Rhizobium multihospitium sp. nov., isolated from multiple legume species native of Xinjiang, China

Tian Xu Han,¹ En Tao Wang,^{1,2} Li Juan Wu,¹ Wen Feng Chen,¹
Jin Gang Gu,³ Chun Tao Gu,¹ Chang Fu Tian¹ and Wen Xin Chen¹

Correspondence

Wen Xin Chen

wenxin@cau.edu.cn

¹Key Laboratory of Agro-Microbial Resource and Application, Ministry of Agriculture/College of Biological Sciences, China Agricultural University, Beijing 100094, PR China

²Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, 11340 México D.F., México

³The Institute of Agricultural Resources and Regional Planning of Chinese Academy of Agricultural Sciences, Beijing 100081, PR China

Thirty-one rhizobial strains isolated from nodules of legumes native of Xinjiang, China, were characterized. These strains were classified as belonging to the genus *Rhizobium* based on amplified 16S rDNA restriction analysis (ARDRA). The strains were distinguished from recognized *Rhizobium* species using analysis of 16S–23S rDNA intergenic spacers (IGS-RFLP), SDS-PAGE analysis of whole proteins and BOX-PCR; the test strains always formed a distinct cluster with patterns that were quite different from those of the reference rhizobial strains used.

According to the phylogenetic analysis based on the 16S rRNA gene, the test strains belonged to the genus *Rhizobium*, with *Rhizobium tropici*, *Rhizobium rhizogenes* and *Rhizobium lusitanum* as the closest related species, with 99.6, 99.2 and 99.4 % sequence similarities, respectively, between the type strains of the three *Rhizobium* species and strain CCBAU 83401^T.

Phylogenetic analyses of the representative strains using IGS and *atpD*, *recA* and *glnII* genes all confirmed the phylogenetic arrangements obtained using the 16S rRNA gene. The DNA–DNA relatedness values between strain CCBAU 83401^T and strains CCBAU 83364, CCBAU 83345 and CCBAU 83523 ranged from 80.8 to 100 %, showing that they belong to the same species. The DNA–DNA relatedness between strain CCBAU 83401^T and *R. tropici* IIB CIAT 899^T, *R. tropici* IIA CFN 299, *R. rhizogenes* LMG 150^T and *R. lusitanum* P1-7^T were 26.9, 27.7, 38.2 and 22.6 %, respectively, clearly indicating that strain CCBAU 83401^T represents a novel species. Phenotypic characterization of four representative strains, CCBAU 83401^T, CCBAU 83364, CCBAU 83345 and CCBAU 83523, showed several distinctive features that differentiated them from closely related species. The 31 strains had identical *nodD* and *nifH* genes, which were very similar to those of the bean-nodulating *R. lusitanum*, *Devosia neptuniae* and *R. tropici* IIB. Based upon these results, the strains from this study are considered to represent a novel species, for which the name *Rhizobium multihospitium* sp. nov. is proposed. The DNA G + C content ranged from 65.3 to 66.0 mol% (*T_m*). The type strain is CCBAU 83401^T (=LMG 23946^T=HMBI 2975^T), which nodulates *Robinia pseudoacacia*, but not *Leucaena leucocephala*, *Phaseolus vulgaris*, *Pisum sativum* or *Medicago sativa*.

Abbreviation: IGS, intergenic spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CCBAU 83401^T, CCBAU 83333, CCBAU 83375, CCBAU 83277, CCBAU 83345, CCBAU 83319, CCBAU 83325, CCBAU 83364, CCBAU 83435, CCBAU 83503 and CCBAU 83523 are EF035074, EF035062, EF035065, EF035067, EF035059, EF490013, EF035069, EF490014, EF035070, EF035076 and EF035077, respectively. Those for partial IGS sequences are EF490015, EF050766, EF490016, EF050762, EF050763, EF050764, EF050761, EF490017, EF490018, EF050760 and EF050765, respectively; those for partial *glnII* gene sequences are EF490040–EF490050, respectively; for partial *recA* gene sequences are EF490029–EF490039, respectively; for partial *atpD* gene sequences are EF490019–EF490024, EU130792 and EF490025–EF490028, respectively; for partial *nodD* gene sequences are EF490051–EF490056, EF549532 and EF490057–EF490060, respectively; and for partial *nifH* gene sequences are EF490070, EF490061–EF490065, EF549533 and EF490066–EF490069, respectively.

