene sequence from strain CCBAU 10071^T had 99·2, 98·2, 96·4 and 95·2 % similarity with 16S rDNA sequences from *B. japonicum* USDA 6^T, *Bradyrhizobium* sp. USDA 110, *B. elkanii* USDA 76^T and *Bradyrhizobium* sp. (*Amorpha*) SH 283012 (Wang *et al.*, 1999), respectively. The phylogenetic relationships between the recognized species in the phylogenetic tree we constructed (Fig. 2) were the same as those reported

Table 2. DNA G+C content and DNA–DNA relatedness values for strains used in this study

Strains: 1, *S. saheli* CCBAU 10091; 2, *B. yuanmingense* CCBAU 10071^T; 3, *B. japonicum* USDA 6^T; 4, *B. elkanii* USDA 3204; 5, *B. elkanii* USDA 76^T. For each strain, the DNA G+C content was determined by the thermal denaturation method (T_m); DNA–DNA relatedness was determined from initial reassociation rates. Data shown represent the mean values of duplicate hybridizations performed using the spectrophotometric method of De Ley *et al.* (1970). ND, Not determined.

Strain	DNA G+C content (mol%)	DNA–DNA homology (%) with				
		1	2	3	4	5
Cluster 1 (*S. saheli*)						
CCBAU 10032		80·8				
CCBAU 10043		91·0				
CCBAU 10063		100				
CCBAU 10171		100				
CCBAU 10091		100				
CCBAU 10113		100				
CCBAU 10143		72·2				
CCBAU 10152		100				
CCBAU 10163		76·5				
CCBAU 13072		100				
CCBAU 13074		92·5				
CCBAU 13145		100				
CCBAU 13146		89·6				
ORS 609^T	64·1	89·6				
Cluster 2 (*B. yuanmingense*)						
CCBAU 10011	62·4		72·6	10·0		
CCBAU 10030	62·2		75·4			
CCBAU 10031	64·1		72·8			
CCBAU 10038	62·4		81·0	47·8		42·7
CCBAU 10040	63·1		90·2	46·2		32·3
CCBAU 10071^T	63·0		100			
CCBAU 10073	61·8		90·6	15·8		11·4
Cluster 3 (*B. japonicum*)						
USDA 3399	62·0			88·6		
USDA 3638	63·9			87·0		
USDA 3651	63·7			72·0		
USDA 3652	62·2			71·4		
USDA 3654	61·8			79·8		
USDA 6^T	63·3		29·8	100	0	
B15	ND		23·9			
USDA 110	ND		45·2			
Cluster 4 (*B. elkanii*)						
USDA 3203	63·8				94·5	
USDA 3204	63·9				100	
USDA 3205	64·5				78·0	
USDA 3211	63·9				79·6	
USDA 3212	62·8				100	
USDA 3220	62·9				86·6	
USDA 3222	63·9				72·0	
USDA 3199	62·1				80·3	
USDA 76^T	63·9		25·3±16·8		91·1	
B. liaoningense SFI 2032^T			17·3			

previously (e.g. Wang *et al.*, 1999; Willems & Collins, 1993; Young & Haukka, 1996). Species of the genera *Mesorhizobium* (Jarvis *et al.*, 1997) and *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994) clustered in two monophyletic groups that were distinct from other genera. The remaining fast-growing, acid-producing bacteria clustered in the genus *Rhizobium* (Young *et al.*, 2001) [formerly comprising the genera *Allorhizo-*

Table 3. Characteristics useful for distinguishing *B. yuanmingense* from related species

Strains: 1, *B. yuanmingense* (Cluster 2, $n = 7$); 2, *B. elkanii* (Cluster 4, $n = 8$); 3, *B. japonicum* (Cluster 3, $n = 6$); 4, *B. liaoningense*. NR, Not reported; ND, not done. Data from this study for *B. yuanmingense*, *B. elkanii* and *B. japonicum* and from Xu *et al.* (1995) for *B. liaoningense*. −, > 95% isolates negative; +, > 95% isolates positive; d, 5–95% isolates positive.

