Rhizobium alkalisoli sp. nov., isolated from *Caragana intermedia* growing in saline-alkaline soils in the north of China

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Three rhizobial strains (CCBAU 01393^T, CCBAU 01389 and CCBAU 03239) isolated from nodules of *Caragana intermedia* grown in saline-alkaline soils in the north of China had identical 16S rRNA genes that showed 99.7 and 99.5 % sequence similarities with those of *Rhizobium huautlense* SO2^T and *Rhizobium galegae* USDA 4128^T, respectively. Phylogenies of the housekeeping genes *atpD*, *recA* and *glnII* confirmed their distinct position, differing from recognized *Rhizobium* species. SDS-PAGE of whole-cell soluble proteins and a series of phenotypic and physiological tests allowed us to differentiate the novel group from all closely related recognized *Rhizobium* species. The levels of DNA–DNA relatedness between strain CCBAU 01393^T and *R. huautlense* SO2^T and *R. galegae* USDA 4128^T were 34.9 and 20.5 %, respectively. Therefore, we propose that strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 represent a novel species, *Rhizobium alkalisoli* sp. nov., with strain CCBAU 01393^T (=LMG 24763^T=HAMBI 3051^T) as the type strain. This strain could form effective nodules on *Caragana microphylla*, *Phaseolus vulgaris* and *Vigna radiata*.

Rhizobia are Gram-negative soil bacteria that mainly cause the formation of root nodules on legumes and fix nitrogen (diazotrophy) inside the nodules. Currently, more than 50 rhizobial species have been described in the classes *Alphaproteobacteria* and *Betaproteobacteria*. *Rhizobium*, a genus in the *Alphaproteobacteria*, was named a century ago and at the time of writing contained at least 16 nitrogen-fixing species including the recently described species *Rhizobium miluonense* (Gu *et al.*, 2008) and *Rhizobium multihospitium* (Han *et al.*, 2008).

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *atpD*, *recA*, *glnII*, *nodC* and *nifH* gene sequences of strain CCBAU 01393^T are EU074168, EU672461, EU672490, EU672475, EU872245 and EU872238, respectively; those of strain CCBAU 01389 are EU872220, EU872226, EU872252, EU872232, EU872246 and EU872239, respectively; and those of strain CCBAU 03239 are EU872221, EU872227, EU872253, EU872233, EU872247 and EU872240, respectively.

Phylogenetic trees based on *nodC* and *nifH* genes, and a phenogram derived from UPGMA/SSM analysis and SDS-PAGE analysis of whole-cell soluble proteins of strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 and related species are available as supplementary material with the online version of this paper.

Caragana is a legume genus with about 100 species and about 70 species have been recorded in China. These *Caragana* species are ecologically important because of their capacity to form nitrogen-fixing nodules with rhizobia.

In the course of a study of rhizobia nodulating *Caragana* species in China, 174 strains were isolated and classified in the genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* by using 16S rRNA gene sequence analysis and other polyphasic taxonomical methods (Lu *et al.*, 2009). Among the strains that were classified in the genus *Rhizobium*, three of them (CCBAU 01393^T, CCBAU 01389 and CCBAU 03239) isolated from saline-alkaline soils in the north of China formed a distinctive 16S rRNA gene type. These three strains together with other related *Rhizobium* species were characterized further in the present study by comparison of their whole-cell soluble proteins using SDS-PAGE, and housekeeping gene (*atpD*, *recA* and *glnII*) sequences, symbiotic gene (*nodC* and *nifH*) sequences, and DNA–DNA hybridization, phenotypic characteristic determination and nodulation tests on various legumes. Rhizobia were isolated from root nodules and were

purified using the standard procedure on yeast-mannitol agar (YMA) (Vincent, 1970). All strains were cultured on YMA (Vincent, 1970) at 28 °C and maintained at 4 °C for temporary storage.