Dendrograms based on IGS–RFLP fingerprints, SDS–PAGE of proteins and BOX-PCR and phylogenetic trees based on sequences of the 16S rRNA gene, IGS and *atpD*, *recA*, *glnII*, *nodD* and *nifH* genes of the test rhizobial strains and reference strains of recognized *Rhizobium* species and a table showing the fatty acid profile of strain CCBAU 83401^T and those of closely related taxa are available as supplementary material with the online version of this paper.

Rhizobia are soil bacteria that cause the formation of root- and/or stem-nodules on leguminous plants. Within the nodules, they reduce N_2 to ammonia, which can be used by the host plants. In the past two decades, the classification of rhizobia has developed rapidly due mainly to the application of a polyphasic approach and the studies of new rhizobial isolates. At the time of writing, about 50 species have been reported in the four main genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium*, and also in other genera including *Azorhizobium*, *Devosia*, *Methylobacterium*, *Ochrobactrum* and *Phyllobacterium* in the *Alphaproteobacteria*, as well as *Burkholderia* and *Cupriavidus* in the *Betaproteobacteria* (Wang *et al.*, 2006). As documented previously, there are more than 19 700 leguminous plant species throughout the world, but only few of them have been investigated for their microsymbionts (Allen & Allen, 1981). As more leguminous species in divergent geographical regions are surveyed it is quite possible that more novel rhizobial species will be discovered.

As reported previously, Xinjiang is an isolated region of China, with unique geographical characteristics and climate conditions (Chen *et al.*, 1997). During a survey of rhizobial resources in Xinjiang, diverse rhizobial populations and complicated relationships among the rhizobial species, host plants and geographical origin were

identified. The main findings of these studies were: (i) the description of two rhizobial species, *Sinorhizobium xinjiangense* (Chen *et al.*, 1988; Peng *et al.*, 2002) and *Mesorhizobium tianshanense* (Chen *et al.*, 1995); (ii) the recording of rhizobial populations that were genetically and phenotypically different from populations of the same species isolated from other regions (Yan *et al.*, 2000) or strains resistant to high salinity and high pH (Wei *et al.*, 2004); and (iii) the demonstration that different plants in the same geographical region could share the same rhizobial species for nodulation (Chen *et al.*, 1995). All of these findings implied that the rhizobial populations in Xinjiang have evolved independently and have high potential in searching for novel bacteria.

In our previous study, 111 rhizobial strains isolated from the nodules of wild legumes native to Xinjiang were characterized by using 16S rDNA restriction analysis (ARDRA) (Han *et al.*, 2008). The strains showed diverse genetic characteristics, among which 31 strains (Table 1) isolated from *Alhagi*, *Astragalus*, *Caragana*, *Halimodendron*, *Lathyrus*, *Lotus*, *Oxytropis*, *Robinia*, *Sophora* and *Vicia* species formed a distinctive rDNA type within the genus *Rhizobium*. With the aim of clarifying their taxonomic relationships, these strains were studied in the present study by using molecular methods. They were maintained in yeast mannitol agar (YMA) at 4 °C for

Table 1. Isolates and reference strains used in this study

CCBAU, Culture Collection of Beijing Agricultural University, Beijing, PR China.

