Mesorhizobium alhagi sp. nov., isolated from wild *Alhagi sparsifolia* in north-western China

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Eleven strains that formed symbiotic root nodules on *Alhagi sparsifolia*, designated previously as genospecies II, were identified as a new lineage of *Mesorhizobium* (Alphaproteobacteria) that could be differentiated from all previously recognized species of the genus *Mesorhizobium* by using 16S rRNA gene sequences (<97.8% similarity), DNA–DNA hybridization (<45%), *dnaJ*, *dnaK*, *recA*, *glnA*, *nifH*, *nodA* and *nodC* gene sequences, fatty acid profiles (C_{18:1}ω7c, 35%; 11-methyl C_{18:1}ω7c, 30%) and numerical taxonomy. These strains are therefore considered to represent a novel species, for which the name *Mesorhizobium alhagi* sp. nov. is proposed, with isolate CCNWXJ12-2^T (=ACCC 15461^T=HAMBI 3019^T) as the type strain.

In north-western China, many legumes grow in poor soils and are dependent on a nitrogen-fixing symbiosis with root-nodule bacteria (rhizobia). Several novel rhizobial taxa have been described from this region: the genus *Sinorhizobium* (Chen *et al.*, 1988) and species *Mesorhizobium tianshanense* (Chen *et al.*, 1995), *Mesorhizobium amorphae* (Wang *et al.*, 1999), *Sinorhizobium xinjiangense* (Chen *et al.*, 1988; Peng *et al.*, 2002), *Sinorhizobium kummerowiae* (Wei *et al.*, 2003), *Rhizobium yanglingense* (Tan *et al.*, 2001), *Rhizobium indigoferae* (Wei *et al.*, 2002) and *Rhizobium loessense* (Wei *et al.*, 2003). In this paper we describe some isolates from the legume *Alhagi sparsifolia*, which grows wild in this region. They represent a novel species of *Mesorhizobium*, a genus that was first defined by Jarvis *et al.* (1997) and which included 15 recognized species at the time of writing.

In a previous study, we characterized 33 rhizobia isolated from *Alhagi sparsifolia* that fell into three genospecies by using restriction analysis and sequencing of the *rrs* (small-subunit rRNA), *dnaK* and *dnaJ* genes (Wei *et al.*, 2009). Their symbiosis-related *nodA* and *nodC* genes also indicated their distinctness from previously described rhizobia. In this paper, we considered genospecies II, which included the largest number of strains, and present additional data comprising sequences of two additional core genes (*recA* and

glnA) and an additional symbiosis-related gene (*nifH*), fatty acid profile, DNA–DNA relatedness and numerical taxonomy based on phenotypic features.

The 16S rRNA gene sequences of six strains of genospecies II, including strain CCNWXJ12-2^T, were identical and formed a novel branch within the genus *Mesorhizobium* (Wei *et al.*, 2009; Fig. 1). They shared less than 97.8% sequence similarity with all recognized *Mesorhizobium* species (the most similar being *Mesorhizobium loti* LMG 6125^T), 96.0% with the genus *Sinorhizobium* and 93.1% with *Rhizobium*.

Nucleotide sequences of *recA*, *glnA* and *nifH* of six representative *Mesorhizobium alhagi* sp. nov. strains were amplified and sequenced (Turner & Young, 2000; Gaunt *et al.*, 2001; Laguerre *et al.*, 2001), and aligned using CLUSTAL_X v.1.81 (Thompson *et al.*, 1997). Phylogenies were constructed by using the neighbour-joining method with the Kimura two-parameter distance correction and 1000 bootstrap replicates using PHYLO_WIN (Galtier *et al.*, 1996), and maximum-likelihood with the GTR model and 100 bootstrap replicates using PhyML (Guindon *et al.*, 2005).

The phylogenetic tree based on 440 bp of the *recA* gene clearly differentiated the six genospecies II strains from other *Mesorhizobium* (Supplementary Fig. S1a, available in IJSEM Online). They formed a clade with two subclades corresponding to the two sampling sites. Strain CCNWXJ12-2^T shared more than 99.7% sequence similarity with the other isolates from Alaer (CCNWXJ05-2, CCNWXJ12-1 and CCNWXJ05-1) and 96.6% sequence similarity with isolates CCNWX44-1 and CCNWX33-1 from Anxi. Similarity to recognized *Mesorhizobium* species, however, was always less than 92.5%. The *glnA* tree

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FJ481873–FJ481890.

Trees based on *recA*, *glnA* and *nifH* genes showing the relationships among the novel isolates and recognized *Mesorhizobium* species, a transmission electron micrograph of a cell of strain CCNWXJ12-2^T and a table showing the major fatty acids of strain CCNWXJ12-2^T and recognized *Mesorhizobium* species are available as supplementary material with the online version of this paper.