About 1350 bp of the 16S rRNA gene was amplified and sequenced for strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 using the primers P1 and P6 according to Tan *et al.* (1997). The sequences were aligned with those of related *Rhizobium* species using the CLUSTAL W program in the MEGA 4.0 software (Tamura *et al.*, 2007). Aligned sequences were analysed using the same software to produce a Jukes–Cantor distance (Munro, 1969) and to construct an optimal unrooted tree using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods. The robustness of the tree topologies was calculated by bootstrap analysis based on 1000 replications of the sequences (Felsenstein, 1985). All the three strains isolated from *Caragana* had identical 16S rRNA gene sequences and were closely related to *R. huautlense* SO2^T

and *R. galegae* USDA 4128^T (Fig. 1a), with 99.7 and 99.5 % gene sequence similarity, respectively (Table 1). The percentage 16S rRNA gene similarities between the new isolates and the type strains of recognized *Rhizobium* species with values equal to or higher than 97 % are shown in Table 1.

SDS-PAGE of whole-cell soluble proteins has been used widely in rhizobial taxonomy and is suitable for grouping strains at species level (Diouf *et al.*, 2000; Tan *et al.*, 1997; Wang *et al.*, 2007). Preparation and electrophoresis of the soluble proteins were performed according to Tan *et al.* (1997). Normalized densitometric traces of the electrophoretic protein patterns were grouped using the GelCompar II software package (Applied Maths). The similarity between each pair of samples (strains) was expressed by using the Dice coefficient and a UPGMA dendrogram was constructed (Vauterin & Vauterin, 1992). The whole-cell soluble proteins of strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 and the four reference

Fig. 1. Comparison of 16S rRNA (a), *atpD* (b), *recA* (c) and *glnII* (d) gene phylogenies, showing the relationships among the novel group (*Rhizobium alkalisolii* sp. nov.) (in bold) and recognized *Rhizobium* species. Trees were constructed by using the neighbour-joining method with a Jukes–Cantor distance matrix. Bootstrap values (%) based on 1000 replicates are shown at each node. Bars, 0.005 or 0.01 expected changes per site.

Table 1. Sequence similarities for 16S rRNA, *atpD*, *recA*, *glnII*, *nodC*, *nifH* genes and DNA–DNA relatedness (%) among strains of the novel group and reference strains

NA, Not available.

Strain	Strain CCBAU 01393 ^T				
	<i>rrs</i>	<i>atpD</i>	<i>recA</i>	<i>glnII</i>	DNA–DNA relatedness
CCBAU 01389	100	100	99.4	99.6	97.2
CCBAU 03239	100	99.8	99.1	98.3	92.4
<i>R. huautlense</i> SO2 ^T	99.7	93.5	92.8	NA	34.9
<i>R. galegae</i> USDA 4128 ^T	99.5	95.4	87.6	87.6	20.5
<i>R. gallicum</i> USDA 2918 ^T	96.8	89.1	86.5	87.9	13.7
<i>R. leguminosarum</i> USDA 2370 ^T	95.7	86.2	86.2	90.8	8.2
<i>R. cellulosilyticum</i> ALA10B2 ^T	98.5	NA	NA	NA	NA
<i>R. loessense</i> CCBAU 7190B ^T	98.3	NA	NA	NA	15.2

strains of related *Rhizobium* species (Table 2) were subjected to SDS-PAGE analysis. The strikingly similar protein patterns observed among the novel strains indicated considerable homogeneity (see Supplementary Fig. S1, available in IJSEM Online), but they were not duplicate isolates because they were isolated from different sites (Table 2). The protein patterns of reference strains of recognized *Rhizobium* species were quite different from those of the novel strains (Supplementary Fig. S1, in IJSEM Online).

Housekeeping genes, including *atpD*, *recA* and *glnII*, are being used increasingly for protein-coding interspersing throughout the genome. These genes were used to investigate the phylogeny of the bacteria in order to complete that of the 16S rRNA gene (Gaunt *et al.*, 2001; Turner & Young, 2000; Vinuesa *et al.*, 2005). In this study, PCR amplification and sequencing of partial *atpD* (510 bp) and *recA* (530 bp) genes were undertaken according to Gaunt *et al.* (2001), and a 660 bp intragenic fragment of *glnII* was amplified according to Turner & Young (2000). Sequence similarities of *atpD*, *recA* and *glnII* genes among the strains isolated in this study and reference strains are given in Table 1. Neighbour-joining trees constructed based on these sequences are shown in Fig. 1. Strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239