CCBAU number	Host plant	Geographical origin
<i>Rhizobium mulithospitium</i>		
83345	<i>Lotus frondosus</i>	Xinjiang, China
83319, 83325, 83347, 83348, 83349	<i>Lotus tenuis</i>	Xinjiang, China
83277	<i>Alhagi toum</i>	Xinjiang, China
83373, 83374, 83375	<i>Astragalus aksuensis</i>	Xinjiang, China
83376, 83377, 83380	<i>Astragalus betetovii</i>	Xinjiang, China
83401 ^T	<i>Halimodendron halodendron</i>	Xinjiang, China
83370	<i>Oxytropis meinshausenii</i>	Xinjiang, China
83332, 83333	<i>Oxytropis glabra</i>	Xinjiang, China
83283	<i>Robinia pseudoacacia</i>	Xinjiang, China
83292	<i>Sophora alopecurioides</i>	Xinjiang, China
83359, 83360, 83364	<i>Caragana jubata</i>	Xinjiang, China
83434, 83435	<i>Lathyrus odoratus</i>	Xinjiang, China
83500, 83501, 83503, 83504, 83523, 83524, 83525	<i>Vicia hirsuta</i>	Xinjiang, China
Reference strains		
<i>Rhizobium etli</i> CFN 42 ^T	<i>Phaseolus vulgaris</i>	Mexico
<i>R. gallicum</i> USDA 2918 ^T	<i>Phaseolus vulgaris</i>	France
<i>R. leguminosarum</i> bv. <i>viciae</i> USDA 2370 ^T	<i>Pisum sativum</i>	USA
<i>R. leguminosarum</i> bv. <i>trifolii</i> 162K68	<i>Trifolium pratense</i>	USA
<i>R. leguminosarum</i> bv. <i>phaseoli</i> 127K17	<i>Phaseolus vulgaris</i>	USA
<i>R. tropici</i> IIA CFN 299	<i>Phaseolus vulgaris</i>	Brazil
<i>R. tropici</i> IIB CIAT 899 ^T	<i>Phaseolus vulgaris</i>	Columbia
<i>R. rhizogenes</i> LMG 150 ^T	<i>Phaseolus vulgaris</i>	
<i>R. lusitanum</i> P1-7 ^T	<i>Phaseolus vulgaris</i>	Portugal

temporary storage and in 20 % glycerol at -80°C for long-term storage.

16S–23S rDNA intergenic spacer (IGS)-RFLP, which is considered to be a useful tool for investigating the relatedness of closely related rhizobial strains (Jensen *et al.*, 1993; Laguerre *et al.*, 1996), was carried out using the primers FGPS1490 (Navarro *et al.*, 1992) and FGPL132' (Ponsonnet & Nesme, 1994) and the restriction endonucleases *Mbo*I, *Msp*I and *Alu*I. The results showed that the test strains formed a cluster with a similarity of 73 %, at which all the reference strains for recognized *Rhizobium* species were distinguished according to their species (see Supplementary Fig. S1, available in IJSEM Online).

SDS-PAGE of whole-cell proteins has been used widely in rhizobial taxonomy (Diouf *et al.*, 2000; Tan *et al.*, 1997; Wang *et al.*, 2007) and is suitable for use to group strains at species level. Methods described previously (Tan *et al.*, 1997; Wang *et al.*, 2006) were applied to extract whole-cell proteins from the 31 test strains and to perform the SDS-PAGE analysis. The 31 strains showed 11 distinct protein patterns and formed a cluster at a similarity level of 80 % in the UPGMA dendrogram constructed with the Dice coefficient (see Supplementary Fig. S2 in IJSEM Online), which was quite different from recognized *Rhizobium* species. Three subclusters could be clearly observed among the test strains.

By using BOX-PCR, fingerprints can be produced that can distinguish closely related isolates, even those of the same species (Vinuesa *et al.*, 1998; Cho & Tiedje, 2000; Healy *et al.*, 2005; Rademaker *et al.*, 2000). The BOX-PCR analysis was performed by using the method described by Versalovic *et al.* (1994) and Nick & Lindström (1994). Among the 31 test strains, 20 BOX patterns were generated, showing that they were not clones (see Supplementary Fig. S3 in IJSEM Online). All the test strains formed a cluster at 71 % similarity, which was obviously separated from all the reference strains in the dendrogram constructed with UPGMA and the Dice coefficient. In accordance with the results from SDS-PAGE, the strains tested formed three distinct subclusters.

Four representative strains, CCBAU 83401^T, CCBAU 83345, CCBAU 83364 and CCBAU 83523, and eight reference strains of recognized species were characterized comparatively. One hundred and five phenotypic features, including the utilization of sole carbon and nitrogen sources; resistance to antibiotics (5, 50, 100 and 300 $\mu\text{g ml}^{-1}$); tolerance to NaCl (1–5.0 %, w/v); production of catalase, urease, L-phenylalaninase and oxidase; methyl blue reduction, nile blue reduction and nitrate reduction; reaction in litmus milk; and pH and temperature ranges for growth were examined using methods described previously (Gao *et al.*, 1994). The results showed that the four representative strains had identical features and could be differentiated from recognized *Rhizobium* species by several characteristics (Table 2).

