Mesorhizobium robiniae sp. nov., isolated from root nodules of *Robinia pseudoacacia*

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Previously, five rhizobial strains isolated from root nodules of *Robinia pseudoacacia* were assigned to the same genospecies on the basis of identical 16S rRNA gene sequences and phylogenetic analyses of the *nodA*, *nodC* and *nifH* genes, in which the five isolates formed a well-supported group that excluded other sequences found in public databases. In this study, the 16S rRNA gene sequence similarities between the isolates and *Mesorhizobium mediterraneum* UPM-Ca36^T and *Mesorhizobium temperatum* SDW018^T were 99.5 and 99.6 %, respectively. The five isolates were also different from defined *Mesorhizobium* species using ERIC fingerprint profiles and they formed a novel *Mesorhizobium* lineage in phylogenetic analyses of *recA* and *atpD* gene sequences. DNA–DNA relatedness values between the representative strain, CCNWYC 115^T, and type strains of defined *Mesorhizobium* species were found to be lower than 47.5 %. These results indicated that the isolates represented a novel genomic species. Therefore, a novel species, *Mesorhizobium robiniae* sp. nov., is proposed, with type strain CCNWYC 115^T (=ACCC 14543^T =HAMBI 3082^T). Strain CCNWYC 115^T can form effective nodules only on its original host.

Rhizobia are Gram-negative soil bacteria and are well known for their ability to perform symbiotic nitrogen fixation in nodules on the roots of legume plants. Novel rhizobial lineages are becoming apparent in further studies to characterize novel isolates with enlarged host ranges and geographical origins. Current rhizobial taxonomy (<http://www.rhizobia.co.nz/taxonomy/rhizobia.html>) describes 76 rhizobial species in the classes *Alphaproteobacteria* and *Betaproteobacteria*. The genus *Mesorhizobium* is classified within the *Alphaproteobacteria* and now contains 19 nitrogen-fixing species including the recently described species *Mesorhizobium caraganae* (Guan *et al.*, 2008), *Mesorhizobium gobiense* (Han *et al.*, 2008), *Mesorhizobium tarimense* (Han *et al.*, 2008) and *Mesorhizobium metallidurans* (Vidal *et al.*, 2009).

Robinia pseudoacacia (black locust) is a North American legume of the subfamily Papilionoideae and is of great economic interest in agroforestry in terms of biomass production, reforestation and site restoration. *R. pseudoacacia* tends to grow in poor or degraded soils (McCray Batzli *et al.*, 1992) so, despite its economic value, the legume poses an ecological threat by outcompeting native

species and reducing overall community richness. *R. pseudoacacia* was first introduced into Europe from North America and then spread from Europe to China about 300 years ago (Ulrich & Zaspel, 2000). It is now very widespread in these areas and is considered one of the top 100 worldwide woody plant invaders. Like most legumes, *R. pseudoacacia* is able to form nitrogen-fixing symbiotic associations with rhizobia.

In our previous study (Wei *et al.*, 2009), 33 isolates from root nodules of *R. pseudoacacia* grown in Yangling, Shaanxi province, China, were grouped into three genospecies on the basis of RFLP analyses and sequencing of PCR-amplified 16S rRNA and symbiotic genes. Among these isolates, five representatives of genospecies I had identical 16S rRNA gene sequences (Fig. 1a). 16S rRNA gene sequence similarities with *Mesorhizobium mediterraneum* UPM-Ca36^T and *Mesorhizobium temperatum* SDW018^T were 99.5 and 99.6 %, respectively, and *nodA*, *nodC* and *nifH* phylogenies showed that these five isolates formed well-supported clusters that excluded other sequences found in the databases.

With the aim of verifying their taxonomic position, the five isolates were further characterized by sequencing of the housekeeping genes *recA* and *atpD*, enterobacterial repetitive intergenic consensus sequence (ERIC) fingerprint analysis, DNA–DNA hybridization, fatty acid analysis and numerical taxonomy. The isolates were cultivated on yeast-mannitol agar (YMA) medium (Vincent, 1970) at 28 °C and maintained at 4 °C for temporary storage.