showed high similarities with each other among the three housekeeping genes (Table 1). They were also closely related to *R. huautlense* and *R. galegae* in the phylogenetic trees based on *atpD* and *recA* genes, with 93.5 and 95.4 % (*atpD*) and 92.8 and 87.6 % (*recA*) similarity, respectively (Table 1), supported by bootstrap values of 97 and 95 %, which were congruent with the 16S rRNA gene tree (Fig. 1a). In the *glnII* tree (Fig. 1d), the three *Caragana* rhizobial strains were adjacent to *R. huautlense* CCBAU 65798 and 65679 (no *glnII* sequence of the type strain *R. huautlense* SO2^T was available from the public database), but were further removed from *R. galegae*. Considering all the sequences, the strains in the novel group formed a phylogenetic cluster distinct from recognized species of *Rhizobium*.

Symbiotic genes (*nodC* and *nifH*) and the formation of effective nitrogen-fixing nodules on legumes are important features of rhizobia. Analyses of the rhizobial symbiotic genes could reveal their evolutionary history and host plant ranges. In this study, partial symbiotic genes *nodC* (about 900 bp) and *nifH* (about 760 bp) of strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 were amplified by using the primer pairs *nodCF/nodCI* and *nifHF/nifHI* with the PCR conditions described by Laguerre *et al.* (2001). In the phylogenetic trees based on *nodC* genes

Table 2. Isolates and reference strains used in this study

Strain	Host plant	Geographical origin	Reference
<i>Rhizobium alkalisoli</i> sp. nov.			
CCBAU 01393 ^T	<i>Caragana intermedia</i>	Inner Mongolia, China	This study
CCBAU 01389	<i>Caragana intermedia</i>	Inner Mongolia, China	This study
CCBAU 03239	<i>Caragana intermedia</i>	Shanxi, China	This study
Reference strains			
<i>R. huautlense</i> SO2 ^T	<i>Sesbania herbacea</i>	Mexico	Wang <i>et al.</i> (1998)
<i>R. galegae</i> HAMBI 540 ^T (=USDA 4128 ^T)	<i>Galega orientalis</i>	Finland	Lindström (1989)
<i>R. gallicum</i> USDA 2918 ^T	<i>Phaseolus vulgaris</i>	France	Amarger <i>et al.</i> (1997)
<i>R. leguminosarum</i> USDA 2370 ^T	<i>Pisum sativum</i>	USA	Frank (1889)

(Supplementary Fig. S2a, available in IJSEM Online), the three *Caragana* rhizobia strains clustered within a separate branch and were most close to *Rhizobium loessense* CCBAU 03019 and *R. mongolense* CCBAU 01546, whose host plants were *Astragalus dahuricus* and *Astragalus membranaceus*, respectively (Zhao *et al.*, 2008). The genus *Astragalus* together with *Caragana* belong to the same subtribe Astragalinae (Polhill, 1994) and this may account for the reason that they could be infected by rhizobia with a similar *nifH* gene. The phylogenetic tree based on *nifH* genes (Supplementary Fig. S2b, in IJSEM Online) also confirmed the close relationship between the rhizobial symbiotic genes and their host ranges. *Gueldenstaedtia multiflora*, a host for *Rhizobium yanglingense* SH 22623^T, is also a member of the subtribe Astragalinae (Polhill, 1994). In addition, data from the symbiotic (*nifH* or *nifD*) and the housekeeping gene analyses agreed with the hypothesis of different evolutionary histories between symbiotic genes and genomic background housekeeping (such as *atpD*, *recA* and *glnII*) and *rrs* genes (Chen *et al.*, 2008; Ueda *et al.*, 1995).

Cross-nodulation tests were performed among strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 with the hosts of origin or related hosts: *Glycyrrhiza uralensis*, *Caragana microphylla*, *Astragalus adsurgens*, *Vigna unguiculata*, *Vigna radiata*, *Glycine max*, *Trifolium pratense*, *Melilotus alba*, *Amorpha fruticosa*, *Lepedeza cuneata*, *Dunbaria rotundifolia*, *Vicia septum* and the recommended plant species *Leucaena leucocephala*, *Phaseolus vulgaris*, *Pisum sativum* and *Medicago sativa* (Graham *et al.*, 1991). The representative strain of the novel group, CCBAU 01393^T, could induce effective nodules on *Caragana microphylla*, *Phaseolus vulgaris* and *Vigna radiata*, but not on the other legumes tested.