Table 2. Characteristics that differentiate *R. multihospitium* sp. nov. and closely related recognized *Rhizobium* species

Taxa: 1, *R. etli* CFN 42^T; 2, *R. leguminosarum* USDA 2370^T; 3, *R. rhizogenes* LMG 150^T; 4, *R. tropici* IIA CFN 299; 5, *R. tropici* IIB CIAT 899^T; 6, *R. lusitanum* P1-7^T; 7, *R. multihospitium* (strains CCBAU 83401^T, CCBAU 83345, CCBAU 83364 and CCBAU 83523). +, Positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Substrates used as sole carbon source							
D-Amygdalin	+	+	+	+	–	+	+
D-Arabinose	+	+	–	–	–	+	+
Calcium gluconate	–	–	–	–	–	+	+
Inulin	–	+	–	–	–	+	–
Melezitose	–	–	+	–	–	–	–
Sodium pyruvate	–	+	–	+	+	+	+
Salicin	+	+	+	–	–	+	+
Sodium acetate	+	+	–	–	–	+	–
Sodium formate	–	–	–	–	–	–	+
D-Sodium gluconate	–	+	–	–	–	+	+
Sodium hippurate	–	+	–	–	–	–	–
Sorbose	–	–	–	+	–	+	+
DL-Arginine	+	–	–	–	–	+	+
DL-Aspartic acid	–	+	–	–	–	+	+
Glycine	–	–	–	–	–	+	–
DL-Proline	+	+	–	–	–	+	+
Substrates used as sole nitrogen source							
DL-Alanine	–	–	+	+	+	+	+
L-Threonine	–	–	–	–	–	–	+
D-Threonine	–	–	–	–	–	–	+
Resistant to ($\mu\text{g ml}^{-1}$):							
Ampicillin (5)	+	+	+	–	+	+	+
Ampicillin (50)	+	+	+	–	+	+	+
Ampicillin (100)	+	+	–	–	+	+	+
Ampicillin (300)	+	+	–	–	–	+	+
Chloramphenicol (5)	+	+	+	+	–	+	+
Chloramphenicol (50)	+	+	–	–	–	+	+
Chloramphenicol (100)	–	–	–	–	–	–	+
Erythromycin (50)	–	–	–	–	–	–	+
Kanamycin (50)	+	+	+	–	–	+	+
Neomycin (50)	+	+	+	+	–	+	+
Streptomycin (5)	+	+	+	–	+	+	+
Streptomycin (50)	+	+	–	–	+	+	+
Streptomycin (100)	+	+	–	–	–	+	+
Other reactions							
Nile blue reduction	+	–	+	+	+	+	+
Methyl blue reduction	–	–	+	–	+	+	–
Acid production in litmus milk	+	+	+	–	+	+	+
Alkaline production in litmus milk	–	–	–	+	–	–	–
Curd in litmus milk	+	–	–	–	+	–	+

To clarify the phylogenetic position of this group of strains, strains representing the three subclusters were analysed based on the 16S rRNA gene. In this study, almost-complete 16S rRNA gene sequences of 11 representative strains including CCBAU 83401^T, CCBAU 83333, CCBAU 83375, CCBAU 83277, CCBAU 83345, CCBAU 83319,

CCBAU 83325, CCBAU 83364, CCBAU 83435, CCBAU 83503 and CCBAU 83523 (all of them were also included in the subsequent sequence analyses of other genes) were amplified using primers P1 and P6 (Chen *et al.*, 1995) and sequenced directly as described by Hurek *et al.* (1997). These sequences were compared with those held in the GenBank database. All sequences obtained from this work and from the GenBank database were aligned using CLUSTAL W software (Thompson *et al.*, 1994). The distances were calculated according to the models of Jukes & Cantor (1969), Kimura two-parameter (Kimura, 1983) and Tajima & Nei (1984) and Tamura & Nei (1993). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Swofford, 1993) methods. Bootstrap analysis was based on 1000 replications. All trees were analysed using the MEGA3 package (Kumar *et al.*, 2004). As no significant topological differences were found among the phylogenetic trees constructed using the different methods, only those trees constructed by using the neighbour-joining method after distance analysis of aligned sequences according to Jukes and Cantor model are shown (and also for the IGS and *atpD*, *recA* and *glnII* genes, see below). The 16S rRNA gene sequences of the 11 representative test strains were identical and therefore only strains CCBAU 83401^T, CCBAU 83345, CCBAU 83364 and CCBAU 83523 are shown in the phylogenetic trees (Fig. 1; see also Supplementary Fig. S4 in IJSEM Online). These sequences showed 99.6% similarity to the 16S rRNA gene of *Rhizobium tropici* IIB CIAT 899^T, 98.6% to *R. tropici* IIA CFN 299, 99.2% to *Rhizobium rhizogenes* LMG 150^T and 99.4% to *Rhizobium lusitanum* P1-7^T.