Characteristic	1	2	3	4
Carbon sources used:				
D-Fructose	−	+	−	±
Inositol	−	+	+	−
Lactose	−	±	+	−
Maltose/sucrose	±	+	+	−
Nitrogen sources used:				
L(+)-Aspartic acid	−	+	+	+
Glycine	−	+	+	NR
Resistant to:				
Erythromycin (100 μg ml^{-1})	−	+	−	−
Polymyxin (300 μg ml^{-1})	+	+	−	NR
0·1% Methyl green/deoxycholate	−	+	−	NR
1·0% NaCl	−	+	+	−
Growth at pH 5·0	−	+	−	NR
Growth at pH 10·0	−	±	−	−
Nitrate reduction	+	−	+	NR
Colony size after 7–10 days incubation (mm)	1·0–2·0	1·0	1·0	0·2–1·0
Generation time (h)	9·5–16·0	> 6	> 6	16·4–39·6
G+C content of DNA (mol%)	62–64	62–64	61–65	60–64
Soybean-nodulating	−	d	d	+
L. cuneata-nodulating	+	+	+	ND
16S rRNA gene PCR-RFLP pattern	QMHE*	PLIE	OKHE	ND

*Four letter codes were given to each cluster to represent the restriction patterns produced upon digestion of the amplified 16S rDNA with *Msp*I, *Hin*fI, *Hha*I and *Sau*3AI, respectively. For each enzyme, the different letters represent the different restriction patterns produced (not shown).

bium (de Lajudie *et al.*, 1998a), *Agrobacterium* and *Rhizobium* (Jordan, 1984)] (Fig. 2). Strain CCBAU 10071^T, representing Cluster 2, was closely related to *B. japonicum* and *B. liaoningense*.

DNA G+C content and DNA–DNA relatedness determinations

Results of these analyses are shown in Table 2. The DNA G+C contents of the *Bradyrhizobium* strains ranged from 61·8 to 64·5 mol%, which is within the range for recognized members of the genus *Bradyrhizobium*. The DNA–DNA homology values seen among strains within each of the four clusters were higher than 70%, indicating that Clusters 1–4 represented four distinct genomic species (Graham *et al.*, 1991; Wayne *et al.*, 1987). The mean DNA–DNA homology values were 92·3, 83·2, 83·1 and 86·9% among the strains within Clusters 1, 2, 3 and 4, respectively. Clusters 1, 3 and 4 were identified as *S. saheli*, *B. japonicum* and *B. elkanii*, respectively, due to the DNA homology values seen between the representative strains of the clusters and the type strains of the aforementioned species (89·6, 100 and 91·1%, respectively). DNA–DNA homology values between the representative strains of Cluster 2 and the reference strains of *B. japonicum* and *B. elkanii* ranged from 10 to 47·8%.

RFLP analysis of 16S rDNA

The RFLP patterns produced for the 16S rDNA from the Cluster 2 strains CCBAU 10011, CCBAU 10031, CCBAU 10038, CCBAU 10040, CCBAU 10071^T and CCBAU 10073 were identical. Digestion of the 16S rDNA of *B. japonicum*, *B. elkanii* and Cluster 2 strains with *Sau*3AI produced identical patterns. Upon digestion of the 16S rDNA from strains of Clusters 1–4, *Hha*I, *Msp*I and *Hin*fI produced distinctive patterns for the strains of the different clusters. The 16S rDNA from Cluster 2 strains and *B. japonicum* USDA 6^T produced the same patterns when digested with *Sau*3AI or *Hha*I, but 1–2 bands differed between them in the *Msp*I- or *Hin*fI-digested PCR products. Four letter codes were given to each of the clusters, which represented the patterns produced upon digestion of the 16S rDNA with each of the four different endonucleases (Table 3; patterns not shown). For each

enzyme, the different letters refer to the different patterns produced.

Determination of cellular plasmid content

Six strains from Cluster 2 were checked; no plasmids were observed from strains CCBAU 10011, CCBAU 10031, CCBAU 10038, CCBAU 10040, CCBAU 10071^T and CCBAU 10073.

