Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the Loess Plateau of China and description of *Rhizobium loessense* sp. nov.

Ge Hong Wei,[1,2] Zhi Yuan Tan,[1] Ming E Zhu,[2] En Tao Wang,[3] Su Zhen Han[1] and Wen Xin Chen[1]

Correspondence
Wen Xin Chen
wenxin_chen@263.net

[1]College of Biological Sciences, China Agricultural University, Beijing 100094, People's Republic of China

[2]College of Resource and Environment Sciences, Northwest Science and Technology University of Agriculture and Forestry, Yangling Shaanxi 712100, People's Republic of China

[3]Departamento de Microbiología, Escuela Nacional de Ciencias biológicas, Instituto Politécnico Nacional, México, DF, Mexico

Twenty-nine rhizobial isolates from root nodules of *Astragalus* and *Lespedeza* spp. growing in the Loess Plateau of China were characterized by numerical taxonomy, RFLP and sequencing of PCR-amplified 16S rRNA genes, measurement of DNA G+C content, DNA–DNA relatedness and cross-nodulation with selected legume species. Based on the results of numerical taxonomy, the isolates formed two clusters (1 and 2) with some single isolates at a similarity level of 82 %. Cluster 1 contained six isolates from *Astragalus* and *Lespedeza* spp. Cluster 2 consisted of nine isolates from *Astragalus* spp. DNA relatedness was greater than 80 % among isolates within cluster 2. Phylogenetic analysis based on 16S rRNA gene sequences showed that CCBAU 7190B^T, representing cluster 2, was closely related to *Rhizobium galegae* and *Rhizobium huautlense*. DNA–DNA relatedness between CCBAU 7190B^T and reference strains of *R. galegae*, *R. huautlense* and other related species ranged from 0 to 48·6 %. The cluster 2 isolates could also be differentiated phenotypically from related species. Based on these data, a novel species, *Rhizobium loessense* sp. nov., is proposed for cluster 2, with the type strain CCBAU 7190B^T (=AS1.3401^T=LMG 21975^T).

INTRODUCTION

To date, more than 30 species have been described for legume-associated symbiotic nitrogen-fixing bacteria within the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* and *Methylobacterium* in the *α-Proteobacteria*, as well as *Burkholderia* and *Ralstonia* in the *β-Proteobacteria* (Chen *et al.*, 2001; Moulin *et al.*, 2001). However, the diversity of these bacteria is still far from clear compared with the great number and vast distribution of their leguminous hosts. Also, in these studies, rhizobia were mainly isolated from a small proportion of legumes, mainly crops, such as soybean, common bean and alfalfa. Recently, some rhizobia have been characterized from wild and tree legumes and several novel taxa were proposed on the basis of these studies (Squartini *et al.*, 2002; Wei *et al.*, 2002). Therefore, the characterization of more isolates from different leguminous species is necessary in order to understand the diversity and evolution of rhizobia.

The genus *Astragalus*, including 1500–2000 species, is one of the largest genera in the family Leguminosae. Many species within this genus are important resources for production of herbal medicines or foliage for wild animals. Nodulation of 90 *Astragalus* species has been reported (Allen & Allen, 1981), although very little taxonomic work has been done on the microsymbionts associated with these species. Rhizobia isolated from *Astragalus sinicus* in China have been assigned to *Mesorhizobium huakuii* (Chen *et al.*, 1991). The genetic diversity among rhizobia from *Astragalus adsurgens* in different geographical regions of China has been investigated, and bacterial lineages related to *Mesorhizobium*,

Published online ahead of print on 9 May 2003 as DOI 10.1099/ijs.0.02031-0.

The GenBank/EMBL/DDBJ accession number of the 16S rDNA sequence of strain CCBAU 7190B^T is AF364069.

Details of the sources of reference strains used in this study and a 16S rDNA-based phylogenetic tree showing a wider selection of reference rhizobial strains are available as supplementary material in IJSEM Online.

Rhizobium and *Sinorhizobium* were reported (Gao *et al.*, 2001). Different rhizobial groups were also defined among isolates from other *Astragalus* species in China (Wang & Chen, 1996) and in other countries (Laguerre *et al.*, 1997; Murooka *et al.*, 1993; Novikova *et al.*, 1994; Wdowiak & Malek, 2000).

About 100 species have been described in the leguminous genus *Lespedeza*, including herbs, semishrubs and shrubs (Allen & Allen, 1981), and 70 *Lespedeza* species have been found in China. The leaves, stems and roots of many of these plants are used as herbal medicines. *Lespedeza cyrtobotrya*, *Lespedeza buergeri*, *Lespedeza bicolor* and some other species are used for treating lung infections, for diuresis or for stopping bleeding; *Lespedeza pilosa*, *Lespedeza tomentosa*, *Lespedeza chinensis* and some others are conducive to the health of the stomach. The flowers of some species are also important honey resources. Some rhizobial isolates from *Lespedeza* species in China and in the USA have previously been characterized (Yao *et al.*, 2002). Most isolates from the USA were identified as *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*. The isolates from China were classified as *Sinorhizobium saheli* and a novel species, *Bradyrhizobium yuanmingense*, as well as some unnamed strains.