It is generally considered that the 16S–23S rRNA gene spacer regions are more aberrant than 16S rRNA genes and are more effective for use among closely related

rhizobial strains (Kwon *et al.*, 2005). The 11 representative strains were amplified with the same primers used in IGS-RFLP and sequenced using the method for the 16S rRNA gene. At the present time, housekeeping genes for protein-coding interspersing throughout the genome, including *atpD*, *recA* and *glnII*, are being used increasingly. These genes were used to investigate the phylogeny of bacteria in order to obtain information besides that using the 16S rRNA gene (Turner & Young, 2000; Gaunt *et al.*, 2001; Maggi Solca *et al.*, 2001; Vinuesa *et al.*, 2005a, b; Valverde *et al.*, 2006). In the present study, *atpD* and *recA* genes were obtained by using the method of Vinuesa *et al.* (2005a, b) and the *glnII* gene by that of Turner & Young (2000). The amplified sequences were sequenced and analysed as that for *rrs* and IGS. The sequence similarities of IGS, *atpD*, *glnII* and *recA* among the representative strains and reference strains are summarized in Table 3. Phylogenetic trees for each locus are available in Supplementary Fig. S5a–d, in IJSEM Online. The results obtained confirmed the phylogenetic position of these strains within the group *R. tropici*–*R. rhizogenes*–*R. lusitanum*. All these results strongly suggest that this group of strains may belong to a novel species within the *R. tropici*–*R. rhizogenes*–*R. lusitanum* phylogenetic branch. In the present study, the 16S rRNA gene was the most conserved among all the sequenced genes or IGS and the protein-coding genes and IGS were more discriminative than the 16S rRNA gene. Considering all the sequences, the strains in the novel group formed a phyletic cluster in *Rhizobium* distinct from recognized species.

DNA–DNA hybridization is considered to be a standard method for use for species definition (Wayne *et al.*, 1987; Graham *et al.*, 1991). In this work, strains CCBAU 83401^T, CCBAU 83345, CCBAU 83364 and CCBAU 83523 and related reference strains were chosen for DNA–DNA hybridization. Total DNA was extracted from each strain

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships among *R. multihospitium* sp. nov. and reference strains of recognized *Rhizobium* species. The neighbour-joining tree was derived from a 16S rRNA gene sequence distance matrix (Jukes–Cantor). Bootstrap confidence levels greater than 50% are indicated at nodes. GenBank accession numbers are shown in parentheses. Bar, 1% nucleotide substitutions. An extended neighbour-joining tree is available in Supplementary Fig. S4 in IJSEM Online. *A.*, *Agrobacterium*.

by using the method of Marmur (1961) and DNA–DNA hybridization was performed using the spectrophotometric method (De Ley *et al.*, 1970). The DNA–DNA relatedness among strain CCBAU 83401^T and the other three strains in the novel group ranged from 80.8 to 100 %, indicating that they represent the same genomic species. The DNA–DNA relatedness values between strain CCBAU 83401^T and *R. tropici* IIB CIAT 899^T, *R. tropici* IIA CFN 299, *R. rhizogenes* LMG 150^T, *R. lusitanum* P1-7^T, *R. etli* CFN 42^T and *R. leguminosarum* USDA 2370^T were 26.9, 27.7, 38.2, 22.6, 18.5 and 18.4 %, respectively. These results indicated that the genomic species represented by strain CCBAU 83401^T was different from recognized species based on the recommendation of a threshold value of 70 % DNA–DNA relatedness for species definition (Wayne *et al.*, 1987; Graham *et al.*, 1991). The G + C content of the DNA was measured using the thermal denaturation method of Marmur & Doty (1962), with *E. coli* K-12 as a standard. The DNA G + C content varied between 65.3 and 66.0 mol%, in the range for *Rhizobium* (Jordan, 1984).