Fig. 1. Phylogeny based on 16S rRNA gene sequences, showing the relationships among the novel isolates and recognized *Mesorhizobium* species. Bootstrap percentages greater than 60 % are indicated for phylogenies constructed by using neighbour-joining (above) and maximum-likelihood (below). GenBank accession numbers are shown in parentheses. *M.*, *Mesorhizobium*; *R.*, *Rhizobium*. Bar, 0.005 nucleotide substitutions per site.

(Supplementary Fig. S1b) was very similar: the sequence of strain CCNWXJ12-2^T was identical to those of CCNWXJ05-2, CCNWXJ12-1 and CCNWXJ05-1, and had 96.2 % similarity to those of CCNWX44-1 and CCNWX33-1, but only 90.3–92.2 % to recognized *Mesorhizobium* species.

Sequences of *nodA* and *nodC* genes have been described previously (Wei *et al.*, 2009): most *Alhagi sparsifolia* symbionts harboured closely related *nodA* and *nodC* genes that formed a new lineage. Within genospecies II, the distinction between Alaer and Anxi strains was maintained, although it was much less marked than for the core genes described above. For the nitrogenase gene *nifH*, which is involved in the symbiosis but frequently has a different phylogenetic history to the nodulation genes, the distinction between Alaer and Anxi strains was more marked, though the two groups still shared 97.1 % sequence identity and were closest neighbours to each other (Supplementary Fig. S1c). However, unlike the core genes, though, the divergence between the *nifH* genes of genospecies II and those of other mesorhizobia was quite limited.

Total DNA was extracted from the strains tested and reference strains using the method of Marmur (1961). The G+C content of DNA was measured using the thermal denaturation method of Marmur & Doty (1962) and with *E. coli* K-12 as a standard. DNA–DNA relatedness was determined using the spectrophotometric method of De Ley *et al.* (1970). The DNA G+C content of strain CCNWXJ12-2^T was 60.4 mol% (Table 1), which was within the range reported for *Mesorhizobium* (59–64 mol%) (Jarvis

et al., 1997), and T_m was 78 °C. The DNA–DNA relatedness of CCNWXJ12-2^T with the other members of genospecies II ranged from 90.8 to 100 %, but was less than 44.3 % with any recognized *Mesorhizobium* species. Therefore, the strains in genospecies II can be regarded as representing a single novel species even though two subgroups were formed in the phylogenetic trees of housekeeping genes.

Fatty acid methyl esters were extracted and measured for CCNWXJ12-2^T by using the standard protocol as recommended by Hewlett Packard (Supplementary Table S1). Based on the fatty acid content, strain CCNWXJ12-2^T should be assigned to *Mesorhizobium* as it lacked $C_{20:3\omega6,9,12cis}$ and summed feature 3 ($C_{12:0}$, unknown ECL 10.928, $C_{16:1iso}$ 1/ $C_{14:0}$ 3-OH) and possessed iso- $C_{17:0}$ fatty acids (Tighe *et al.*, 2000). Strain CCNWXJ12-2^T differed from recognized *Mesorhizobium* species based on the high concentrations of $C_{18:1\omega7c}$, 11-methyl $C_{18:1\omega7c}$, $C_{20:1\omega7c}$ and a relatively high concentration of $C_{17:0}$ iso.

One hundred and fifty-three phenotypic features covering utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance to dyes and chemicals, tolerance to various concentrations of NaCl, temperature and pH ranges for growth, and other tests useful for diagnostic purposes with rhizobia were tested for these strains. Phenotypic features were analysed by using the unweighted pair group method with arithmetic averages (UPGMA) (Sneath & Sokal, 1973). The eleven isolates fell into one cluster at 85 % similarity level and were further divided into two subgroups, one group consisting of seven isolates from Alaer, and the other four isolates from Anxi (Fig. 2). Thus the phenotypic features reveal the same split into two subgroups that was apparent from the gene sequences. The distinctive features of the representative strains are shown in Table 2 and additional features are presented in the description of the novel species.

In nodulation tests, all 11 strains tested formed pink or brown nodules on the roots of *Alhagi sparsifolia*, which indicated that nodules were effective for nitrogen fixation, and no nodules formed on the uninoculated control plants in all experiments. In cross-infection tests using legumes that can be found in the sampling areas, CCNWXJ12-2^T could nodulate *Sophora alopecuroides*, *Glycyrrhiza inflata*, *Medicago sativa*, *Indigofera endecaphylla*, *Vicia cracca* and *Sophora flavescens*, but not *Cassia tora* or *Vigna sesquipedalis*. This indicates that strain CCNWXJ12-2^T has a wide host range, even though isolates with similar *nodA* and *nodC* genes have not yet been reported from any host other than *Alhagi sparsifolia*.