Legume species within the genera *Astragalus* and *Lespedeza*, as well as many others, are found in the Loess Plateau, which includes parts of the provinces of Shanxi, Shaanxi, Gansu and Ningxia. Located in the north-western region of China, these areas have semi-arid climates and soils poor in organic matter. The leguminous plants that grow there are normally drought-enduring and are held in high esteem as foliage, green-manure crops or honey resources and play an important role in preventing soil erosion and in improving the adverse environment. In recent years, we have investigated rhizobial resources and characterized rhizobial isolates from *Amorpha fruticosa*, *Glycyrrhiza* spp., *Gueldenstaedtia* spp. and some other plants in this region according to their geographical origin or host origin (Peng *et al.*, 2002; Tan *et al.*, 1999; Wang *et al.*, 1999). Novel species within the genera *Rhizobium* (Tan *et al.*, 2001; Wei *et al.*, 2002), *Sinorhizobium* (Wei *et al.*, 2002) and *Mesorhizobium* (Wang *et al.*, 1999) have been described for some of these isolates. This research indicates that rhizobia in temperate regions are as diverse as those in tropical areas.

Considering the potential value of *Astragalus* and *Lespedeza* species in sustainable agriculture and the insufficient study on the diversity of rhizobia associated with these plants, we decided to collect and characterize rhizobia associated with these two plant genera in the Loess Plateau. In this research, 29 nodule isolates from *Astragalus* and *Lespedeza* species growing in the Loess Plateau were characterized. The aims of the research were to examine the diversity and to clarify the taxonomic position of the isolates by both phenotypic and genetic analyses.

METHODS

Isolates and strains. Root nodules were collected from 11 leguminous members of *Astragalus*, *Lespedeza* and *Hedysarum* in fields of Shaanxi, Gansu and Ningxia provinces (Table 1). Nodules were collected from wild plants growing in soils poor in organic matter and without the addition of fertilizer. Rhizobia were isolated from fresh nodules by the standard method on YMA medium (Vincent, 1970). Single colonies were picked and checked for purity by repeated streaking and by microscopic examination. Nodulation on the original host plant of each isolate was checked in glass tubes half-filled with vermiculite as described by Vincent (1970). Inoculated plants were grown in a greenhouse at 23 °C during the day and 12 °C during the night and were illuminated with 10 000–20 000 lux for 14 h day^{-1}. Details of the 37 reference strains used in this study are available as supplementary material in IJSEM Online.

Phenotypic characterization and numerical taxonomy. One hundred and thirty-six phenotypic features, including utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance of dyes and chemicals, temperature and pH ranges for growth and some physiological and biochemical reactions described previously (Gao *et al.*, 1994) were examined. Generation times of the isolates were determined spectrophotometrically (Yelton *et al.*, 1983) in YM broth (Vincent, 1970).

UPGMA (Sneath & Sokal, 1973) was used for clustering analysis of phenotypic features. The mean similarity for each isolate within a cluster was estimated to present the phenotypic variation in the cluster, as described previously, and the central isolate, representative of the cluster, was identified as the highest mean similarity holder (Sneath & Sokal, 1973).

PCR-based RFLP of 16S rRNA genes. Procedures described previously (Wang *et al.*, 1998) were used for PCR amplification of almost-complete 16S rRNA genes with primers P1 and P6 (Tan *et al.*, 1997), for digestion of the PCR products with restriction endonuclease *Msp*I, *Hinf*I, *Hae*I or *Rsa*I and for separation of the digested fragments in 3·0 % agarose gels. Similarities (S_j) among bacteria were estimated using the formula $S_j=(a+b)/(a+b+2c)$, where a is the number of bands unique to strain A, b is the number of bands unique to strain B and c is the number of bands common to the two strains. A dendrogram showing phylogenetic relationships among the bacteria was constructed by using the UPGMA method (Sneath & Sokal, 1973).

Phylogenetic analysis of 16S rRNA genes. Full-length 16S rRNA genes (1500 bp) were amplified, cloned and sequenced as described previously (Tan *et al.*, 1997). The 16S rDNA sequences obtained and those of related bacteria from the GenBank database were aligned using the PILEUP program in the GCG package (Genetics Computer Group, 1995). Sequence similarities among the 1500 bp sequences were calculated and a phylogenetic tree was generated and bootstrapped with 1000 subsamples using CLUSTAL W version 1.7 (Thompson *et al.*, 1994). The tree was visualized with the TreeView program (Page, 1996).

Estimation of DNA G+C content and DNA–DNA relatedness. DNAs were extracted and purified by a standard method (Marmur, 1961). DNA base compositions were determined using the thermal melting protocol (De Ley, 1970) with *Escherichia coli* K-12 as standard. Levels of DNA relatedness were estimated by measurement of the initial reassociation rate (De Ley *et al.*, 1970).

