Rhizobium giardinii was proposed as a species by Amarger et al. (1997) for a group of bacteria recovered from root nodules of Phaseolus vulgaris grown in France. On the basis of symbiotic characteristics (host range and nitrogen-fixation effectiveness) and genotypic data, this species was then subdivided into two biovars, R. giardinii bv. giardinii and R. giardinii bv. phaseoli (Amarger et al., 1997). So far, several reports have claimed that this species can form root nodules on many other legume species besides P. vulgaris (Amarger et al., 1997; Herrera-Cervera et al., 1999; Mhamdi et al., 2002), including Desmanthus illinoensis (Beyhaut et al., 2006), Arachis hypogaea (Taurian et al., 2002, 2006), Kummerovia stipulacea, Aechnonomene indica (Kwon et al., 2005), Desmanthus leptolobus, Dalea purpurea, Psoralea esculenta, Prosopis juliflora (Benata et al., 2008; Beyhaut et al., 2006) and Prosopis alba (Iglesias et al., 2007).

As R. giardinii was clearly separated by both phenotypic and molecular characteristics from all other Rhizobium species (Amarger et al., 1997), it was believed to deserve genus status. This hypothesis has been confirmed by 16S rRNA gene phylogeny (Laguerre et al., 2001; Wang et al., 2006), which indicated that the sequence similarities between R. giardinii H152 and other Rhizobium (including Agrobacterium) species were as low as those between the genera Rhizobium and Ensifer (Sinorhizobium).

In our previous studies, several strains associated with the leguminous plants Albizia lebbeck (Wang et al., 2006), Oxytropis cashmiriana, Caragana sinica (Hou et al., 2009), Astragalus membranaceus, Astragalus danicus (Zhao et al., 2008) and Kummerovia stipulacea (Lin et al., 2007) grown in different regions of China were demonstrated to be closely related to R. giardinii by amplified 16S rRNA gene restriction analysis (ARDRA).

Taking into account the special taxonomic position of R. giardinii, these novel strains were interesting in the taxonomy of the genus Rhizobium. In the present study, seven strains related to R. giardinii and reference strains for related Rhizobium species were characterized by genomic, phylogenetic and phenotypic analyses. The aims of this study were to clarify their taxonomic status.

Abbreviations: ARDRA, amplified 16S rDNA restriction analysis; IGS, intergenic spacer; YMA, yeast mannitl agar.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, atpD, recA and glnI gene sequences of the seven strains are GU565531–GU565537, GU565538–GU565544, GU565545–GU565551 and GU565552–GU565558, respectively.

Three supplementary figures and a supplementary table are available with the online version of this paper.
and investigate the relationship between the *R. giardinii*-related group and the other recognized *Rhizobium* species.

Seven novel strains (Table 1) previously isolated from root nodules of *Oxytropis cashmiriana*, *Caragana sinica*, *Astragalus membranaceus*, *Astragalus danicus*, *Albizia kalkora* and *Kummerowia stipulacea* grown in different regions of China were used in the present study. Routine methods and yeast mannitol agar (YMA) (Vincent, 1970) were used for growing and maintenance of the bacteria.

In the present study, total DNA from each strain was prepared as described by Terefeework et al. (2001) and used as the template to amplify the DNA fragments mentioned below.

In this study, RFLP analysis of amplified 16S rRNA gene and 16S–23S rRNA intergenic spacer (IGS) was used to group the strains, as in other studies (Hou et al., 2009; Zhao et al., 2008). The 16S rRNA gene was amplified with the primers P1 and P6 and procedures described by Tan et al. (1997). The IGS regions were amplified with the primers FGPS6 and 23S-38 (Rasolomampianina et al., 2005) and the PCR protocol described by Kwon et al. (2005). Aliquots (5–10 µl) of the PCR products were digested separately with restriction endonucleases (MspI, HaeIII, HinfI and Alul for 16S RNA; MspI, HaeIII and Hhal for IGS) for 4–6 h at 37 °C. The digested fragments were separated by electrophoresis in 3% (w/v) agarose gels at 100 V [5 V (cm of the gel)−1] for 4 h, followed by ethidium bromide (0.5 µg ml−1) staining and photographing under UV light. The restriction patterns were normalized, combined and clustered using the GelCompar II version 3.0 software package (Applied Maths) with the Dice coefficient and the UPGMA method.