The symbiotic property is an important feature of rhizobia and the symbiotic genes (*nod* and *nif*) are adaptive genes that in many cases have an evolutionary history independent of the other genes in the genome (Wang *et al.*, 2007). The comparison of their phylogenies with those derived from housekeeping genes may reveal events of lateral gene transfer among rhizobia (Haukka *et al.*, 1998). In the present study, partial *nodD* and *nifH* genes of the representative strains were amplified and sequenced as described previously (Rivas *et al.*, 2002). Interestingly, the sequences of both *nodD* and *nifH* among all the 11 strains were identical and had 100 % sequence similarities with those of *R. lusitanum* P1-7^T and *Devosia neptuniae* J1^T, and 98.0 % with that of *R. tropici* IIB CIAT 899^T for *nodD*, and 100 % similarities with those of the three strains for *nifH* (see Supplementary Fig. S6 in IJSEM Online). These results demonstrated that the *nodD* and *nifH* genes might have the same origin in this novel group and the species *R. lusitanum* and *Devosia neptuniae*, and are similar to those of *R. tropici*.

Cross-nodulation tests were performed among the 11 representative strains with the hosts of origin or related hosts *Alhagi sparsifolia*, *Astragalus membranaceus*, *Caragana korshinskii*, *Glycyrrhiza uralensis*, *Halimodendron halodendron*, *Lathyrus odoratus*, *Lotus corniculatus*, *Oxytropis glabra*, *Robinia pseudoacacia*, *Sophora alopecuroides*, *Vicia angustifolia*, *Vicia multicaulis*, *Vicia sepium*, and the recommended plant species *Leucaena leucocephala*, *Phaseolus vulgaris*, *Pisum sativum* and *Medicago sativa* (Graham *et al.*, 1991). Only strain CCBAU 83401^T formed nodules on *Robinia pseudoacacia* in addition to its original host, *Halimodendron halodendron*. Because these strains were isolated from legumes grown in alkaline soils with high salt concentration, they may require special soil conditions to form nodules.

Based on all the results obtained in this study, we believe that the 31 strains represent a novel species of the genus *Rhizobium*. According to the current criteria of rhizobial species description (Graham *et al.*, 1991; Wayne *et al.*, 1987), we propose a novel species, *Rhizobium multihospitium*, for the strains in the group. This species can be differentiated from closely related *Rhizobium* species by several phenotypic characteristics (Table 2) and fatty acid composition (see Supplementary Table S1 in IJSEM Online).

Description of *Rhizobium multihospitium* sp. nov.

Rhizobium multihospitium (mul.ti.ho.spi'ti.um. L. adj. *multus* many, numerous; L. n. *hospes* -itis, he who entertains a stranger, a host; N.L. gen. pl. n. *multihospitium* of numerous hosts, referring to the isolation of the bacterium from various legume species).

Cells are Gram-negative, aerobic, motile, non-spore-forming rods, 0.3–0.5 µm wide and 1–3 µm long. Colonies on YMA are circular, convex, white, opaque and usually have a diameter of 1–2 mm within 2–3 days at 28 °C. Grows on YMA with 2 % NaCl. Growth occurs at 12–37 °C and pH 5–10 on YMA. Utilizes D-amygdalin, D-arabinose, calcium gluconate, *meso*-erythritol, D-fructose,

Table 3. Sequence similarities (%) of housekeeping and symbiotic genes among strain CCBAU 83401^T and the other 10 representative strains of the novel group and reference strains of recognized species

NA, Not available; –, not analysed.

Strain	CCBAU 83401 ^T						
	<i>rrs</i>	IGS	<i>atpD</i>	<i>recA</i>	<i>glnII</i>	<i>nifH</i>	<i>nodD</i>
Other 10 strains in the group	100	99.9–100	99.8–100	99.8–100	99.6–100	100	100
<i>R. tropici</i> IIB CIAT 899 ^T	99.6	91.3	96.8	93.6	94.3	100	99.3
<i>R. tropici</i> IIA CFN 299	98.6	83.2	95.5	93.3	91.8	NA	NA
<i>R. rhizogenes</i> LMG 150 ^T	99.2	80.4	95.0	91.0	(94.5)*	86.7	72.5
<i>R. lusitanum</i> P1-7 ^T	99.4	75.8	92.4	89.6	90.7	100	100
<i>Devosia neptuniae</i> J1 ^T	–	–	–	–	–	100	100

*Value was obtained by comparison with *R. rhizogenes* LMG 152.