Distinctive features and host range of the novel group. Distinctive features were chosen by comparing the phenotypic features of the nine isolates within cluster 2 with those of the most closely related species, *Rhizobium galegae* and *Rhizobium huautlense*. In order to test the host range of the novel group, plants inoculated with the rhizobial isolates were grown in pots filled with vermiculite

Table 1. Isolates used in this research

CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China. All isolates were obtained from locations in north-western China. Details of reference strains are available as supplementary material in IJSEM Online.

Strain/isolate	Host plant	Geographical origin
Cluster 1 (*Rhizobium* sp.)		
CCBAU 71291	*Astragalus complanatus*	Gansu
CCBAU 71394, CCBAU 71395	*Astragalus adsurgens*	Shaanxi
CCBAU 71218	*Lespedeza davidii*	Ningxia
CCBAU 71323	*Lespedeza cyrtobotrya*	Shaanxi
CCBAU 71199	*Hedysarum scoparium*	Ningxia
Cluster 2 (*Rhizobium loessense* sp. nov.)		
CCBAU 71264, CCBAU 7190A, CCBAU 7190B^T	*A. complanatus*	Gansu
CCBAU 71288, CCBAU 71309	*A. complanatus*	Shaanxi
CCBAU 71240	*Astragalus scobwerrimus*	Gansu
CCBAU 71289	*A. scobwerrimus*	Ningxia
CCBAU 71295	*Astragalus chrysopterus*	Shaanxi
CCBAU 71291	*A. chrysopterus*	Gansu
Other rhizobial isolates		
CCBAU 71148, CCBAU 71169	*A. complanatus*	Shaanxi
CCBAU 71272	*A. complanatus*	Gansu
CCBAU 71145	*A. scobwerrimus*	Shaanxi
CCBAU 71236	*A. scobwerrimus*	Gansu
CCBAU 71155	*Astragalus mongholicus*	Shaanxi
CCBAU 671286	*A. adsurgens*	Gansu
CCBAU 71152	*A. adsurgens*	Shaanxi
CCBAU 71Y12	*Lespedeza bicolor*	Shaanxi
CCBAU 71069	*Lespedeza davurica*	Shaanxi
CCBAU 71312	*Lespedeza virgata*	Shaanxi
CCBAU 71308, CCBAU 71075	*Lespedeza cyrtobotrya*	Shaanxi
CCBAU 71263	*Lespedeza* sp.	Gansu

moistened with N-free plant nutrient solution (Vincent, 1970) for 1 month before nodulation and nitrogen fixation was recorded. Cross-nodulation was tested among the isolates within the novel cluster and their host plants, as well as among representatives of the novel groups and recommended legume species (Graham *et al.*, 1991), including *Astragalus sinicus*, *Medicago sativa*, *Galega orientalis*, *Lotus corniculatus*, *Vigna sinensis*, *Glycine max*, *Trifolium repens*, *Phaseolus vulgaris* and *Caragana korshinskii*.

RESULTS AND DISCUSSION

Thanks to the application of molecular biological methods, the taxonomy of root- and/or stem-nodulating bacteria has changed dramatically in the last two decades. Polyphasic taxonomy covering phenotypic, genomic and phylogenetic characteristics has generally been used. In this study, we characterized 29 nodule isolates from *Astragalus* and *Lespedeza* species in the Loess Plateau of China by means of a polyphasic approach.

Phenotypic characterization and numerical taxonomy

In this study, the 29 isolates used were fast-growing rhizobia that formed single colonies with diameters of 2–3 mm within 3 days on YMA. All of them were effective symbionts, as evidenced by the formation of pink nodules on their original hosts. None of the tested strains could use adipate, inulin, sorbitol or syringic acid as sole carbon sources. All of the strains were sensitive to kanamycin (100–300 μg ml^{-1}) and neomycin (100 and 300 μg ml^{-1}). No strain could grow in medium supplied with 0·1 % gentian violet or methylene blue or 5 % NaCl. They could not grow at pH 4·0 or at 4 °C. All strains used L-arginine as a sole nitrogen source and were resistant to the antibiotics bacitracin (5–300), erythromycin (5), neomycin (5) and polymyxin (5) (all μg ml^{-1}). All strains grew in medium supplemented with 0·1 % Bismarck brown, Congo red, erythrosin, neutral red, sodium deoxycholate and sodium nitrite and with 1·0 (w/v) NaCl.