According to the results of the ARDRA analysis, the seven test strains were grouped with *R. giardinii* H152T (see Supplementary Fig. S1, available in IJSEM Online). Strains CCBAU 83011, CCBAU 85032 and CCBAU 85050 had identical restriction patterns and shared 93% similarity with H152T; the remaining four strains had restriction patterns identical to those of H152T. In the IGS-RFLP analysis, the seven strains were divided into four groups at similarity levels of 75% (Fig. 1), at which the reference strains for defined *Rhizobium* species were distinguished. The three strains in group I, CCBAU 85040, CCBAU 41230 and CCBAU 45226, had different patterns and showed 70.5% similarity to *R. giardinii* H152T. The two strains in group II, CCBAU 85032 and CCBAU 83011T, had identical patterns and showed 66% similarity to *R. giardinii* H152T and *Agrobacterium rubi* LMG 156T. Group III (strain CCBAU 01209) showed 73% similarity to group II and 66% to H152T. Group IV (strain CCBAU 85050) showed 62.5% similarity to H152T and reference strains for other *Rhizobium* species. These data indicated that the seven test strains were most closely related to, but differed from, *R. giardinii* H152T. It is clear that the high sequence variation of the IGS region allows discrimination between closely related strains (Kwon et al., 2005).

The taxonomic position of the novel strains was studied further by phylogenetic analyses of the 16S rRNA gene and other housekeeping genes. The 16S rRNA gene was

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**Table 1. Rhizobial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host plant</th>
<th>Geographical origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R. herbae (group II)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCBAU 83011T</td>
<td>Astragalus membranaceus</td>
<td>Xinjiang, China</td>
<td>Zhao et al. (2008)</td>
</tr>
<tr>
<td>CCBAU 85032</td>
<td>Oxytropis cashmiriana</td>
<td>Tibet</td>
<td>Hou et al. (2009)</td>
</tr>
<tr>
<td><strong>R. giardinii (group I)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCBAU 85040</td>
<td>Caragana sinica</td>
<td>Tibet</td>
<td>Hou et al. (2009)</td>
</tr>
<tr>
<td>CCBAU 45226</td>
<td>Albizia kalkora</td>
<td>Henan, China</td>
<td>Wang et al. (2006)</td>
</tr>
<tr>
<td>CCBAU 41230</td>
<td>Kummerowia stipulacea</td>
<td>Hunan, China</td>
<td>Lin et al. (2007)</td>
</tr>
<tr>
<td>Group III CCBAU 01209</td>
<td>Astragalus danicus</td>
<td>Inner Mongolia, China</td>
<td>Zhao et al. (2008)</td>
</tr>
<tr>
<td>Group IV CCBAU 85050</td>
<td>Oxytropis cashmiriana</td>
<td>Tibet</td>
<td>Hou et al. (2009)</td>
</tr>
<tr>
<td><strong>Reference strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. giardinii</em> H152T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. tropici</em> A LMG 9517</td>
<td>Phaseolus vulgaris</td>
<td>France</td>
<td>Amarger et al. (1997)</td>
</tr>
<tr>
<td><em>R. daejeonense</em> L61T</td>
<td>Phaseolus vulgaris</td>
<td>Brazil</td>
<td>Martinez-Romero et al. (1991)</td>
</tr>
<tr>
<td><em>R. selenitireducens</em> B1T</td>
<td>Bioreactor</td>
<td>Korea</td>
<td>Quan et al. (2005)</td>
</tr>
<tr>
<td><em>R. leguminosarum</em> USDA 2370T</td>
<td>Pisum sativum</td>
<td>USA</td>
<td>Hunter et al. (2007)</td>
</tr>
<tr>
<td><em>R. galegae</em> ATCC 43677T</td>
<td>Galega orientalis</td>
<td>USA</td>
<td>Frank (1889)</td>
</tr>
<tr>
<td><em>R. huautlense</em> SO2T</td>
<td>Sesbania herbacea</td>
<td>Finland</td>
<td>Lindström (1989)</td>
</tr>
<tr>
<td><em>R. alkalisoli</em> CCBAU 01393T</td>
<td>Caragana intermedia</td>
<td>Mexico</td>
<td>Wang et al. (1998)</td>
</tr>
<tr>
<td><em>R. celluloviticosum</em> ALA108B2T</td>
<td>Populus alba</td>
<td>Inner Mongolia, China</td>
<td>Lu et al. (2009)</td>
</tr>
<tr>
<td><em>R. vitis</em> LMG 8750T</td>
<td>Vitis sp.</td>
<td>Spain</td>
<td>García-Fraile et al. (2007)</td>
</tr>
<tr>
<td><em>Ag. tumefaciens</em> ATCC 19358</td>
<td>Malus sp.</td>
<td>Australia</td>
<td>Ophel &amp; Kerr (1990)</td>
</tr>
<tr>
<td><em>Ag. rubi</em> LMG 156T</td>
<td>Rubus sp.</td>
<td>USA</td>
<td>Sawada et al. (1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>USA</td>
<td>Kersters &amp; De Ley (1984)</td>
</tr>
</tbody>
</table>
amplified as described above for ARDRA, and partial
housekeeping genes atpD, recA and glnII were amplified
according to Gaunt et al. (2001) and Turner & Young
(2000), with primer pairs atpD255F/atpD782R, recA41F/
recA640R and glnII12F/glnII689R, respectively. All ampli-
fied DNAs were sequenced directly (Hurek et al., 1997).
The acquired sequences were aligned with those in
GenBank by using the CLUSTAL W program in the MEGA
4.0 software (Tamura et al., 2007). Maximum-parsimony
and neighbour-joining (Saitou & Nei, 1987) trees under
the Jukes–Cantor model (Jukes & Cantor, 1969) were
constructed. The robustness of the tree topology was
calculated with bootstrap analysis using 1000 replications
of the sequences (Felsenstein, 1985). For all of the genes,
the phylogenetic trees constructed with the maximum-
parsimony (not shown) and neighbour-joining methods
were almost the same in topology.