The novel species can be clearly differentiated from other species in the genus by using both genetic and phenotypic methods, including sequences of core genes (*rrs*, *recA*, *glnA*, *dnaK* and *dnaJ*) and symbiosis genes (*nifH*, *nodA* and *nodC*), fatty acid composition and DNA–DNA hybridization. Therefore the 11 strains are considered to represent a novel species for which the name *Mesorhizobium alhagi* sp. nov. is proposed.

Table 1. Strains used in this study and DNA–DNA relatedness among strains representing the candidate species and the type strains of some recognized species in the genus *Mesorhizobium*

ND, Not determined.

Strain	Host plant	Geographical origin	T_m	DNA G+C content (mol%)	DNA–DNA relatedness with strain CCNWXJ12-2 ^T (%)
CCNWXJ12-2 ^T	<i>Alhagi sparsifolia</i>	Alaer, Xinjiang, China	78.0	60.4	100
CCNWXJ05-2	<i>Alhagi sparsifolia</i>	Alaer, Xinjiang, China	78.0	60.4	97.2
CCNWXJ12-1	<i>Alhagi sparsifolia</i>	Alaer, Xinjiang, China	77.8	59.9	100
CCNWXJ36-1	<i>Alhagi sparsifolia</i>	Alaer, Xinjiang, China	77.6	59.5	91.0
CCNWXJ05-1	<i>Alhagi sparsifolia</i>	Alaer, Xinjiang, China	78.0	60.4	96.8
CCNWXJ02-1	<i>Alhagi sparsifolia</i>	Alaer, Xinjiang, China	78.2	60.8	94.6
CCNWXJ12-3	<i>Alhagi sparsifolia</i>	Alaer, Xinjiang, China	79.0	62.4	93.9
CCNWAX44-1	<i>Alhagi sparsifolia</i>	Anxi, Gansu, China	79.0	62.4	90.7
CCNWAX23-1	<i>Alhagi sparsifolia</i>	Anxi, Gansu, China	79.4	63.3	97.9
CCNWAX33-1	<i>Alhagi sparsifolia</i>	Anxi, Gansu, China	79.4	63.3	97.9
CCNWAX39-1	<i>Alhagi sparsifolia</i>	Anxi, Gansu, China	79.4	63.3	90.8
<i>M. temperatum</i> SDW018 ^T	<i>Astragalus adsurgens</i>	Liaoning, China	ND	ND	15.3
<i>M. plurifarium</i> LMG 11892 ^T	<i>Acacia senegal</i>	Senegal	ND	ND	0.6
<i>M. amorphae</i> ACCC 19665 ^T	<i>Amorpha fruticosa</i>	Beijing, China	ND	ND	9.7
<i>M. loti</i> NZP2213 ^T	<i>Lotus corniculatus</i>	New Zealand	ND	ND	34.1
<i>M. huakuii</i> CCBAU2609 ^T	<i>Astragalus sinicus</i>	Nanjing, China	ND	ND	44.3
<i>M. ciceri</i> USDA 3383 ^T	<i>Cicer arietinum</i>	Spain	ND	ND	4.9
<i>M. septentrionale</i> SDW014 ^T	<i>Astragalus adsurgens</i>	Liaoning, China	ND	ND	2.2
<i>M. albiziae</i> CCBAU 61158 ^T	<i>Albizia kalkora</i>	Sichuan, China	ND	ND	37.8
<i>M. chacoense</i> LMG 19008 ^T	<i>Prosopis alba</i>	Argentina	ND	ND	27.1
<i>M. mediterraneum</i> USDA 3392 ^T	<i>Cicer arietinum</i>	Spain	ND	ND	34.4
<i>M. tianshanense</i> CCBAU 3306 ^T	<i>Glycyrrhiza uralensis</i>	Xingjiang, China	ND	ND	34.8

Description of *Mesorhizobium alhagi* sp. nov.

Mesorhizobium alhagi (al.ha'gi. N.L. gen. n. *alhagi* of *Alhagi*, a genus of leguminous plants, referring to the host from which the type strain was isolated).

Gram-negative, non-spore-forming rods, aerobic, motile, 0.5–0.7 µm wide by 1.3–1.5 µm long (Supplementary

Fig. S2). Colonies on YMA (10.0 g mannitol, 3.0 g yeast extract, 0.25 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 20 g agar, 1000 ml distilled water; pH 6.8–7.0) are white, opaque, circular and convex with diameter of 1–2 mm within 7 days at 28 °C. Generation time ranges from 4.8 to 6.6 h in TY broth (5.0 g tryptone, 3.0 g yeast extract, 0.7 g CaCl₂·2H₂O, 1000 ml distilled water; pH 6.8–7.0). Produces

Fig. 2. Dendrogram based on the results of numerical taxonomy showing the phenotypic similarities among the rhizobial isolates and reference strains. Clustering analysis was performed using the UPGMA method.