DNA–DNA hybridization is considered as a necessary and standard method for defining bacterial species (Graham *et al.*, 1991; Wayne *et al.*, 1987). In this study, total DNA extracted from each strain using the method of Marmur (1961) was used for hybridization with the spectrophotometric method (De Ley *et al.*, 1970). DNA–DNA hybridization was performed between strain CCBAU 01393^T and related reference strains (Table 1). All the experiments were performed three times and the mean DNA–DNA relatedness values were used. The range of the standard deviation was 1.47–5.54% and the mean value was 3.68%. Strain CCBAU 01393^T showed similarities of 92–97% with strains CCBAU 01389 and CCBAU 03239 and had similarities of 8.2–34.9% with the reference strains of recognized species (Table 1), indicating that the three strains represent a single genomic species but differ from recognized species based on the recommendation of a threshold value of 70% DNA–DNA relatedness for definition of novel genomic species (Graham *et al.*, 1991; Wayne *et al.*, 1987). The DNA G+C contents were determined using the thermal denaturation method (*T_m*) with *Escherichia coli* K-12 as a standard (Marmur & Doty, 1962). Strains CCBAU 01393^T, CCBAU 01389 and CCBAU

03239 had DNA G+C contents of 65.47, 65.21 and 64.87 mol%, respectively, in the range expected for *Rhizobium* (Jordan, 1984).

Colony morphologies of the isolates as well as morphology of Hucker's Gram- and Shaeffer-Fulton's spore stained smears were examined. Phenotypic features of the novel group (strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239) were determined in comparison with related *Rhizobium* species according to method described by Gao *et al.* (1994). The features determined included the utilization of sole carbon and nitrogen sources, resistance to antibiotics and tolerance to NaCl, and pH and temperature ranges for growth (Gao *et al.*, 1994). Biochemical tests including catalase and oxidase production, methyl red and Voges–Proskauer reaction, and hydrolysis of casein, gelatin, starch, DNA and Tween 80 were performed according to Smibert & Krieg (1994). Production of hydrogen sulfide (H₂S) from cysteine and of indole from tryptophan were determined as described by Barrow & Feltham (1993). Differential characteristics of strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 are shown in Table 3 and in the species description.

Based on all the results obtained, strains CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 are considered to represent a novel species that could be differentiated from closely related *Rhizobium* species by means of the polyphasic taxonomic methods used, including SDS-PAGE of cellular soluble proteins, biochemical characteristics, DNA–DNA relatedness and genotypic comparison of housekeeping and symbiotic genes. The name *Rhizobium alkalisoli* sp. nov. is proposed for this novel symbiotic bacterial species.

Description of *Rhizobium alkalisoli* sp. nov.

Rhizobium alkalisoli [al.ka.li.so'li. N.L. n. *alkali* (from Arabic al-qaliy), alkali; L. n. *solum* soil; N.L. gen. n. *alkalisoli* of alkaline soil, referring to the fact that the bacterium was isolated from *Caragana intermedia* grown in saline-alkali soil].

Cells are Gram-negative, aerobic, motile, non-spore-forming rods, 0.3–0.5 µm wide and 1–3 µm long. Colonies on YMA medium are circular, convex, white and opaque, with a diameter of 1–2 mm within 2–3 days at 28 °C. Growth occurs on YMA agar in 0–3% NaCl (optimum, 1%). Growth occurs at 12–37 °C and pH 5–10 on YMA, with optimum growth at 28 °C and pH 7.0. Catalase, urease and oxidase are present, but not L-phenylalaninase. Nitrate is not reduced and reduction of methyl blue is not observed. Nile blue reduction is observed. Lactic acid production, reduction of litmus and acid curd formation are produced in litmus milk tests. Does not hydrolyse gelatin, casein, starch, DNA or Tween 80. Indole is produced, but not H₂S, and the Voges–Proskauer and methyl red reaction are negative. Resistant to (µg ml^{−1}): ampicillin (300), kanamycin sulfate (50), neomycin sulfate (5), streptomycin sulfate (5), bacitracin (300), erythromycin (50) and chloramphenicol (5). Growth