Abbreviation: ERIC, enterobacterial repetitive intergenic consensus.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA* and *atpD* gene sequences of strain CCNWYC 115^T are EU849582, GQ856501 and GQ856506, respectively.

A table of fatty acid compositions of strain CCNWYC 115^T and selected *Mesorhizobium* taxa is available with the online version of this paper.

Fig. 1. Neighbour-joining phylogenetic trees based on 16S rRNA gene (a), *recA* (b) and *atpD* (c) sequences, showing the relationships between strains CCNWCY 115^T and CCNWCY 124 and type strains of recognized species of the genus *Mesorhizobium*. Bootstrap values (>50%) are indicated at branch nodes. Bars, 10% nucleotide substitution rate.

recA and *atpD* phylogenies support the small-subunit rRNA gene-based classification of rhizobia (Gaunt *et al.*, 2001) and can be used to verify and compensate the phylogeny of the 16S rRNA gene, as suggested by Martens *et al.* (2008). In this study, PCR amplification and sequencing of *recA* and *atpD* were undertaken according to Gaunt *et al.* (2001) and Turner & Young (2000). The *atpD* sequence for strain CCNWCY 124 was not successfully determined. Neighbour-joining trees were constructed from sequences acquired in this study and from the GenBank database using a Jukes–Cantor distance matrix. The phylogenies of *recA* and *atpD* showed that the five isolates formed a cluster within the genus *Mesorhizobium* (Fig. 1b, c) and supported the conclusion from 16S rRNA gene sequence analysis. For *recA*, strains CCNWCY 115^T, CCNWCY 147, CCNWCY 120 and CCNWCY 132 had identical sequences and showed 98.0% sequence similarity to strain CCNWCY 124. Strain CCNWCY 115^T showed the highest *recA* sequence similarities to *Mesorhizobium tianshanense* CCBAU 3306^T (95.4%) and *M. temperatum* SDW018^T (95.0%). For *atpD*, strains CCNWCY 115^T, CCNWCY 120, CCNWCY 132 and CCNWCY 147 had identical sequences and showed *atpD* sequence similarities of 97.9 and 96.7%, respectively, to *M. tianshanense* GA11P and *M. temperatum* SDW018^T.

ERIC fingerprint profiles have been used to distinguish and classify different rhizobial strains in population studies (Madrzak *et al.*, 1995). ERIC fingerprint profiles were obtained by using primers ERIC1R and ERIC2R (de Bruijn, 1992). Cluster analysis was performed using the Bionumerics program (Applied Mathematics), with the unweighted pair group method with arithmetic means algorithm and the Jaccard coefficient (Sneath & Sokal, 1973). The analysis of ERIC patterns showed that the five isolates were quite different from the type strains of closely related *Mesorhizobium* species and that strains CCNWCY 115^T, CCNWCY 120, CCNWCY 132 and CCNWCY 147 were identical, while strain CCNWCY 124 had 75% similarity to the other four isolates (Fig. 2).

Fig. 2. Cluster analysis of ERIC fingerprints, showing the relationships between the five isolates and the type strains of closely related *Mesorhizobium* species, based on the unweighted pair group method with arithmetic means.

The G+C content of DNA was measured using the thermal denaturation method of Marmur & Doty (1962) and using *Escherichia coli* K-12 as a standard. The G+C content of the representative strain CCNWYC 115^T was 61.5 mol%, which is within the range reported for *Mesorhizobium* (59–64 mol%; Jarvis *et al.*, 1997). DNA–DNA hybridization is another standard method to define novel bacterial species (Graham *et al.*, 1991). Hybridizations were performed using the thermal melting protocol of De Ley *et al.* (1970). DNA–DNA relatedness varied from 90.1 to 96.9% between strain CCNWYC 115^T and the other four isolates and from 20.9 to 47.5% between strain CCNWYC 115^T and the type strains of defined *Mesorhizobium* species (Table 1).