Cross-inoculation tests

Strain CCBAU 10071^T, representing Cluster 2, nodulated *V. unguiculata* and *Glycyrrhiza uralensis*, but it did not nodulate *Glycine max*, *Phaseolus vulgaris*, *Pisum sativum*, *Galega officinalis*, *T. repens* or *Leucaena leucocephala*. *S. saheli* USDA 4102^T, *B. japonicum* USDA 6^T and *B. elkanii* USDA 76^T nodulated *Lespedeza cuneata*. Two other strains isolated from *Lespedeza* spp., USDA 3204 (Cluster 4) and USDA 3654 (Cluster 3), did not nodulate *Glycine max*.

DISCUSSION

The diversity and classification of rhizobial species associated with *Lespedeza* spp. have not been investigated systematically. In this study, we characterized rhizobial strains isolated from *Lespedeza* spp. using a polyphasic approach, which included numerical taxonomic analysis, SDS-PAGE analysis of soluble proteins, DNA–DNA hybridization analysis, 16S rRNA gene sequence analyses and nodulation tests with selected host legumes. The results obtained from the different methods used were in general agreement, and four clusters were recognized among the strains on the basis of these results. Besides the four clusters, some single strain or small groups of (two to three) strains were also identified. Some of the strains studied were distinctive from all the type or reference strains used here for recognized species, while others had more than 80% phenotypic similarity, as determined by numerical taxonomical analysis, to *B. japonicum*, *S. saheli*, *S. terangae*, *S. fredii*, *R. galegae* or *Mesorhizobium tianshanense*. These results indicate that *Lespedeza* spp. are associated with a number of different species belonging to the genera *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*; however, the single strain branches and small groups of strains were not classified in this study. Therefore, *Lespedeza* spp. are non-selective hosts for the microsymbionts described here.

Bradyrhizobium strains were the predominant microsymbionts, nodulating *Lespedeza* spp. from the USA soils (Table 1; Fig. 1). Most of the American strains grouped within Clusters 3 and 4 and were identified as *B. japonicum* and *B. elkanii*, respectively, on the basis of the results from the different analyses performed. Only four of the American *Bradyrhizobium* strains did not belong to these two clusters. The *Lespedeza*-nodulating strains *B. japonicum* USDA 3204 and *B. elkanii* USDA 3399 did not nodulate soybean, but the soybean-derived strains *B. japonicum* USDA 6^T and *B. elkanii* USDA 76^T formed nodules on *L. cuneata*. These results may imply the existence of biovars within *Bradyrhizobium* spp., but further data regarding the host ranges of the different strains are needed before biovars can be described. It has been reported that the host ranges of *Bradyrhizobium* spp. are rather complicated. Strains isolated from a wide range of hosts in China (Chen *et al.*, 1991; Gao *et al.*, 1994) have been classified as *B. japonicum*.

Fast-growing, acid-producing rhizobial species were isolated mainly from Chinese soils associated with nine *Lespedeza* spp. (Table 1; Fig. 1). Most of these strains grouped in Cluster 1 (*S. saheli*). *S. saheli* was originally described for strains nodulating *Sesbania* spp. in Africa (de Lajudie *et al.*, 1994). The identification of Cluster 1 strains as *Sinorhizobium saheli* indicated a wide distribution of this bacterium, and added to it new hosts native to the temperate regions of China. The nodulation observed upon inoculation of *L. cuneata* with the type strain of *S. saheli* demonstrated that the strains isolated from *Sesbania* spp. and the strains isolated from *Lespedeza* spp. represent a cross-nodulating group of bacteria. It is interesting that species of *Sesbania* and *Lespedeza* can share their microsymbionts, since these two groups of plants have geographical origins and habitats that are very different from each other. In this case, it appears that the symbiotic determinants of the plants may not relate to the phylogeny of the legume species.

