Rhizobium miluonense sp. nov., a symbiotic bacterium isolated from *Lespedeza* root nodules

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Seventeen strains of *lespedeza* rhizobia, a previously defined novel group, were further characterized by comparison with phylogenetically related species using a polyphasic approach, including SDS-PAGE of whole-cell proteins, PCR-based restriction fragment length polymorphism analysis of ribosomal intergenic spacer sequences, determination of DNA G+C contents, DNA–DNA hybridization and analysis of phenotypic features. Based on data obtained in the present and previous studies, a novel species, *Rhizobium miluonense* sp. nov., is proposed for these strains. The type strain of *R. miluonense* is CCBAU 41251^T (=LMG 24208^T=HAMBI 2971^T). Strain CCBAU 41251^T could form ineffective nodules on *Phaseolus vulgaris* in addition to its original host *Lespedeza chinensis*.

Rhizobia are soil bacteria that can form nodules in symbiosis with leguminous plants. Currently, about 50 rhizobial species within more than 10 genera of the alpha- and betaproteobacteria have been described. Among these genera, *Rhizobium* was the first described species, named 100 years ago. After a series of taxonomic changes since the 1980s, the genus *Rhizobium* now contains about 20 species, including three recently described species: *Rhizobium daejeonense* (Quan *et al.*, 2005), *Rhizobium lusitanum* (Valverde *et al.*, 2006) and *Rhizobium cellulosilyticum* (García-Fraile *et al.*, 2007).

In our previous study (Gu *et al.*, 2007), diverse rhizobia associated with *Lespedeza* spp. in temperate and subtropical regions of China were characterized and two novel *Rhizobium* groups (6 and 7) were identified based on 16S rRNA gene sequence analysis and DNA–DNA hybridization. In the present study, the main novel group, group 7, was further studied to clarify its taxonomic status.

Seventeen test strains in group 7 (Gu *et al.*, 2007) and six reference strains were used in this study (Table 1). These test strains were isolated from root nodules of nine *Lespedeza* species grown in Hunan province, China.

Abbreviations: IGS, intergenic spacer; RFLP, restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Rhizobium miluonense* CCBAU 41251^T is EF061096.

Phenotypic characteristics of strains of *Rhizobium miluonense* sp. nov. are given in Supplementary Table S1, which is available with the online version of this paper.

Nodulation on the original host plant of each isolate has been confirmed previously (Gu *et al.*, 2007). All strains were maintained on yeast-mannitol agar (YMA) medium at 4 °C during the study.

Previously, these 17 strains were defined as group 7 since they formed a cluster using SDS-PAGE of proteins that was distinct from reference strains and other *lespedeza* rhizobia at a similarity of 88 %. These 17 strains also clustered in PCR-based ribosomal intergenic spacer restriction fragment length polymorphism (IGS-RFLP) analysis, showing 99.5–100 % similarity within the group and 70 % similarity with other rhizobia (Gu *et al.*, 2007). 16S rRNA gene sequencing and phylogenetic analysis revealed that three representative strains of group 7 had identical sequences and were closely related to *Rhizobium tropici* type B CIAT 899^T, *R. tropici* type A LMG 9517, *R. lusitanum* P1-7^T and *Rhizobium rhizogenes* IFO 13257^T, with 16S rRNA gene sequence similarities of 99.3, 98.8, 99.6 and 99.4 %, respectively (Gu *et al.*, 2007).