Cellular fatty acid profiles have also been used to discern species of *Mesorhizobium* accurately and to distinguish between related genera (Tighe *et al.*, 2000). The fatty acid composition of strain CCNWYC 115^T was determined according to the standard protocol of the Sherlock Microbial Identification System (Hewlett Packard). The composition indicated that strain CCNWYC 115^T should be assigned to *Mesorhizobium* because the isolate contained 16:0, 17:0, iso-17:0, 17:1 ω 8c, 18:0 and 19:0 cyclo ω 8c and lacked 20:3 ω 6,9,12c (Tighe *et al.*, 2000). On the other hand, strain CCNWYC 115^T differed from other members of the genus *Mesorhizobium* on the basis of the presence of iso-15:1 F and anteiso-15:1 A and the concentrations of anteiso-17:0, 17:0 cyclo, 18:1 ω 9c and 19:0 cyclo ω 8c being the highest observed to date for the genus *Mesorhizobium* (Supplementary Table S1, available in IJSEM Online).

Phenotypic features of the novel group of isolates were determined and compared with those of the type strains of closely related *Mesorhizobium* species, according to the method described by Wei *et al.* (2003). The tested features included the use of sole carbon and nitrogen sources, resistance to antibiotics, tolerance of NaCl and low pH and temperature ranges for growth. The distinctive features of

the novel group are shown in Table 2 and the species description.

Host range is an important feature of root nodule bacteria and cross-nodulation tests with selected hosts are required for the description of novel rhizobial species (Graham *et al.*, 1991). In nodulation tests with *R. pseudoacacia*, all five isolates formed pink root nodules, which indicated that the nodules were effective for nitrogen fixation. Strain CCNWYC 115^T was used for cross-nodulation tests with seven common leguminous plant species that grow naturally in the Yangling region: *Sophora alopecuroides*, *Sophora flavescens*, *Vicia cracca*, *Vigna sesquipedalis*, *Phaseolus vulgaris*, *Melilotus officinalis* and *Trifolium pratense*. No nodules were induced on these plants when inoculated with strain CCNWYC 115^T. No nodules were formed on the uninoculated control plants in any experiment.

On the basis of the results obtained in this study and our previous work (Wei *et al.*, 2009), we believe that the five isolates represent a novel species in the genus *Mesorhizobium*. This species can be differentiated from defined *Mesorhizobium* species by ERIC fingerprint profiles, phenotypic characteristics, fatty acid composition, DNA–DNA relatedness and 16S rRNA gene, *recA*, *atpD*, *nodA* and *nifH* sequences. We propose the name *Mesorhizobium robiniae* sp. nov., with CCNWYC 115^T as the type strain.

Description of *Mesorhizobium robiniae* sp. nov.

Mesorhizobium robiniae (ro.bi'ni.æ. N.L. gen. n. *robiniae* of *Robinia*, a genus of leguminous plants, referring to the host from which this species was first isolated).

Gram-negative, aerobic, motile, non-spore-forming rods, 0.3–0.6 µm wide and 1–2.5 µm long. Colonies on YMA medium are circular, convex, white and opaque and usually have a diameter of 1–2 mm after 7–10 days at 28 °C. Optimum growth occurs at 25–30 °C and growth

Table 1. *Mesorhizobium* strains used in this study

The five novel strains were all collected at Yangling, Shaanxi, China. ND, Not determined.

Strain	Geographical origin	Host plant	DNA G+C content (mol%)	DNA–DNA relatedness with CCNWYC 115 ^T (%)
CCNWYC 115 ^T	North slope, Houhe River	<i>Robinia pseudoacacia</i>	61.5	(100)
CCNWYC 124	North slope, Houhe River	<i>R. pseudoacacia</i>	61.4	90.1 ± 1.1
CCNWYC 120	South slope, Houhe River	<i>R. pseudoacacia</i>	62.1	96.7 ± 1.4
CCNWYC 132	Flat area, north gate outside university	<i>R. pseudoacacia</i>	63.3	96.9 ± 1.9
CCNWYC 147	Depression, Houhe River	<i>R. pseudoacacia</i>	62.4	96.5 ± 1.5
<i>M. tianshanense</i> A-1BS ^T	Xinjiang, China	<i>Glycyrrhiza pallidiflora</i>	ND	20.9 ± 1.5
<i>M. temperatum</i> SDW018 ^T	Liaoning, China	<i>Astragalus adsurgens</i>	ND	40.4 ± 1.5
<i>M. tarimense</i> CCBAU 83306 ^T	Xinjiang, China	<i>Lotus frondosus</i>	ND	47.5 ± 1.3
<i>M. mediterraneum</i> UPM-Ca36 ^T	Spain	<i>Cicer arietinum</i>	ND	30.7 ± 1.5