In 16S rRNA gene phylogeny (Fig. 2), the seven test strains
formed a cluster with bootstrap values of 100% and were
related closely to R. giardinii H152T, with sequence
similarities ranging from 99.3 to 99.9% (Table 2), which
were well consistent with the IGS-RFLP results. They were
further grouped with Agrobacterium species, Ensifer
(Sinorhizobium) species and other Rhizobium species, but
the bootstrap values were <46%.

For the housekeeping genes, the phylogenies of the atpD
(Fig. 3) and recA (see Supplementary Fig. S2, available in
IJSEM Online) trees were similar to that of the 16S rRNA
gene, indicating that the R. giardinii-related bacteria
formed a clade differing from both the Ensifer species
and the remaining Rhizobium species. The phylogeny of the
glnII gene was a little different from those of the 16S rRNA,
atpD and recA genes, in which the R. giardinii-related
bacteria were divided into two phyletic groups inter-
mingled with other Rhizobium species (see Supplementary
Fig. S3, available in IJSEM Online). These results were
similar to those of previous studies in that the phylogenies
of the atpD and recA genes support that of the 16S rRNA
gene (Gaunt et al., 2001), but the phylogeny of glnII differs
slightly (Turner & Young, 2000).

In the phylogenetic trees constructed separately from the
atpD, recA and glnII sequences (Fig. 3, Supplementary Figs
S2 and S3), it was clear that the seven test strains and R.
giardinii formed a distinctly coherent phylogenetic group,
but their relationships with other bacteria (Ensifer, Agro-
bacterium and other Rhizobium species) were only
supported by bootstrap values <50%, indicating that the
topology of these trees was not stable. Sequence similarities
of atpD, recA and glnII among the seven strains and R.
giardinii H152T are summarized in Table 2. CCBAU 85040,

Fig. 1. Ribosomal IGS-RFLP patterns showing the relationships among the seven R. giardinii-related strains and
phylogenetically related reference strains. The dendrogram was constructed with the Dice coefficient and the UPGMA method.
CCBAU 41230 and CCBAU 45226 (IGS-RFLP group I) all showed high similarities (>97%) to *R. giardinii* H152\(^\mathrm{T}\) for the three housekeeping genes. Similarities between the IGS-RFLP group II strains (CCBAU 83011\(^\mathrm{T}\) and CCBAU 85032) and *R. giardinii* H152\(^\mathrm{T}\) were 93.8 and 93.5% (*atpD*), 89.6 and 90.6% (*recA*) and 86.4 and 86.9% (*glnII*). Both CCBAU 01209 (group III) and CCBAU 85050 (group IV) showed much lower similarities to strain H152\(^\mathrm{T}\) (Table 2). These results suggest strongly that IGS-RFLP groups II, III and IV belong to different novel genomic species.

To further estimate the relationships between the novel strains and the defined species, total DNA extracted from each strain with the method of Marmur (1961) was used to determine the DNA G+C content with the thermal-denaturation method (*T_m*) using *Escherichia coli* K-12 as standard (Marmur & Doty, 1962) and to determine DNA relatedness among the strains using the spectrophotometric method (De Ley *et al.*, 1970). In this experiment, the DNA G+C content of the seven strains varied between 57.6 and 59.1 mol% (*T_m*), which were within the range of *Rhizobium* species (Jordan, 1984). According to the DNA