Based on our results, geographical restrictions exist for the different rhizobial species that nodulate *Lespedeza* spp. Although *L. bicolor* and *L. daurica* are found in the USA and China, they nodulate with fast-growing, acid-producing rhizobia in Chinese soils and with *Bradyrhizobium* spp. in American soils. The differences between the USA strains and the Chinese strains might indicate a geographical isolation for these rhizobial populations. In other words, there may be a lack of natural transmission of these rhizobial species between China and the USA. Strains associated with *L. inschanica* were from China and were identified mainly as *Sinorhizobium saheli*, but two strains grouped with *S. terangae* and *S. fredii* (Fig. 1). *L. juncea* was found to nodulate with both *B. elkanii* and *B. japonicum* in the American soils. Although only strains of *Bradyrhizobium* spp. were isolated from *L. juncea* and *L. cuneata* and strains of *Sinorhizobium* spp. were the dominant organisms isolated from *L. inschanica*, complex relationships should be expected between the *Lespedeza* spp. and rhizobial species when more rhizobial strains are investigated.

Diverse *Bradyrhizobium* populations and many novel groups or lineages belonging to this genus have been reported, including strains isolated from peanut (van Rossum *et al.*, 1995), shrubby legumes (Lafay & Burdon, 1998), *Acacia albida* (Dupuy *et al.*, 1994), *Aeschynomene* spp. (van Berkum *et al.*, 1995; Ladha &

So, 1994; So *et al*., 1994) and *Lupinus* spp. (Barrera *et al*., 1997). These reports indicated that novel taxa should be described for these slow-growing, alkali-producing rhizobia. In this study, *Bradyrhizobium* strains isolated from *L. cuneata* growing in Beijing formed a unique group (Cluster 2) based upon the results of numerical taxonomic, SDS-PAGE, 16S rDNA RFLP and DNA–DNA hybridization analyses. The high 16S rRNA gene sequence similarity between strain CCBAU 10071^T (the representative strain of Cluster 2) and *B. japonicum* LMG 6138^T (Fig. 2) indicated that Cluster 2 represented a lineage within the genus *Bradyrhizobium*.

We have noticed that the criterion of 70 % DNA–DNA homology as the cut-off value for strains of the same species has been heavily criticized (Ward, 1998); strains showing DNA–DNA homology values of much less than 70 % have been included in some single species, e.g. *R. tropici* type A and type B (Martínez-Romero *et al*., 1991), *Mesorhizobium plurifarium* (de Lajudie *et al*., 1998b) and *R. mongolense* (van Berkum *et al*., 1998). These cases offer examples where the definition of a species was not based solely on DNA–DNA homology data, but also on phenotypic distinctiveness and results from other genetic analyses. In this study, strains within Clusters 1, 2, 3 and 4 had DNA–DNA homology values of more than 72·2, 72·6, 71·4 and 72·0 %, respectively. Although DNA–DNA homology values of up to 47·8 % were obtained among the different clusters, there was a big gap between the homologies within a genomic group and among different groups. Therefore, it is clear from our results that Cluster 2 represents a distinct genomic species that is different from *B. japonicum*, *B. liaoningense* and *B. elkanii*. Since the strains within Cluster 2 could be distinguished from the three recognized members of the genus *Bradyrhizobium* on the basis of phenotypic features, SDS-PAGE analysis of proteins and RFLP analysis of 16S rDNA, we propose that the strains within this cluster represent a novel species of the genus *Bradyrhizobium*, *Bradyrhizobium yuanmingense*. Features useful in distinguishing this novel species from other *Bradyrhizobium* spp. can be found in Table 3; another unique feature of this novel species is that it can form ineffective nodules on *Medicago sativa* and *Melilotus albus*, but not on soybean.

Description of *Bradyrhizobium yuanmingense* sp. nov.

Bradyrhizobium yuanmingense (yu.an.ming.en′se. N.L. neut. adj. *yuanmingense* referring to the royal garden Yuanmingyuan in Beijing, China, from where the bacterium was isolated).