Table 3. Differential characteristics of *R. alkanisoli* sp. nov. and closely related recognized *Rhizobium* species

Strains: 1, CCBAU 01393^T, CCBAU 01389 and CCBAU 03239 (*R. alkanisoli* sp. nov.); 2, *R. huautlense* SO2^T; 3, *R. galegae* USDA 4128^T; 4, *R. gallicum* USDA 2918^T; 5, *R. leguminosarum* USDA 2370^T. +, Positive; –, negative. Concentrations of carbon and nitrogen sources are 0.1 % (w/v).

Characteristic	1	2	3	4	5
Substrates used as sole carbon source					
D-Arabinose	+	+	+	+	–
Calcium gluconate	–	+	+	–	–
Calcium malonate	–	+	–	–	–
Dulcitol	–	+	–	+	–
meso-Erythritol	+	–	–	+	+
Melibiose	–	+	+	–	–
Melezitose	+	–	–	–	–
Sodium pyruvate	+	+	+	+	–
Salicin	+	+	–	+	–
D-Ribose	+	+	+	+	–
Sodium formate	–	–	–	+	–
D-Xylose	–	+	+	+	+
Glycine	–	+	+	–	–
L-Proline	+	+	–	+	–
Substrates used as sole nitrogen source					
D-Glutamic acid	+	+	+	–	–
D-Threonine	+	–	+	–	–
L-Methionine	+	+	+	+	–
Resistant to (µg ml ^{–1}):					
Ampicillin (50)	+	–	+	+	–
Ampicillin (100)	+	–	+	–	–
Ampicillin (300)	+	–	+	–	–
Kanamycin sulfate (50)	+	–	+	–	–
Streptomycin sulfate (5)	+	–	+	+	–
Bacitracin (300)	+	–	+	+	+
Erythromycin (50)	+	+	+	–	–
Bismarck brown (0.1 %)	+	+	+	–	–
Erythrosin B (0.1 %)	+	–	+	+	–
Neutral red (0.1 %)	+	–	+	–	–
Sodium nitrite (0.1 %)	–	+	+	–	–
NaCl (1 %)	+	+	+	–	+
NaCl (2 %)	+	+	+	–	–
NaCl (3 %)	+	–	–	–	–
pH 10.0	+	+	–	–	+

occurs on YMA with Bismarck brown (0.1 %), congo red (0.1 %), erythrosin B (0.1 %), neutral red (0.1 %) and sodium deoxycholate (0.1 %). Utilizes D-arabinose, D-fructose, D-galactose, D-mannose, melezitose, D-ribose, D-sorbitol, DL-malate, glucose, inositol, L-methionine, L-rhamnose, L-proline, meso-erythritol, raffinose, salicin, sodium citrate, D-sodium gluconate, sodium maltose, sodium pyruvate, sodium succinate, sucrose and trehalose as sole carbon sources, and (+)-L-aspartic acid, (+)-L-glutamic acid, D-glutamic acid, DL-alanine, D-threonine, glycine, hypoxanthine, L-arginine, L-cystine, L-hydroxyproline, L-isoleucine, L-lysine, L-phenylalanine, L-threonine and

L-valine as sole nitrogen sources. Does not utilize adipic acid, vanillic acid, calcium gluconate, calcium malonate, D-amygdalin, melibiose, D-xylose, dextrin, DL-asparagine, dulcitol, glycine, inulin, L-arginine, L-threonine, sodium acetate, sodium formate, sodium tartrate, soluble starch, sorbose or syringic acid as sole carbon sources. The G+C content of the type strain is 65.47 mol% (T_m).

The type strain, CCBAU 01393^T (=LMG 24763^T=HAMBI 3051^T), was isolated from nodules of *Caragana intermedia* grown in saline-alkaline soils in Qingshuihe city, Inner Mongolia, China.