Since the reference strains for the *Rhizobium* species most related to group 7 were not included in SDS-PAGE of whole-cell proteins and IGS-RFLP analysis, these two methods were used in this study to compare the group 7 strains and the related species. For SDS-PAGE, all strains were incubated in yeast-mannitol (YM) broth at 28 °C for 2 days. The preparation of protein samples, SDS-PAGE and silver-staining of the gels were performed as described previously (Tan *et al.*, 1997). For IGS-RFLP, the ribosomal IGS between the 16S and 23S rRNA genes was amplified using primers FGPS6 and 23S-38 and the

Table 1. Bacterial strains used in this study

Strain	Host plant	Geographical origin
Cluster 7 (<i>R. miluonense</i>):		
CCBAU 41005, CCBAU 41018, CCBAU 41141	<i>Lespedeza davidii</i>	Hunan
CCBAU 41029, CCBAU 41231	<i>Lespedeza formosa</i>	Hunan
CCBAU 41031, CCBAU 41126	<i>Lespedeza cuneata</i>	Hunan
CCBAU 41041, CCBAU 41114, CCBAU 41132	<i>Lespedeza pilosa</i>	Hunan
CCBAU 41068	<i>Lespedeza virgata</i>	Hunan
CCBAU 41108, CCBAU 41128, CCBAU 41251 ^T	<i>Lespedeza chinensis</i>	Hunan
CCBAU 41164	<i>Lespedeza cyrtobotrya</i>	Hunan
CCBAU 41200	<i>Lespedeza bicolor</i>	Hunan
CCBAU 41216	<i>Lespedeza tomentosa</i>	Hunan
<i>R. tropici</i> type A CFN 299 (=LMG 9517)	<i>Phaseolus vulgaris</i>	Brazil
<i>R. tropici</i> type B LMG 9503 ^T (=CIAT 899 ^T)	<i>Leucaena leucocephala</i>	Colombia
<i>R. lusitanum</i> LMG 22705 ^T (=P1-7 ^T)	<i>Phaseolus vulgaris</i>	Portugal
<i>R. lusitanum</i> P3-13	<i>Phaseolus vulgaris</i>	Portugal
<i>R. rhizogenes</i> LMG 150 ^T (=IFO 13257 ^T)		
<i>R. rhizogenes</i> LMG 152		

protocol of Rasolomampianina *et al.* (2005). The PCR products were digested separately with *Msp*I, *Cfo*I and *Hae*III as described by Rasolomampianina *et al.* (2005). Restriction fragments were separated by electrophoresis in 3% agarose gels and photographed under UV light after ethidium bromide staining. Cluster analyses of the protein and RFLP patterns were performed as described previously (Gu *et al.*, 2007).

In both SDS-PAGE of proteins and ribosomal IGS-RFLP analysis, the 17 strains of group 7 showed almost identical patterns (99–100% similarity). Similarities between the group 7 strains and *R. tropici* type B LMG 9503^T, *R. tropici* type A CFN 299, *R. lusitanum* LMG 22705^T and *R. rhizogenes* LMG 150^T were $\leq 64.5\%$ in SDS-PAGE of whole-cell proteins (Fig. 1) and $\leq 81\%$ in ribosomal IGS-RFLP analysis (Fig. 2). These data demonstrated that members of group 7 differed from phylogenetically related *Rhizobium* species.

Previously, DNA–DNA hybridization has been performed between group 7 strain CCBAU 41251^T and the reference strains of several defined species. To determine the relationships between group 7 strains and all the most related species, DNA G+C contents were determined and further DNA–DNA hybridizations were performed. The DNA was prepared according to the method of Marmur (1961). DNA G+C contents were measured using the thermal melting protocol of De Ley (1970) with *Escherichia coli* K-12 as the standard. DNA relatedness was determined by the initial renaturation rate method (De Ley *et al.*, 1970). The DNA G+C contents of the three group 7 strains CCBAU 41251^T, CCBAU 41029 and CCBAU 41128 were 58.4, 58.8 and 58.2 mol%, respectively. The DNA–DNA relatedness data are presented in Table 2. Low to medium DNA–DNA relatedness (15–45%) was obtained between strain CCBAU 41251^T and six reference strains of *R. tropici* type B, *R. tropici* type A, *R. lusitanum* and *R. rhizogenes* (Table 2). DNA–DNA

Fig. 1. Electrophoretic protein patterns showing the relationships between strain CCBAU 41251^T and reference strains of phylogenetically related species. Patterns for the other 16 strains in group 7 (Table 1) were almost identical to that of strain CCBAU 41251^T.