The 102 features that varied among the tested strains were used for cluster analysis. Based on these results (Fig. 1), most of the defined species could be separated at a similarity level of 82 %. The exceptions were *Rhizobium tropici* and *Rhizobium hainanense*, which were separated at 86 % similarity. Similarities of 96–65 % were found among the novel isolates (Fig. 1), indicating that these isolates represented phenotypically diverse populations. Since the characteristics covered physiological, biochemical and

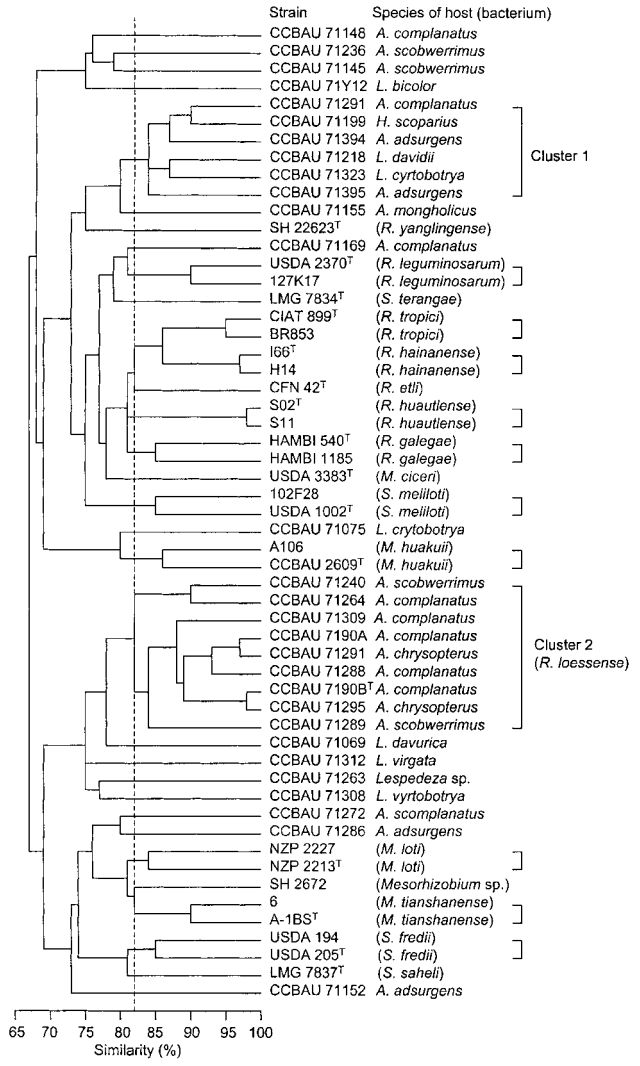

Fig. 1. Dendrogram showing the results of numerical taxonomy. The coefficient S_{sm} and UPGMA (Sneath & Sokal, 1973) were used for clustering analysis of phenotypic features.

environmental factors and resistance to chemicals and antibiotics, the phenotypic diversity also indicated that the isolates represented a diverse gene pool. The diversity within the rhizobial population could offer advantages in survival and adaptation of nodulation in different environments.

Fifteen of the 29 isolates formed two clusters at a similarity level of 83 %, while the other isolates were single branches (Fig. 1). These groups and single branches were different from the reference species, and they might infer different species or genera. Some important aspects of the two clusters are described below.

Cluster 1 consisted of six fast-growing isolates from five host species within three genera, *Astragalus complantus*, *Astragalus adsurgens*, *Hedysarum scoparium*, *Lespedeza davidii* and *Lespedeza cyrtobotrya*. The mean similarity for the isolates ranged from 84 % for CCBAU 71395 to 91 % for CCBAU 71199; the latter was chosen as the central isolate. Colonies were 2–3 mm in diameter after 3 days on YMA at 28 °C. All isolates in this cluster produced acid in YMA, and all isolates grew in Luria–Bertani (LB) broth.

Cluster 2 contained nine isolates from *Astragalus scobwerrimus*, *Astragalus complanatus* and *Astragalus chrysopterus*. The mean similarities ranged from 83 to 99 %. CCBAU 7190B^T was chosen as the central isolate. They were fast-growing, acid-producing bacteria with a generation time of 2·2–3·4 h in YM broth, as determined spectrophotometrically. No growth of these isolates was observed in LB broth.

Numerical taxonomy has been used for grouping bacteria and for offering descriptive features for species. Clusters formed in numerical taxonomy often correspond to genomic groups defined by DNA–DNA hybridizations and other genomic analyses (Chen *et al.*, 1991, 1995, 1997; de Lajudie *et al.*, 1994). Therefore, the grouping results obtained in this approach could be a basis for further taxonomic study. Since cluster 1 consisted of only a few isolates, we did not characterize it further and instead focused on cluster 2.

PCR-RFLP of 16S rRNA genes

As a rapid method, this technology has been used for grouping and identifying rhizobia. The grouping results of PCR-RFLP patterns of 16S rRNA genes agree well with those of multilocus enzyme electrophoresis, numerical taxonomy and sequencing of 16S rRNA genes (Laguerre *et al.*, 1994; Tan *et al.*, 1999; Wang *et al.*, 1998, 1999). Following the grouping results from numerical taxonomy, three cluster 2 isolates, CCBAU 7190B^T, CCBAU 7190A and CCBAU 71240, and reference strains from defined species were chosen for this technique. The three representative isolates of cluster 2 had very similar patterns, with one or two bands different in one or another digestion with *Msp*I, *Hinf*I, *Hae*I or *Rsa*I (not shown). Distinctive patterns were observed among the rhizobia in cluster 1 and other species (not shown). In a dendrogram constructed based upon the PCR-RFLP patterns of 16S RNA genes (Fig. 2), four groups corresponding to the genera *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Rhizobium* could be divided at the level of 80 % similarity. Isolates of cluster 2 formed a subgroup within the genus *Rhizobium*, together with *R. galegae* and *R. huautlense*. These results indicated that cluster 2 was also a group based upon 16S rRNA gene sequences and it was probably related to *R. galegae*.