G+C content, DNA–DNA hybridization was performed under an optimum temperature of 76.5 °C in a solution of 2 × SSC (De Ley et al., 1970).

The group I strains CCBAU 45226, CCBAU 85040 and CCBAU 41230 shared DNA relatedness of 77.1–81.3 %, and had relatedness of 64.3 % with Rhizobium giardinii H152T (Table 2), indicating a close genomic relationship to R. giardinii. The two group II strains shared 82.2 % DNA relatedness and shared 26.8 % relatedness with R. giardinii H152T. Groups III and IV had DNA relatedness of 15.4 and relatedness and shared 26.8 % relatedness with R. giardinii H152T, further confirming their distinct taxonomic positions based on the recommended threshold value of 70 % DNA relatedness for species definition (Graham et al., 1991; Wayne et al., 1987).

Phenotypic features of the seven strains were determined in comparison with those of R. giardinii H152T according to the method described by Gao et al. (1994). The tested features included the utilization of sole carbon and nitrogen sources, resistance to antibiotics (5–300 μg ml⁻¹) and tolerance to NaCl (1–5 %, w/v), pH and temperature ranges for growth, and other physiological and biochemical tests (Gao et al., 1994). In this analysis, some differences were observed among the seven strains, even within the group I and group II strains (Table 3), indicating that they represent phenotypically divergent rhizobial populations. Some phenotypic characters could also differentiate the seven strains from R. giardinii H152T (Table 3).

Although several differences between the two strains of group II were found, they showed common features in most of the characteristics, as presented in the subsequent description. The ability to use meso-erythritol, but not melezitose, as sole carbon source could differentiate group II from R. giardinii H152T and the group I strains. Meanwhile, the three strains in group I could use citrate, but R. giardinii H152T could not, although Amarger et al. (1997) reported that 52 % of the R. giardinii strains analysed were able to use citrate.

To further reveal the phenotypic relationship between group II and R. giardinii, profiles of cellular fatty acids were analysed, as in other studies of rhizobial taxonomy (Quan et al., 2005; Schutter & Dick, 2000; Tighe et al., 2000). The two strains of group II and R. giardinii USDA 2914T were incubated for 48 h at 28 °C on YMA. Cells were harvested and fatty acids were extracted and identified according to the method of the Microbial Identification system (MIDI). Common fatty acids were observed among the two strains of group II and R. giardinii H152T, in both the presence and proportion of 10:0 iso, 12:0, 14:0, 16:0, 17:0 cyclo, summed feature 1, summed feature 3 and summed feature 8 (see Supplementary Table S1, available in IJSEM Online, for detailed information). The novel group could be differentiated from R. giardinii H152T by the absence of 10:0 and 15:1 anteiso A; the presence of 18:0, 18:0 3OH, 18:1 07c 11-methyl and summed feature 7; and distinctive proportions of 10:0 iso, 12:0, 14:0, 16:0, 17:0, 19:0 cyclo 08c and summed feature 2. Meanwhile, differences in the presence of several other minor fatty acids in the two strains of group II might be further evidence that they were not clones of the same strain.