Fig. 2. Ribosomal IGS-RFLP patterns showing the genomic relationships between strains of *R. miluonense* sp. nov. and phylogenetically related reference strains.

relatedness of 96–100 % was obtained between strain CCBAU 41251^T and two other strains that were randomly selected as representatives of group 7 (Table 2). The large gap between the DNA–DNA relatedness values within the group and with the reference strains obviously indicated that the group 7 strains formed a genomic species that differed from related *Rhizobium* species.

To clarify whether the group 7 strains could be defined as a species, phenotypic features that differentiated group 7 from related species were analysed according to the current criterion of bacterial species definition (Stackebrandt *et al.*, 2002). In the present study, phenotypic features, including

the utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance to NaCl and pH and temperature ranges for growth (see Supplementary Table S1, available in IJSEM Online) were analysed as described previously (Gao *et al.*, 1994). Among the 96 tested features, 68 were the same (positive or negative) for all the strains. In the cluster analysis (Fig. 3) using the S_{SM} coefficient ($S_{SM} = \Sigma I / 96$, where ΣI is the number of features identical for the compared strain pair; 96 is the total number of tested features) and unweighted pair-group method with arithmetic averages (UPGMA) (Sneath & Sokal, 1973), the 17 strains in group 7 showed 98.5 % or greater similarity and clearly differed from the reference strains of recognized

Table 2. DNA–DNA relatedness between strain CCBAU 41251^T and phylogenetically related reference strains

Data are mean values of at least three repetitions.

Strain	DNA relatedness (%) with CCBAU 41251 ^T
<i>R. miluonense</i> sp. nov.	
CCBAU 41251 ^T	100
CCBAU 41029	100
CCBAU 41128	96
<i>R. tropici</i> A CFN 299	29
<i>R. tropici</i> B LMG 9503 ^T	34
<i>R. rhizogenes</i> LMG 150 ^T	24
<i>R. rhizogenes</i> LMG 152	20
<i>R. lusitanum</i> LMG 22705 ^T	45
<i>R. lusitanum</i> P3-13	15

Fig. 3. Dendrogram showing the phylogenetic relationships among strains of *R. miluonense* sp. nov. and phylogenetically related reference strains. The dendrogram was constructed based upon 96 phenotypic features using S_{SM} coefficients and the UPGMA method (Sneath & Sokal, 1973).

species (Fig. 3). Distinctive features of group 7 are shown in Table 3 and more features are presented in the species description. In this analysis, colony morphology (i.e. wet and translucent) could distinguish group 7 strains from *R. tropici* type A and *R. rhizogenes*, whereas no growth in Luria–Bertani (LB) medium and sensitivity to 1 % NaCl could differentiate group 7 strains from *R. tropici* type B and *R. lusitanum*.

To check the host range, seeds of *Phaseolus vulgaris*, *Medicago sativa*, *Pisum sativum* and *Leucaena leucocephala*

were surface-sterilized, germinated and inoculated with the representative strain CCBAU 41251^T according to standard protocols (Vincent, 1970). The growth conditions of the plants were as described previously (Yao *et al.*, 2002). After 7 weeks of growth, inefficient (white) nodules were observed on *Phaseolus vulgaris* inoculated with strain CCBAU 41251^T, but no nodules were found on *Medicago sativa*, *Pisum sativum* or *Leucaena leucocephala*. This failure to nodulate *P. vulgaris* effectively might be an additional phenotypic feature to enable the differentiation of group 7 members from related species since *R. tropici*, *R. rhizogenes*

Table 3. Distinctive features of *R. miluonense* sp. nov. and phylogenetically related species

Strains: 1, *R. miluonense* CCBAU 41251^T sp. nov.; 2, *R. tropici* A CFN 299; 3, *R. tropici* B LMG 9503^T; 4, *R. lusitanum* LMG 22705^T; 5, *R. rhizogenes* LMG 150^T. –, No growth; +, growth; ±, light growth.