(+)-D-galactose, D-glucose, inositol, lactose, sodium malate, maltose, D-mannose, turanose, sodium pyruvate, raffinose, L-rhamose, salicin, sodium citrate, sodium formate, D-sodium gluconate, sodium succinate, sorbose, sucrose, trehalose, D-xylose, DL-arginine, DL-aspartic acid and DL-proline as sole carbon sources, and DL-alanine, L-arginine, (+)-L-aspartic acid, L-cystine, D-glutamic acid, (+)-L-glutamic acid, hypoxanthine, L-isoleucine, L-lysine, L-phenylalanine, D-threonine, L-valine, glycine, L-threonine and L-hydroxyproline as sole nitrogen sources. Does not use adipic acid, inulin, melezitose, sodium acetate, sodium hippurate, soluble starch, syringic acid, sodium tartrate, vanillic acid, glycine or L-methionine as sole carbon sources. Resistant to kanamycin (50 µg ml⁻¹), erythromycin (50 µg ml⁻¹), neomycin (50 µg ml⁻¹), streptomycin (100 µg ml⁻¹), chloramphenicol (100 µg ml⁻¹) and ampicillin (300 µg ml⁻¹). 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. Acid and curd are produced in litmus milk. The G+C content of the DNA ranges from 65.3 to 66.0 mol%.

The type strain, CCBAU 83401^T (=LMG 23946^T=HAMBI 2975^T), was isolated from nodules of legumes native of Xinjiang, China.

Acknowledgements

We thank Professor Ge Hong Wei and Professor Fu Suo Zhang for their kind help with the seed and nodule collections, and our colleagues Xue Rui Yan, Li Li Han and Chao Xin Man as well as Hai Sheng Wang and Yu Shi for their help in the experiments and data analysis. This work was supported by the National Basic Research Program of China (2006CB100206), National Program for Basic S & T Platform Construction (2005DKA21201-10) and the National Natural Science Foundation of China (30670001 and 30400001). E. T. W. was supported by grants CGPI20060213 and CGPI20070538 authorized by IPN, Mexico.

References

- Allen, O. N. & Allen, E. K. (1981). *The Leguminosae. A Source Book of Characteristics, Uses and Nodulation*. Madison, USA: University of Wisconsin Press.
- Chen, W. X., Yan, G. H. & Li, J. L. (1988). Numerical taxonomy 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., Wang, E., Wang, S., Li, Y., Chen, X. Q. & 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.
- Cho, J.-C. & Tiedje, J. M. (2000). Biogeography and degree of endemism of fluorescent *Pseudomonas* strains in soil. *Appl Environ Microbiol* **66**, 5448–5456.
- 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.
- 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 of 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.
- Han, T. X., Wang, E. T., Han, L. L., Chen, W. F., Sui, X. H. & Chen, W. X. (2008). Molecular diversity and phylogeny of rhizobia associated with wild legumes native to Xinjiang, China. *Syst Appl Microbiol* (in press).
- Haukka, K., Lindström, K. & Young, J. P. (1998). Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. *Appl Environ Microbiol* **64**, 419–426.
- Healy, M., Huong, J., Bittner, T., Lising, M., Frye, S., Raza, S., Schrock, R., Manry, J., Renwick, A. & other authors (2005). Microbial DNA typing by automated repetitive-sequence-based PCR. *J Clin Microbiol* **43**, 199–207.
- Hurek, T., Wagner, B. & Reinhold-Hurek, B. (1997). Identification of N₂-fixing plant- and fungus-associated *Azoarcus* species by PCR-based genomic fingerprints. *Appl Environ Microbiol* **63**, 4331–4339.
- Jensen, M. A., Webster, J. A. & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* **59**, 945–952.
- Jordan, D. C. (1984). Family III. *Rhizobiaceae* Conn 1938, 321^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–242. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Kwon, S. W., Park, J. Y., Kim, J. S., Kang, J. W., Cho, Y. H., Lim, C. K., Parker, M. A. & Lee, G. B. (2005). Phylogenetic analysis of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* on the basis of 16S rRNA gene and internally transcribed spacer region sequences. *Int J Syst Evol Microbiol* **55**, 263–270.
- Laguerre, G., Mavingui, P., Allard, M. R., Charnay, M. P., Louvrier, P., Mazurier, S. I., Rigottier-Gois, L. & Amarger, N. (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl Environ Microbiol* **62**, 2029–2036.