Nodulation of the seven test strains on their hosts of origin has been confirmed previously (Hou et al., 2009; Lin et al., 2007; Wang et al., 2006; Zhao et al., 2008). In the present study, a cross-nodulation test was performed using the standard methods (Vincent, 1970) for seed treatment and inoculation. All plants were maintained in glass tubes containing sterile vermiculite moistened with nitrogen-free plant nutrition solution (Vincent, 1970) under natural sunlight. After 4–8 weeks growth, all novel strains could form nodules on Albizia julibrissin, but not on seedlings of Phaseolus vulgaris, Medicago sativa, Pisum sativum or Trifolium pratense. Furthermore, CCBAU 45226 (group I) could fix nitrogen with Albizia julibrissin, as evidenced by the large size (2–4 mm in diameter) and red color (leghaemoglobin) of nodules, as well as dark-green leaves of the seedlings, whereas the other six strains induced nodules with small size (1–2 mm in diameter) and white inside, indicating that ineffective symbiosis had been established.

In this study, the amplified 16S rRNA gene RFLP patterns and nucleotide sequences clearly verified that the seven test strains belonged to the genus Rhizobium, and were related closely to R. giardinii. As shown in Table 1, the seven strains originated from six shrubbery and herbal legume species grown in five Chinese provinces or autonomous regions covering the temperate and subtropical regions.
selected from 381 strains used in four independent studies on diversity of rhizobia (Hou et al., 2009; Lin et al., 2007; Wang et al., 2006; Zhao et al., 2008). These data, as well as those in the other mentioned reports, demonstrated that the *R. giardinii*-related bacteria are minor groups in root nodules of various legumes in divergent regions.

According to the consensus of all analyses, the seven test strains were divided into four groups, demonstrating the existence of different genomic species related to *R. giardinii*. The formation of a clade, by *R. giardinii* and the novel groups, differing from the other *Rhizobium* species in the phylogenies of both the 16S rRNA gene and the housekeeping genes is strong evidence to support the hypothesis that *R. giardinii* represents an independent genus (Amarger et al., 1997; Lagueree et al., 2001; Wang et al., 2006). Considering the argument about the combination of *Rhizobium* and *Agrobacterium* species (Farrand et al., 2003; Young et al., 2001), further revision of the current *Rhizobium* classification should be considered; however, more data, particularly genomic data, are necessary before further decisions are taken.

In the present study, group I strains (CCBAU 85040, CCBAU 41230 and CCBAU 45226) had high similarities...
(>97%) to R. giardinii H152T in the phylogenetic trees of the 16S rRNA and housekeeping genes, and showed relatively high DNA relatedness (64.3–67.5%) to H152T, indicating that these three strains belonged to R. giardinii. Considering their low similarities in the housekeeping genes (Table 2) and low DNA relatedness (70%), strains in groups II, III and IV could represent three novel species.

The different phylogenetic positions of these strains were consistent with their divergent phenotypic characteristics. The great divergence among the strains within groups I and II might be related to the fact that they were isolated from different hosts and geographical regions. The two strains in group II, CCBAU 85032 and CCBAU 83011T, were isolated from two legume genera grown in two geographical regions, whilst the three strains in group I were isolated from three legume genera grown in three regions. Previously, the Sinorhizobium (Ensifer) meliloti (Yan et al., 2000) and Sinorhizobium (Ensifer) fredii (Peng et al., 2002) populations in Xinjiang, China, have been defined as phena differing from reference strains of the same species that originated from other regions. It could be hypothesized that rhizobial strains in different regions might have evolved genetically and phenotypically diverse populations adapted to the local conditions.