Table 2. Differentiating phenotypic features of strain CCNWC 115^T and the type strains of closely related *Mesorhizobium* species

Strains: 1, *Mesorhizobium robiniae* sp. nov. CCNWC 115^T; 2, *M. tarimense* CCBAU 83306^T; 3, *M. tianshanense* A-1BS^T; 4, *M. temperatum* SDW018^T; 5, *M. mediterraneum* UPM-Ca36^T.

Characteristic	1	2	3	4	5
Growth at:					
40 °C	+	+	+	–	–
pH 11	+	+	+	+	–
Sole carbon sources					
L-Arabinose	+	+	+	+	–
Sodium malonate	+	+	+	+	–
Sodium pyruvate	+	+	+	+	–
Sole nitrogen sources					
L-Arginine	+	+	+	–	–
L-Glutamic acid	+	+	+	–	–
Hypoxanthine	+	+	+	–	+
L-Phenylalanine	+	+	+	–	+
Resistant to (µg ml ^{–1}):					
Ampicillin (5)	+	+	+	+	–
Ampicillin (50)	+	–	–	+	–
Ampicillin (100)	+	–	–	+	–
Ampicillin (300)	+	–	–	–	–
Penicillin (50)	+	+	–	+	–
Penicillin (100)	+	–	–	–	–
Streptomycin (5)	+	+	–	+	–
Tolerance of (%):					
Eosin Y (0.2)	+	+	+	–	–
Phenol red (0.2)	+	+	+	+	–
Bromothymol blue (0.2)	+	+	–	+	+
Helianthine (0.2)	+	–	–	–	–
Deoxysodium cholate (0.2)	+	–	+	+	–
Malachite green (0.1)	+	+	–	–	–
NaCl (4)	+	–	–	+	+
NaCl (5)	+	–	–	–	–
Tolerance of (mM):					
Zn ²⁺ (0.4)	+	+	–	–	–
Zn ²⁺ (0.8)	+	–	–	–	–
Zn ²⁺ (1.2)	+	–	–	–	–
Cu ²⁺ (0.8)	+	+	+	–	+
Cu ²⁺ (1.2)	+	+	–	–	–
Cu ²⁺ (1.6)	+	+	–	–	–

continues at 40 °C. Growth is inhibited at 10 or 60 °C. Growth occurs at pH 9–11 (optimum pH 6–8). Produces acid on YMA. Grows on YMA in the presence of 5 % (w/v) NaCl and with 0.2 % each of crocus T, eosin Y, phenol red, bromothymol blue, helianthine, deoxysodium cholate, bromocresol purple and Sudan black B. As sole carbon source, utilizes mannose, sodium malonate, D-xylose, sodium succinate, L-arabinose, D-ribose, D-galactose, raffinose, sodium hippurate, fructose, maltose, sucrose, rhamnose, sodium pyruvate, sorbitol, glucose, lactose and chitosan, but not L-sorbose or sodium tartrate. As sole nitrogen source, utilizes L-cystine, L-methionine, L-lactamine, L-cysteine, L-tryptophan, hypoxanthine, L-tyrosine,

L-aspartic acid, L-glutamic acid, L-asparagine, L-threonine, L-leucine, L-histidine, L-adenine, L-arginine, L-phenylalanine and L-proline. Sensitive to (µg ml^{–1}) streptomycin (50) and azithromycin (5). The G + C content of the type strain is 61.5 mol% (*T_m*).

The type strain, CCNWC 115^T (=ACCC 14543^T =HAMBI 3082^T), was isolated from a root nodule of *Robinia pseudoacacia* grown in Yangling, Shaanxi province, China.

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