Cell morphology is characteristic for the genus. Colonies < 1·0 mm in diameter after 7 days incubation at 28 °C. Produces alkali on YMA. Generation time is 9·5–16 h in PY broth. Optimum growth temperature is between 25 and 30 °C; no growth at 10 or 40 °C. Optimum growth pH is between 6·5 and 7·5; no growth at pH 5·0 or 10·0. Sensitive to 1·0 % (w/v) NaCl in YMA. Reduces nitrate. Uses mannitol, pyruvate and xylose, but not D-ribose, sorbose, starch, D-arginine, glycine or L-methionine as sole carbon source. Nitrate, DL-alanine, L-glutamine, L-isoleucine, L-threonine and hypoxanthine are utilized as sole nitrogen source. Sensitive to treatment at 60 °C. Catalase-positive and L-phenylalaninase-negative. Does not produce 3-keto-lactose. Symbiotic genes are chromosomal-borne; no plasmids observed. Other characteristics of the species can be found in Table 3. Isolated from *L. cuneata*, but also nodulates *V. unguiculata* and *Glycyrrhiza uralensis*. DNA G+C content is 61·8–64·1 mol % (T_m). The type strain of *Bradyrhizobium yuanmingense* is CCBAU 10071^T (= CFNEB 101^T); generation time of the type strain is 10·2 h and its DNA G+C content is 63·0 mol % (T_m).

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NOTE ADDED IN PROOF

While this manuscript was in preparation for publication, Young *et al*. (2001) proposed a revision of the genus *Rhizobium*, so that it would include all species of the genus *Agrobacterium* and *Allorhizobium undicola*. Fig. 2 has been changed to reflect the amended classification, as has the text of the manuscript. The almost-complete 16S rRNA gene sequence of *B. liaoningense* was also deposited within GenBank during this time (Willems *et al*., 2001).

REFERENCES

Allen, O. N. & Allen, E. K. (1981). *The Leguminosae: A Source Book of Characteristics, Uses, and Nodulation*. Madison, WI: University of Wisconsin.

Barrera, L. L., Trujillo, M. E., Goodfellow, M., García, F. J., Hernández-Lucas, I., Dávila, G., van Berkum, P. & Martínez-Romero, E. (1997). Biodiversity of bradyrhizobia nodulating *Lupinus* spp. *Int J Syst Bacteriol* **47**, 1086–1091.

Campanacci, L., Romagnoli, G. F., Borsatti, A., Mendola, R., Maschio, G., Poli, D. & Todesco, S. (1965). Effects of the purified fraction of '*Lespedeza capitata*' on kidney function in the normal subject and in the kidney disease patient. *Minerva Med* **56**, 4288–4298 (English translation).

Chen, W., Wang, E., Wang, S., Li, Y., Chen, X. & Li, Y. (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.

Chen, W. X., Yan, G. H. & Li, J. L. (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.

Chen, W. X., Li, G. S., Qi, Y. L., Wang, E. T., Yuan, H. L. & Li, J. L. (1991). *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *Int J Syst Bacteriol* **41**, 275–280.

de Lajudie, P., Willems, A., Pot, B. & 7 other authors (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.

de Lajudie, P., Laurent-Fulele, E., Willems, A., Tork, U., Coopman, R., Collins, M. D., Kersters, K., Dreyfus, B. & Gillis, M. (1998a). *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *Int J Syst Bacteriol* **48**, 1277–1290.

de Lajudie, P., Willems, A., Nick, G. & 9 other authors (1998b). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *Int J Syst Bacteriol* **48**, 369–382.

De Ley, J. (1970). Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. *J Bacteriol* **101**, 738–754.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Dupuy, N., Willems, A., Pot, B. & 7 other authors (1994). Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*. *Int J Syst Bacteriol* **44**, 461–473.

Eckhardt, T. (1978). A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1**, 584–588.

Gamble, G. R., Akin, D. E., Makkar, H. P. S. & Becker, K. (1996). Biological degradation of tannins in sericea lespedeza (*Lespedeza cuneata*) by the white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus* analyzed by solid-state ^{13}C nuclear magnetic resonance spectroscopy. *Appl Environ Microbiol* **62**, 3600–3604.

Gao, J. L., Sun, J. G., Li, Y., Wang, E. T. & Chen, W. X. (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, China. *Int J Syst Bacteriol* **44**, 151–158.

Genetics Computer Group (1995). *Program Manual for the Wisconsin Package, Version 8*. Genetics Computer Group, 575 Science Drive, Madison, WI, USA.

Graham, P. H., Sadowsky, M. J., Keyser, H. H. & 8 other authors (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.

He, S. Y. (1993). *Beijing Flora*, vol. 1. Beijing: Beijing Press (in Chinese).