Sequencing of 16S rDNA and its phylogeny

Two independent clones of PCR-amplified 16S rDNA from the central isolate CCBAU 7190B^T of cluster 2 were sequenced. Sequences of the two clones were identical. In the reconstructed phylogenetic tree (Fig. 3), the phylogenetic relationships among the defined species are similar to those

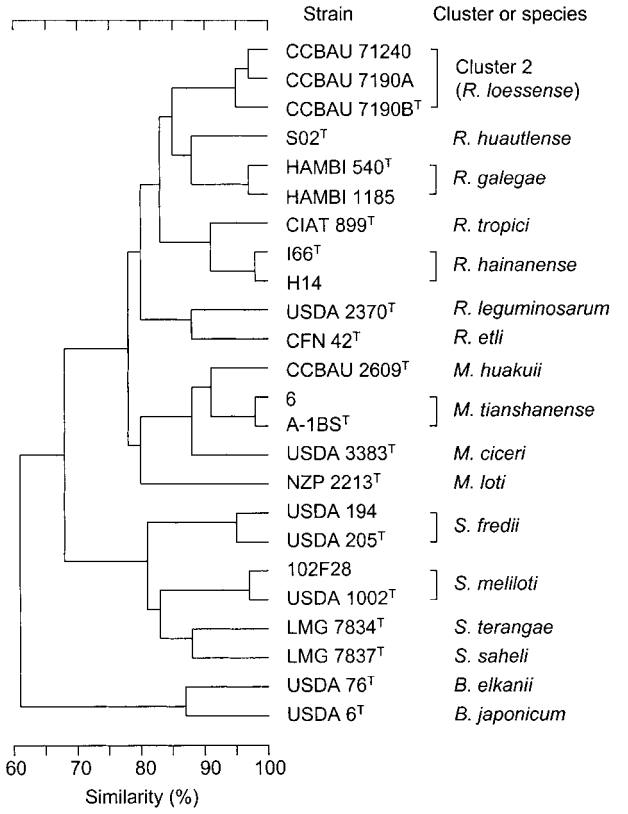

Fig. 2. Dendrogram showing relationships among the novel isolates and reference strains revealed by PCR-RFLP of 16S rRNA genes. The coefficient S_j and the UPGMA method were used for the estimation of similarity and clustering analysis (Sneath & Sokal, 1973).

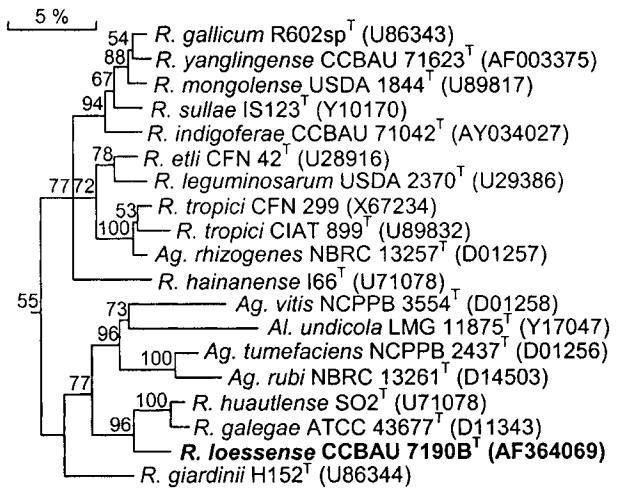

Fig. 3. Phylogenetic tree based on 16S rDNA sequences showing the position of the novel species. Nucleotide sequences of 16S rRNA genes obtained in this research and from the GenBank database were aligned, sequence similarities were calculated and a phylogenetic tree generated, bootstrapped with 1000 subsamples and visualized as described in Methods. Abbreviations: *R.*, *Rhizobium*; *Ag.*, *Agrobacterium*; *Al.*, *Allorhizobium*. An extended tree including a wider range of reference sequences is available as supplementary material in IJSEM Online.

in previously reported trees (de Lajudie *et al.*, 1994, 1998a, b; Tan *et al.*, 1997; Wang *et al.*, 1999). The genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* are monophyletic groups; an extended tree including these genera is available as supplementary material in IJSEM Online. The species within the genus *Rhizobium* form a polyphyletic group. Nine *Rhizobium* species, including the type species, *Rhizobium leguminosarum*, formed a monophyletic group. The cluster 2 isolate CCBAU 7190B^T, *R. galegae* and *R. huautlense* formed another monophyletic group and linked more closely to *Agrobacterium* species than to other *Rhizobium* species. This *Rhizobium–Agrobacterium* group was also polyphyletic, because *Agrobacterium vitis* and *Allorhizobium undicola* formed two long branches within the group. The 16S rDNA sequence similarities between CCBAU 7190B^T and *R. galegae* and *R. huautlense* were respectively 96·8 and 97·5 %, and were greater than those between CCBAU 7190B^T and other species within the genera *Agrobacterium* and *Rhizobium* ($<$95·0 %).