Table 2. Distinctive features of strains of *Mesorhizobium alhagi* sp. nov. and recognized species of the genus *Mesorhizobium*

Strains: 1, *M. alhagi* sp. nov. CCNWXJ12-2^T; 2, *M. alhagi* CCNWX44-1; 3, *M. ciceri* USDA 3383^T; 4, *M. chacoense* LMG 19008^T; 5, *M. huakuii* CCBAU 2609^T; 6, *M. albiziae* CCBAU 61158^T; 7, *M. amorphae* ACCC 19665^T; 8, *M. loti* NZP 2213^T; 9, *M. mediterraneum* USDA 3392^T; 10, *M. tianshanense* CCBAU 3306^T; 11, *M. septentrionale* SDW014^T; 12, *M. temperatum* SDW018^T; 13, *M. plurifarum* LMG 11892^T. +, Growth or resistant; –, no growth or sensitive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Utilization as sole carbon source													
D-Arabinose	+	+	+	–	–	–	–	–	–	–	–	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	–	+	+	+	+	–
Maltose	+	+	+	–	+	–	+	+	+	–	+	–	+
Melibiose	+	–	–	–	–	–	+	–	–	–	–	–	–
L-Rhamnose	+	+	+	–	+	–	–	+	+	–	+	+	+
Sodium citrate	+	+	–	–	–	–	+	–	–	–	–	–	+
Sodium hippurate	–	–	–	–	+	–	–	–	–	–	+	–	+
Sodium succinate	+	+	+	–	–	+	+	+	–	+	+	–	+
D-Sorbitol	+	+	+	–	+	–	+	+	–	+	+	+	+
Sodium tartrate	+	–	–	+	–	–	–	–	–	–	–	–	–
D-Xylose	+	+	+	–	+	–	–	–	–	–	+	+	+
Utilization as sole nitrogen source													
D-Arginine	+	+	+	–	+	+	+	+	–	+	–	–	+
D-Aspartate	–	–	+	–	+	+	+	+	–	–	+	–	+
L-Cystine	+	+	+	–	+	+	+	+	–	+	–	–	+
L-Lysine	+	+	+	+	+	+	+	+	–	–	–	+	+
L-Phenylalanine	+	+	+	–	+	+	+	+	+	+	+	+	+
DL-Histidine	–	–	–	–	–	–	–	–	–	–	+	–	–
Glycine	+	+	–	–	–	–	–	–	+	+	+	+	–
Growth at:													
pH 5	–	–	+	–	–	–	–	–	–	–	–	–	–
pH 10	+	+	–	–	–	–	–	–	–	–	–	–	–
1 % NaCl	+	+	+	–	+	–	–	–	–	–	–	–	–
Resistance to (µg ml ^{–1}):													
Ampicillin (50)	–	–	–	–	–	+	–	–	–	–	+	–	–
Erythromycin (5)	+	+	+	–	+	+	+	+	+	+	+	–	+
Erythromycin (50)	–	–	+	–	+	+	+	+	–	+	+	–	+
Streptomycin sulfate (5)	–	–	+	–	+	+	–	+	–	–	+	–	+
Gentamicin (5)	–	–	+	–	+	+	+	+	–	–	–	–	+
Chloramphenicol (5)	–	+	+	–	–	+	–	–	–	–	–	–	–
Neutral red (0.2 %)	+	–	–	–	–	–	–	+	+	+	–	–	–
Bromothymol blue (0.2 %)	–	–	+	+	+	+	+	–	+	+	+	+	+

acid on YMA and utilizes sodium citrate, D-sorbitol, rhamnose, L-arabinose, D-xylose and galactose as sole carbon source. Does not utilize sodium oxalate, glycogen or sodium hippurate as sole carbon source, or utilize DL-histidine as sole nitrogen source. Growth occurs on YMA at pH 10.0, 1 % NaCl and with 0.1 % neutral red and 0.1 % NaNO₂. Sensitive to streptomycin, gentamicin sulfate, amikacin sulfate (50 µg ml^{–1}) and 5 µg ampicillin ml^{–1}. The DNA G + C content of the type strain is 59.5–63.3 mol%.

The type strain, CCNWXJ12-2^T (=ACCC 15461^T =HAMBI 3019^T), was isolated from a root nodule of *Alhagi sparsifolia* in Alaer, Xinjiang, China.

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