Hynes, M. F. & McGregor, N. F. (1990). Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. *Mol Microbiol* **4**, 567–574.

Jarvis, B. D. W., van Berkum, P., Chen, W. X., Nour, S. M., Fernandez, M. P., Cleyet-Marel, J. C. & Gillis, M. (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int J Syst Bacteriol* **47**, 895–898.

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* Conn 1938, 321^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–254. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

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

Ladha, J. K. & So, R. B. (1994). Numerical taxonomy of photosynthetic rhizobia nodulating *Aeschynomene* species. *Int J Syst Bacteriol* **44**, 62–73.

Lafay, B. & Burdon, J. J. (1998). Molecular diversity of rhizobia occurring on native shrubby legumes in Southeastern Australia. *Appl Environ Microbiol* **64**, 3989–3997.

Lindström, K. (1989). *Rhizobium galegae*, a new species of legume root nodule bacteria. *Int J Syst Bacteriol* **39**, 365–367.

Marmur, J. (1961). A procedure for the isolation of DNA from microorganisms. *J Mol Biol* **3**, 208–218.

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

Nour, S. M., Fernandez, M. P., Normand, P. & Cleyet-Marel, J.-C. (1994). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *Int J Syst Bacteriol* **44**, 511–522.

Page, R. D. (1996). TREE VIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

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

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

Sneath, P. H. A. & Sokal, R. B. (1973). *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. San Francisco: W. H. Freeman.

So, R. B., Ladha, J. K. & Young, J. P. W. (1994). Photosynthetic symbionts of *Aeschynomene* spp. form a cluster with bradyrhizobia on the basis of fatty acid and rRNA analyses. *Int J Syst Bacteriol* **44**, 392–403.

Tan, Z.-Y., Xu, X.-D., Wang, E.-T., Gao, J.-L., Martínez-Romero, E. & Chen, W.-X. (1997). Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. *Int J Syst Bacteriol* **47**, 874–879.

Tan, Z. Y., Wang, E. T., Peng, G. X., Zhu, M. E., Martínez-Romero, E. & Chen, W. X. (1999). Characterization of bacteria isolated from wild legumes in the north-western regions of China. *Int J Syst Bacteriol* **49**, 1457–1469.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

van Berkum, P., Tully, R. E. & Keister, D. L. (1995). Nonpigmented and bacteriochlorophyll-containing bradyrhizobia isolated from *Aeschynomene indica*. *Appl Environ Microbiol* **61**, 623–629.

van Berkum, P., Beyene, D., Bao, G., Campbell, T. A. & Eardly, B. D. (1998). *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. *Int J Syst Bacteriol* **48**, 13–22.

van Rossum, D., Schuurmans, F. P., Gillis, M., Muyotcha, A., van Verseveld, H. W., Stouthamer, A. H. & Boogerd, F. C. (1995). Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. *Appl Environ Microbiol* **61**, 1599–1609.

Vincent, J. M. (1970). *A Manual for the Practical Study of Root Nodule Bacteria*. Oxford: Blackwell Scientific.

Wang, E. T., van Berkum, P., Sui, X. H., Beyene, D., Chen, W. X. & Martínez-Romero, E. (1999). Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. *Int J Syst Bacteriol* **49**, 51–65.

Ward, D. M. (1998). A natural species concept for prokaryotes. *Curr Opin Microbiol* **1**, 271–277.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

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

Willems, A., Coopman, R. & Gillis, M. (2001). Phylogenetic and DNA–DNA hybridization analyses of *Bradyrhizobium* species. *Int J Syst Evol Microbiol* **51**, 111–117.

Xu, L. M., Ge, C., Cui, Z., Li, J. & Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* **45**, 706–711.

Young, J. P. W. & Haukka, K. E. (1996). Diversity and phylogeny of rhizobia. *New Phytol* **133**, 87–94.

Young, J. P. W., Downer, H. L. & Eardly, B. D. (1991). Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *J Bacteriol* **173**, 2271–2277.

Young, J. M., Kuykendall, L. D., Martínez-Romero, E., Kerr, A. & Sawada, H. (2001). A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.* 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int J Syst Evol Microbiol* **51**, 89–103.