Since *R. galegae* has been grouped into the phylogenetic branch of *Agrobacterium* species, the taxonomic position of *R. galegae* and related rhizobia has been discussed. One opinion is to give this branch independent genus status (Young & Haukka, 1996). Another, opposing, suggestion is to combine all the species of *Rhizobium*, *Agrobacterium* and *Allorhizobium* into an emended genus *Rhizobium* (Young *et al.*, 2001). Our results in this paper show clearly that the *R. galegae*, *R. huautlense* and cluster 2 are a monophyletic group, separated from both the other *Rhizobium* species and *Agrobacterium* species. The phylogenetic distances shown in Fig. 3 indicate that the relationships among this group and the *Agrobacterium* species are as distant as those among the genera *Mesorhizobium* and *Sinorhizobium*. Moreover, *R. galegae* and *R. huautlense* have been grouped with *Rhizobium mongolense* and *Rhizobium gallicum* in some previous reports (Wang *et al.*, 1998; Peng *et al.*, 2002). Considering the low bootstrap values at the node separating the *R. galegae* branch and *Agrobacterium* species in Fig. 3, characterization of more *R. galegae*-related bacteria is needed in order to make a confident taxonomic conclusion about *R. galegae* and related taxa.

DNA G+C contents and DNA–DNA hybridization

DNA–DNA hybridization was done among reference strains chosen according to the clustering results from numerical taxonomy and the phylogenetic relationships in 16S rDNA analyses. DNA G+C contents and DNA–DNA relatedness values are shown in Table 2. The DNA G+C content for the isolates from cluster 2 was 59·1–60·3 mol% (T_m), which is

Table 2. DNA G+C content and DNA–DNA relatedness among the novel isolates and defined species

G+C contents were determined by the T_m method. Results of DNA–DNA hybridizations are mean percentages ± standard error from three replicates. ND, Not determined.

Species or cluster	DNA G+C content (mol%)	DNA relatedness with strain CCBAU 7190B^T (%)
Cluster 2 (*R. loessense* sp. nov.)		
CCBAU 71240	59·1	81·5 ± 3·8
CCBAU 71264	60·3	81·9 ± 4·0
CCBAU 71309	60·3	91·0 ± 2·9
CCBAU 7190A	59·1	86·2 ± 2·6
CCBAU 71291	59·5	89·0 ± 5·0
CCBAU 71288	60·2	88·6 ± 4·6
CCBAU 7190B^T	59·5	100
CCBAU 71295	59·1	84·7 ± 3·2
Agrobacterium tumefaciens IAM 13129^T	ND	0
Agrobacterium rubi IAM 13569^T	ND	6·2 ± 1·0
Agrobacterium vitis IAM 14140^T	ND	0
R. galegae		
HAMBI 540^T	ND	40·1 ± 2·1
HAMBI 1185	ND	34·2 ± 1·6
R. huautlense		
S02^T	ND	9·3 ± 0·6
S03	ND	10·5 ± 1·2
R. giardinii USDA 2914^T	ND	12·0 ± 1·8
R. yanglingense SH 22623^T	ND	28·2 ± 2·2
R. indigoferae CCBAU 71042^T	ND	48·6 ± 1·3
R. sullae IS123^T	ND	21·1 ± 3·0
Rhizobium sp. SDW014	ND	28·3 ± 1·9
Rhizobium sp. SDW018	ND	37·6 ± 3·8

within the range for *Rhizobium* (Jordan, 1984). The DNA–DNA relatedness among the isolates within cluster 2 ranged from 81·5 to 91·0 % with a mean of 86·1 %, indicating that these isolates were also a genomic group.

The DNA–DNA relatedness between the cluster 2 isolate CCBAU 7190B^T and reference strains, including the most closely related species *R. galegae* and *R. huautlense*, the recently described species *Rhizobium yanglingense* (Tan *et al.*, 2001) and *Rhizobium indigoferae* (Wei *et al.*, 2002) from the Loess Plateau and strains SDW014 and SDW018, representing unnamed rhizobial groups associated with *A. adsurgens* (Gao *et al.*, 2001), varied from 0 to 48·6 %. Although an intermediate level of DNA–DNA relatedness (40 to 48 %) was detected between isolate CCBAU 7190B^T and some reference strains, these values are still much lower than those among strains within the cluster (more than 81 %). From these results, we conclude that the isolates in cluster 2 formed a single genomic group different from the related species.