Considering all of the results and the current criteria for the definition of Rhizobium species, we identified the group I strains as members of R. giardinii, which might be a novel species.
biovar as they could form nodules on *Albizia julibrissin*. This identification extended the host range of *R. giardinii* to *Caragana sinica* and *Albizia kalkora*.

Among the novel genomic species (groups II, III and IV), we propose the two strains in group II as a novel species named *Rhizobium herbae* sp. nov., referring to the fact that both strains were isolated from herbaceous legumes. As groups III and IV contain only one strain, it is desirable to gather additional strains to clarify their taxonomic status.

**Description of *Rhizobium herbae* sp. nov.**

*Rhizobium herbae* (her’ba.e. L. gen. n. *herbae* of a herb, because this bacterium was originally isolated from root nodules of herb legumes).

Gram-negative rods (0.5–0.7 × 1.2–1.9 µm); aerobic; non-spore-forming. Colonies on YMA are circular, convex, white and opaque and usually have a diameter of 2–3 mm within 2–3 days at 28 °C. Can grow on YMA supplemented with 0–2% (w/v) NaCl, with an optimum of 1%. Growth occurs at 10–40 °C and pH 7–9 on YMA plates, with optima of 28 °C and pH 7.0. Catalase, urease and oxidase are present, but not l-phenylalaninase. Nitrate is reduced and reduction of methyl blue and Nile blue are observed. Acid reaction, reduction and curd are produced in litmus milk. Strains are resistant to 5 µg ml⁻¹ of kanamycin sulfate, neomycin sulfate, streptomycin sulfate, chloramphenicol and gentamicin sulfate; 50 µg ml⁻¹ of ampicillin and erythromycin; and 300 µg bacitracin ml⁻¹. They can grow on YMA supplemented with 0.1% Bismarck brown, Congo red and sodium deoxycholate. Strains CCBAU 83011ᵀ and CCBAU 85032 can both use D-fructose, D-galactose, D-mannose, D-sorbitol, D-xyllose, sodium Dl-malate, glucose, inositol, L-rhamnose, raffinose pentaehydrate, maltose, sodium succinate, sucrose, trehalose, glycine and meso-erythritol as sole carbon sources for growth, and almost all of the tested sole nitrogen sources, including L-aspartic acid, (+)-L-glutamic acid, D-glutamic acid, L-alanine, hypoxanthine, L-arginine, L-cysteine, L-isoleucine, L-lysine, L-threonine and L-methionine. Some differences were also observed between the strains within the novel group. CCBAU 85032 differed from CCBAU 83011ᵀ by failure to use D-arabinose, dulcitol, D-amygdalin, melibiose, sodium pyruvate, salicin, sodium acetate, sodium formate, sodium citrate, L-arginine, L-threonine, Dl-asparagine and Dl-proline as sole carbon sources; L-phenylalanine, L-valine and Dl-threonine as sole nitrogen sources; sensitivity to kanamycin sulfate (50 µg ml⁻¹); growth in sodium nitrite (0.1%) and at pH 10; and resistance to neomycin sulfate and streptomycin sulfate (both at 50 µg ml⁻¹).

The type strain, CCBAU 83011ᵀ (=LMG 25718ᵀ=HAMBI 3117ᵀ), was isolated from nodules of *Astragalus membranaceus* grown in Xinjiang, China; its DNA G+C content is 58.9 mol% (*Tm*).

**Acknowledgements**

We thank the BCCM/LMG, HAMBI and MIAE culture collections for providing reference strains *R. daejeonense* L6¹, *R. vitis* LMG 8750⁴ and *R. giardinii* H152¹. This study was supported financially by the Foundation of the State Key Basic Research and Development Plan of China (grant 2010CB126300) and by the National Natural Science Foundation of China (project no. 30970004). E. T. W. was supported by projects authorized by ICyT D. F. (PICS08-3) and IPN (SIP2009179 and 20100667), Mexico.

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