- Maggi Solca, N., Bernasconi, M. V., Valsangiacomo, C., Van Doorn, L. J. & Piffaretti, J. C. (2001). Population genetics of *Helicobacter pylori* in the southern part of Switzerland analysed by sequencing of four housekeeping genes (*atpD*, *ghnA*, *scoB* and *recA*), and by *vacA*, *cagA*, *iceA* and IS605 genotyping. *Microbiology* **147**, 1693–1707.
- 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.
- Navarro, E., Simonet, P., Normand, P. & Bardin, R. (1992). Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. *Arch Microbiol* **157**, 107–115.
- Nick, G. & Lindström, K. (1994). Use of repetitive sequences and the polymerase chain reaction to fingerprint the genomic DNA of *R. galegae* strains and to identify the DNA obtained by sonicating liquid cultures and root nodules. *Syst Appl Microbiol* **17**, 265–273.
- 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.
- Ponsonnet, C. & Nesme, X. (1994). Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. *Arch Microbiol* **161**, 300–309.
- Rademaker, J. L., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P. & de Bruijn, F. J. (2000). Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. *Int J Syst Evol Microbiol* **50**, 665–677.
- Rivas, R., Velázquez, E., Willems, A., Vizcaino, N., Subba-Rao, N. S., Mateos, P. F., Gillis, M., Dazzo, F. B. & Martínez-Molina, E. (2002). A new species of *Devosia* that forms a unique nitrogen-fixing root nodule symbiosis with the aquatic legume *Neptunia natans* (L. f.) Druce. *Appl Environ Microbiol* **68**, 5217–5222.
- Rzhetsky, A. & Nei, M. (1993). Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Mol Biol Evol* **10**, 1073–1095.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Swofford, D. L. (1993). PAUP: Phylogenetic Analysis Using Parsimony, version 3.1.1. Champaign, IL: Illinois Natural History Survey.
- Tajima, F. & Nei, M. (1984). Estimation of evolutionary distance between nucleotide sequences. *Mol Biol Evol* **1**, 269–285.
- Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512–526.
- 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.
- 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.
- Turner, S. L. & Young, J. P. (2000). The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. *Mol Biol Evol* **17**, 309–319.
- Valverde, A., Igual, J. M., Peix, A., Cervantes, E. & Velázquez, E. (2006). *Rhizobium lusitanum* sp. nov., a bacterium that nodulates *Phaseolus vulgaris*. *Int J Syst Evol Microbiol* **56**, 2631–2637.
- Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**, 25–40.
- Vinuesa, P., Rademaker, J. L., de Bruijn, F. J. & Werner, D. (1998). Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA and 16S–23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. *Appl Environ Microbiol* **64**, 2096–2104.
- Vinuesa, P., Silva, C., Lorite, M. J., Izaguirre-Mayoral, M. L., Bedmar, E. J. & Martínez-Romero, E. (2005a). Molecular systematics of rhizobia based on maximum likelihood and Bayesian phylogenies inferred from *rrs*, *atpD*, *recA* and *nifH* sequences, and their use in the classification of *Sesbania* microsymbionts from Venezuelan wetlands. *Syst Appl Microbiol* **28**, 702–716.
- Vinuesa, P., Silva, C., Werner, D. & Martínez-Romero, E. (2005b). 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, F. Q., Wang, E. T., Zhang, Y. F. & Chen, W. X. (2006). Characterization of rhizobia isolated from *Albizia* spp. in comparison with microsymbionts of *Acacia* spp. and *Leucaena leucocephala* grown in China. *Syst Appl Microbiol* **29**, 502–517.
- 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 nodulating *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.
- Wei, W., Jiang, J., Li, X., Wang, L. & Yang, S. S. (2004). Isolation of salt-sensitive mutants from *Sinorhizobium meliloti* and characterization of genes involved in salt tolerance. *Lett Appl Microbiol* **39**, 278–283.
- Yan, A. M., Wang, E. T., Kan, F. L., Tan, Z. Y., Sui, X. H., Reinhold-Hurek, B. & Chen, W. X. (2000). *Sinorhizobium meliloti* associated with *Medicago sativa* and *Melilotus* spp. in arid saline soils in Xinjiang, China. *Int J Syst Evol Microbiol* **50**, 1887–1891.