Based upon the results of numerical taxonomy, 16S rRNA sequence analysis and DNA–DNA hybridization, we conclude that cluster 2 represents a unique group within the genus *Rhizobium*. Considering the definition of other rhizobial species and the current criteria for description of new rhizobial taxa (Graham *et al.*, 1991), we propose a novel species, *Rhizobium loessense* sp. nov., for the isolates within cluster 2. The distinctive features and host range of this bacterium were investigated for identification purposes.

Distinctive features and host range of the novel group

Distinctive phenotypic features for cluster 2, *R. huautlense*, *R. galegae* and some other related species are presented in Table 3. In addition, PCR-RFLP of 16S rRNA genes and host origin are also valuable for distinguishing the novel species from related bacteria.

In cross-nodulation tests, the nine isolates in cluster 2 could share host plants and also nodulated *Astragalus adsurgens*. Isolate CCBAU 7190B^T did not nodulate *Astragalus sinicus*, *Medicago sativa*, *Galega orientalis*, *Lotus corniculatus*, *Vigna sinensis*, *Glycine max*, *Trifolium repens*, *Phaseolus vulgaris* or *Caragana korshinskii*. Since host specificity is an important feature for rhizobia and cross-nodulation has been recommended for definition of novel rhizobia taxa (Graham *et al.*, 1991), we did the cross-nodulation tests.

Table 3. Distinctive features of *Rhizobium loessense* sp. nov. and related species

Species/cluster: 1, cluster 1 ($n=6$); 2, *R. loessense* sp. nov. ($n=9$); 3, *R. galegae* ($n=2$); 4, *R. huautlense* ($n=2$); 5, *R. leguminosarum* ($n=2$); 6, *R. tropici* ($n=2$); 7, *R. hainanense* ($n=2$); 8, *R. etli* ($n=2$). Numbers are percentages of positive strains: +, all strains positive; −, all strains negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Utilization as sole carbon source:								
D-Amygdalin	+	−	50	ND	+	−	−	−
D-Arabinose	50	−	+	+	+	+	+	+
D(+)-Arabitol	+	67	−	+	−	50	+	+
Calcium malonate	−	−	+	ND	+	−	−	−
Dulcitol	+	+	+	+	+	−	+	−
meso-Erythritol	−	67	50	−	−	+	+	−
Sodium citrate	67	−	+	−	+	+	+	+
Salicin	+	90	+	ND	+	−	−	+
D-Ribose	33	−	−	ND	+	+	+	+
D-Gluconate	16	−	50	+	−	−	+	+
D-Sorbitol	+	−	+	+	+	+	+	+
Sucrose	+	67	+	+	−	−	+	+
Tartrate	−	−	50	ND	50	50	−	+
Trehalose	+	−	+	+	+	+	+	+
Xylose	67	−	−	+	+	+	+	+
Glycine	+	78	50	−	−	+	+	+
Utilization as sole nitrogen source:								
L-Glutamic acid	+	+	+	+	−	+	+	−
Glycine	+	+	−	ND	−	+	+	+
L-Valine	+	−	+	ND	−	−	+	−
Resistance to (μg ml^{-1}):								
Ampicillin (100)	67	21	+	−	+	+	+	+
Chloramphenicol (5)	+	+	−	−	+	+	+	+
Chloramphenicol (100)	50	−	−	−	−	+	+	−
Erythromycin (100)	+	−	+	+	−	−	+	−
Kanamycin (50)	−	−	+	+	−	−	−	−
Streptomycin (300)	50	−	−	−	−	+	−	+
Tolerance of (%):								
Acridine hydrochloride (0·1)	16	−	−	−	−	+	+	−
Methyl green (0·1)	+	−	+	−	+	−	+	+
NaCl (2·0)	+	+	−	−	−	+	+	−
Growth at/in:								
pH 5·0	−	−	+	−	−	+	+	−
pH 10·0	+	+	−	+	+	+	+	+
40 °C	ND	ND	−	+	−	−	ND	ND
LB broth	+	−	−	−	−	+	+	−

However, we believe that the association between rhizobia and their hosts under laboratory conditions is less important than in the natural environment. Some rhizobia can form nodules with a legume under laboratory conditions, such as *R. huautlense* on *Leucaena leucocephala* (Wang *et al.*, 1998), from which they have never been isolated in the field.

Description of *Rhizobium loessense* sp. nov.

Rhizobium loessense (lo.es.sen′se. N.L. neut. adj. *loessense* referring to the Loess Plateau of China, where the bacterium was isolated).