Characteristic	1	2	3	4	5
Resistance to ($\mu\text{g ml}^{-1}$):					
Ampicillin (300)	–	–	+	–	–
Chloramphenicol (50)	–	–	+	±	–
Kanamycin sulfate (5)	–	–	–	+	+
Streptomycin sulfate (50)	–	–	+	–	–
Growth on LB	–	–	+	±	–
Tolerance to 1 % NaCl (w/v)	–	–	+	±	–
Colony morphology on YMA	Wet, translucent	Opaque	Wet, translucent	Wet, translucent	Opaque

and *R. lusitanum* strains could form effective root nodules with this plant (Valverde *et al.*, 2006).

In conclusion, the 17 strains in group 7 were very similar in all the genomic and phenotypic analyses. They were identified as members of the genus *Rhizobium* and were related to *R. tropici*, *R. rhizogenes* and *R. lusitanum* by 16S rRNA gene sequencing (Gu *et al.*, 2007), but formed a group that differed from the recognized species of the genus *Rhizobium* in SDS-PAGE of proteins (Fig. 1), ribosomal IGS-RFLP (Fig. 2), DNA–DNA hybridization (Table 2) and numerical taxonomy (Fig. 3). Taking the recently reported *Rhizobium* species as references (García-Fraile *et al.*, 2007; Quan *et al.*, 2005; Valverde *et al.*, 2006), the strains in group 7 could be defined as a novel species of the genus *Rhizobium* based on the results presented in this study and in our previous work (Gu *et al.*, 2007); the name proposed for this species is *Rhizobium miluonense* sp. nov.

Description of *Rhizobium miluonense* sp. nov.

Rhizobium miluonense (mi.lu.o.nen'se. N.L. fem. adj. *miluonense* pertaining to the Miluo River, a famous river located in Hunan Province, where the bacterium was isolated).

Aerobic, Gram-negative, non-spore-forming rods, 0.6–0.8 × 1.8–3.2 µm. Colonies on YMA are circular, convex, translucent and usually 2–3 mm in diameter after 3 days incubation at 28 °C. Amygdalin, D-arabinose, calcium gluconate, calcium malonate, meso-erythritol, D-fructose, D-galactose, D-glucose, inositol, lactose, sodium DL-malate, maltose, D-mannose, turanose, raffinose, L-rhamnose, salicin, sodium citrate, sodium D-gluconate, sodium succinate, sorbose, sucrose, trehalose, D-xylose, L-arginine, L-proline and L-aspartic acid are used as sole carbon sources for growth, but adipic acid, dextrin, inulin, melezitose, sodium acetate, sodium formate, sodium hippurate, soluble starch, syringic acid, potassium sodium tartrate, vanillic acid, glycine, L-threonine and L-methionine are not utilized. DL-α-Aminopropionic acid, L-arginine, L-aspartic acid, L-cystine, D-glutamic acid, L-glutamic acid, hypoxanthine, L-isoleucine, L-lysine, L-phenylalanine, L-valine, glycine, L-threonine and L-hydroxyproline are used as sole nitrogen sources for growth, but D-threonine is not utilized. Resistant to 100 µg ampicillin ml⁻¹ and 5 µg ml⁻¹ each of chloramphenicol, neomycin sulfate and streptomycin sulfate. Sensitive to 300 µg ampicillin ml⁻¹, 5 µg ml⁻¹ each of gentamicin sulfate, erythromycin and kanamycin sulfate, and 50 µg ml⁻¹ each of chloramphenicol, neomycin sulfate and streptomycin sulfate. Optimum growth temperature is 25–30 °C; can grow at 37 °C, but not 4 °C. Optimum pH is 6–8. Sensitive to 1 % (w/v) NaCl in YMA. Does not grow in Luria–Bertani medium.

The type strain is CCBAU 41251^T (=LMG 24208^T =HAMBI 2971^T), isolated from root nodules of *Lespedeza* species grown in Hunan province, China. The DNA G+C content of strain CCBAU 41251^T is 58.4 mol%.

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