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References

- Amarger, N., Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* **47**, 996–1006.
- Barrow, G. I. & Feltham, R. K. A. (1993). In *Cowan and Steel's Manual for the Identification of Medical Bacteria*, pp. 230–231. Cambridge: Cambridge University Press.
- Chen, W. F., Guan, S. H., Zhao, C. T., Yan, X. R., Man, C. X., Wang, E. T. & Chen, W. X. (2008). Different *Mesorhizobium* species associated with *Caragana* carry similar symbiotic genes and have common host ranges. *FEMS Microbiol Lett* **283**, 203–209.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Diouf, A., de Lajudie, P., Neyra, M., Kersters, K., Gillis, M., Martinez-Romero, E. & Gueye, M. (2000). Polyphasic characterization of rhizobia that nodulate *Phaseolus vulgaris* in West Africa (Senegal and Gambia). *Int J Syst Evol Microbiol* **50**, 159–170.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Frank, B. (1889). Über die Pilzsymbiose der Leguminosen. *Ber Dtsch Bot Ges* **7**, 332–346 (in German).
- 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.
- Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. W. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037–2048.
- Graham, P. H., Sadowsky, M. J., Keyser, H. H., Barnett, Y. M., Bradley, R. S., Cooper, J. E., De Ley, D. J., Jarvis, B. D. W., Roslycky, E. B. & 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.

Gu, C. T., Wang, E. T., Tian, C. F., Han, T. X., Chen, W. F., Sui, X. H. & Chen, W. X. (2008). *Rhizobium miluonense* sp. nov., a symbiotic bacterium isolated from *Lespedeza* root nodules. *Int J Syst Evol Microbiol* **58**, 1364–1368.

Han, T. X., Wang, E. T., Wu, L. J., Chen, W. F., Gu, J. G., Gu, C. T., Tian, C. F. & Chen, W. X. (2008). *Rhizobium multihospitium* sp. nov., isolated from multiple legume species native of Xinjiang, China. *Int J Syst Evol Microbiol* **58**, 1693–1699.

Jordan, D. C. (1984). Family III. *Rhizobiaceae* Conn 1938, 321^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–256. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Laguerre, G., Nour, S. M., Macheret, V., Sanjuan, J., Drouin, P. & Amarger, N. (2001). Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology* **147**, 981–993.

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

Lu, Y. L., Chen, W. F., Wang, E. T., Guan, S. H., Yan, X. R. & Chen, W. X. (2009). Genetic diversity and biogeography of rhizobia associated with *Caragana* species in three ecological regions of China. *Syst Appl Microbiol* **32**, 351–361.

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

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

Munro, H. N. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, 21–132. Edited by H. N. Munro. New York: Academic Press.

Polhill, R. M. (1994). Classification of the Leguminosae and synopsis of legume genera. In *Phytochemical Dictionary of the Leguminosae*, vol. 1, pp. xxxv–lvii. Edited by F. A. Bisby, J. Buckingham & J. B. Harborne. London: Chapman and Hill.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

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

Turner, S. L. & Young, J. P. W. (2000). The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. *Mol Biol Evol* **17**, 309–319.

Ueda, T., Suga, Y., Yahiro, N. & Matsuguchi, T. (1995). Phylogeny of Sym plasmids of rhizobia by PCR-based sequencing of a *nodC* segment. *J Bacteriol* **177**, 468–472.

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

Vincent, J. M. (1970). The cultivation, isolation and maintenance of rhizobia. In *A Manual for the Practical Study of the Root-Nodule Bacteria*, pp. 1–13. Edited by J. M. Vincent. Oxford: Blackwell Scientific.

Vinuesa, P., Silva, C., Werner, D. & Martínez-Romero, E. (2005). Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Mol Phylogenet Evol* **34**, 29–54.

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, F. Q., Wang, E. T., Liu, J., Chen, Q., Sui, X. H., Chen, W. F. & Chen, W. X. (2007). *Mesorhizobium albiziae* sp. nov., a novel bacterium that nodulates *Albizia kalkora* in a subtropical region of China. *Int J Syst Evol Microbiol* **57**, 1192–1199.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & 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.

Zhao, C. T., Wang, E. T., Chen, W. F. & Chen, W. X. (2008). Diverse genomic species and evidences of symbiotic gene lateral transfer detected among the rhizobia associated with *Astragalus* species grown in the temperate regions of China. *FEMS Microbiol Lett* **286**, 263–273.