Short, aerobic, Gram-negative, non-spore-forming rods, 0·5–0·7 μm wide by 1·8–2·1 μm long. Colonies are 2–3 mm in diameter after 2–3 days on YMA at 28 °C. Strains produce acid in YMA and do not grow in LB medium. Generation time is 2·2–3·4 h in PY broth as determined spectrophotometrically. Utilizes D-arabinose, *meso*-erythritol, D-mannose, sodium citrate, D-ribose, D-sorbitol and dulcitol, but not D-amygdalin, calcium malonate, dulcitol, salicin, D-sodium gluconate or sucrose as sole carbon sources. Utilizes L-glutamic acid and glycine but not L-valine as sole nitrogen sources. Sensitive to 100 μg erythromycin ml^{-1}. Cannot grow in medium supplemented with 0·1 % methyl green or

4·0 % NaCl. Strains have been isolated from *Astragalus scobwerrimus*, *Astragalus complanatus* and *Astragalus chrysopterus* and they can nodulate *Astragalus adsurgens* under laboratory conditions. The DNA G+C content is 59·1–60·3 mol% (T_m).

The type strain is CCBAU 7190B^T (=AS1.3401^T=LMG 21975^T). Its generation time is 3·1 h and its DNA G+C content is 59·5 mol% (T_m).

ACKNOWLEDGEMENTS

This research was a part of projects no. 39730010 and 3000005 supported by the National Science Foundation of China.

REFERENCES

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

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.

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., Tan, Z.-Y., Gao, J.-L., Li, Y. & Wang, E.-T. (1997). *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *Int J Syst Bacteriol* **47**, 870–873.

Chen, W.-M., Laevens, S., Lee, T.-M., Coenye, T., De Vos, P., Mergeay, M. & Vandamme, P. (2001). *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol* **51**, 1729–1735.

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

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

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.

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.

Gao, J., Terefework, Z., Chen, W. & Lindström, K. (2001). Genetic diversity of rhizobia isolated from *Astragalus adsurgens* growing in different geographical regions of China. *J Biotechnol* **91**, 155–168.

Genetics Computer Group (1995). *Program Manual for the Wisconsin Package, Version 8.* Madison, WI: Genetics Computer Group.

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-nodulation bacteria. *Int J Syst Bacteriol* **41**, 582–587.

Jordan, D. C. (1984). Family III. *Rhizobiaceae* Conn 1938, 321^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–235. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

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

Laguerre, G., van Berkum, P., Amarger, N. & Prévost, D. (1997). Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astragalus*, *Oxytropis*, and *Onobrychis*. *Appl Environ Microbiol* **63**, 4748–4758.

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

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature* **411**, 948–950.

Murooka, Y., Xu, Y. & Sanada, K. (1993). Formation of root nodules by *Rhizobium huakuii* biovar renge, bv. nov. on *Astragalus sinicus* cv. Japan. *Ferment Bioeng* **78**, 38–44.

Novikova, N. I., Pavlova, E. A., Vorobjev, N. I. & Limeshchenko, E. V. (1994). Numerical taxonomy of *Rhizobium* strains from legumes of the temperate zone. *Int J Syst Bacteriol* **44**, 734–742.

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

Peng, G. X., Tan, Z. Y., Wang, E. T., Reinhold-Hurek, B., Chen, W. F. & Chen, W. X. (2002). Identification of isolates from soybean nodules in Xinjiang Region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*. *Int J Syst Evol Microbiol* **52**, 457–462.

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

Squartini, A., Struffi, P., Döring, H. & 10 other authors (2002). *Rhizobium sullae* sp. nov. (formerly '*Rhizobium hedysari*'), the root-nodule microsymbiont of *Hedysarum coronarium* L. *Int J Syst Evol Microbiol* **52**, 1267–1276.

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.

Tan, Z. Y., Kan, F. L., Peng, G. X., Wang, E. T., Reinhold-Hurek, B. & Chen, W. X. (2001). *Rhizobium yanglingense* sp. nov., isolated from arid and semi-arid regions in China. *Int J Syst Evol Microbiol* **51**, 909–914.

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.

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

Wang, S. Y. & Chen, W. X. (1996). Numerical taxonomy and DNA relatedness of rhizobia isolated from *Astragalus* spp. In *Diversity and Taxonomy of Rhizobia*, pp. 79–84. Edited by F. D. Li, D. Lie, W. X. Chen & J. C. Zhou. Beijing: China Agricultural Scientech Press.

Wang, E. T., van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X. & Martínez-Romero, E. (1998). *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int J Syst Bacteriol* **48**, 687–699.

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.

Wdowiak, S. & Malek, W. (2000). Numerical analysis of *Astragalus cicer* microsymbionts. *Curr Microbiol* **41**, 142–148.

Wei, G. H., Wang, E. T., Tan, Z. Y., Zhu, M. E. & Chen, W. X. (2002). *Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov., respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea*. *Int J Syst Evol Microbiol* **52**, 2231–2239.

Yao, Z. Y., Kan, F. L., Wang, E. T, Wei. G. H. & Chen, W. X. (2002). Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. *Int J Syst Evol Microbiol* **52**, 2219–2230.

Yelton, M. M., Yang, S. S., Edie, S. A. & Lim, S. T. (1983). Characterization of an effective salt-tolerant, fast-growing strain of *Rhizobium japonicum*. *J Gen Microbiol* **129**, 1537–1547.

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

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.