Revision of the taxonomic status of the species *Rhizobium lupini* and reclassification as *Bradyrhizobium lupini* comb. nov.

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The species *Rhizobium lupini* was isolated from *Lupinus* nodules and included in the Approved Lists of Bacterial Names in 1980. Nevertheless, on the basis of the analysis of the type strain of this species available in DSMZ, DSM 30140T, whose 16S rRNA gene was identical to that of the type strain of *Bradyrhizobium japonicum*, *R. lupini* was considered a later synonym of this species. In this study we confirmed that the strain DSM 30140T belongs to the species *B. japonicum*, but also that it cannot be the original strain of *R. lupini* because this species effectively nodulated *Lupinus* whereas strain DSM 30140T was able to nodulate soybean but not *Lupinus*. Since the original type strain of *R. lupini* was deposited into the USDA collection by L. W. Erdman under the accession number USDA 3051T we analysed the taxonomic status of this strain showing that although it belongs to the genus *Bradyrhizobium* instead of genus *Rhizobium*, it is phylogenetically distant from *B. japonicum* and closely related to *Bradyrhizobium canariense*. The type strains *R. lupini* USDA 3051T and *B. canariense* BTA-1T share 16S rRNA, recA and glnII gene sequences with similarities of 99.8%, 96.5% and 97.1%, respectively. They presented a DNA–DNA hybridization value of 36% and also differed in phenotypic characteristics and slightly in the proportions of some fatty acids. Therefore we propose the reclassification of the species *Rhizobium lupini* as *Bradyrhizobium lupini* comb. nov. The type strain is USDA 3051T (=CECT 8630T =LMG 28514T).

The species *Rhizobium japonicum* (Kirchner, 1896) Buchanan 1926 and *Rhizobium lupini* (Eckhardt et al., 1931) were included in the Approved Lists of Bacterial Names from Skerman et al. (1980). They are slow-growing rhizobia, with *R. japonicum* and *R. lupini* being able to nodulate members of the genera *Glycine* and *Lupinus*, respectively (Jordan & Allen, 1974). When the slow-growing rhizobia were reclassified in a different genus, *Bradyrhizobium* (Jordan, 1982), the species *R. lupini* was not included in this genus since the major difference with respect to the novel and single recognized species at that time, *Bradyrhizobium japonicum*, was a high degree of nodulation affinity for members of the genera *Lupinus* and *Ornithopus* (Jordan & Allen, 1974; Jordan, 1982, 1984). Nevertheless, as this species was not specifically rejected in the publication of Jordan (1982) or in later works, it has been maintained with the original name *Rhizobium lupini* on the list of validly published prokaryotic names (http://www.bacterio.net/rhizobium.html).

When the 16S rRNA gene sequence of the type strain *R. lupini* DSM 30140T, named *Bradyrhizobium* sp. (*Lupinus*) following the recommendations of Jordan (1984), was analysed it showed 100% identity with respect to that of the type strain *B. japonicum* LMG 6138T, apparently confirming the identity of these two species (supplementary material of Vinuesa et al., 2005). Nevertheless, when the nodulation test was carried out on *Lupinus albus* with strain DSM 30140T using the methodology described by...
Velázquez et al. (2010), negative results were obtained, whereas effective nodulation was found in Glycine max (Fig. S1a, available in the online Supplementary Material). Therefore it was clear that the culture DSM 30140T cannot be a subculture of the original strain of R. lupini because this species effectively nodulated members of the genus Lupinus.

The original type strain of R. lupini was deposited into the USDA collection by L. W. Erdman (USDA 3051T) and hence in the present work we analysed the taxonomic status of this strain. In the first place we also carried out nodulation experiments with this strain that confirmed the effective nodulation of Lupinus albus (Fig. S1b).

Amplification and sequencing of the 16S rRNA gene were done as described previously (Rivas et al., 2007) and partial sequences of the recA (500 nt) and glnII (670 nt) genes were obtained using the primers described by Vinuesa et al. (2008). The nodC gene sequences (700 nt) were obtained according to the method of Velázquez et al. (2010). The sequences obtained were compared to those in the GenBank database using the BLASTN program (Altschul et al., 1990) and also to the sequences held in the EzTaxon-e server (Kim et al., 2012). Alignments were performed using the CLUSTAL W software (Thompson et al., 1997). Distances were calculated according toKimura’s two-parameter model (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining (NJ) and maximum-likelihood models (Kimura, 1980). Phylogenetic analysis showed that both strains formed a well-defined cluster supported by 84% of bootstrap (Fig. 1), different to that formed by the type strain B. japonicum LMG 6138T and strain DSM 30140T with 100% similarity between their 16S rRNA gene sequences in agreement with the results of Vinuesa et al. (2005). The similarity of this gene between strains USDA 3051T and DSM 30140T was 99.4%, which is a value equal or even lower than those commonly found among different species of the genus Bradyrhizobium (Fig. 1). Despite the high similarity values presented by strains USDA 3051T and BTA-1T, they can belong to different species since in the genus Bradyrhizobium there are recognized species with even 100% 16S rRNA gene sequence similarity as occurs with the recently described species Bradyrhizobium icense and Bradyrhizobium paxllaeri (Durán et al., 2014). Nevertheless, all these species can be differentiated on the basis of housekeeping gene analysis that also allows the selection of the species necessary for DNA–DNA hybridization experiments (Ramírez-Bahena et al., 2009; Chahboune et al., 2012; Wang et al., 2013; Durán et al., 2014).

The recA and glnII genes have been commonly used for species delineation within the genus Bradyrhizobium and they have been sequenced in all described species of the genus Bradyrhizobium. The similarities in these genes between strains USDA 3051T and B. canariense BTA-1T were 96.5% and 97.1%, respectively. The results of the phylogenetic analysis of these concatenated gene sequences confirmed that the culture DSM 30140T belongs to the species B. japonicum with nearly identical recA and glnII genes to those of the type strain of this species, whereas strain USDA 3051T was related to B. canariense but occupied a separated branch (Fig. 2). The distance found between strain USDA 3051T and B. canariense BTA-1T was similar to that found between the type strains of Bradyrhizobium jicamae and the recently described species B. paxllaeri (Fig. 2). The similarity value for the recA gene sequences between strain USDA 3051T and B. canariense BTA-1T was 96.5%, lower than the 98% found between the type strains of B. jicamae and B. paxllaeri (Durán et al., 2014). In the case of the glnII gene the similarity value between strain USDA 3051T and B. canariense BTA-1T was identical (97%) to the value found between B. japonicum and the recently described Bradyrhizobium diaeofficiens USDA 110T (Delamuta et al., 2013) and was lower than the 97.3% found between the type strains of B. jicamae and B. paxllaeri. These results support that R. lupini USDA 3051T represents a species different from B. canariense.

This result was confirmed after DNA–DNA hybridization experiments carried out as described by Ezaki et al. (1989) and Willems et al. (2001) showing a relatedness value between strains Bradyrhizobium lupini comb. nov. USDA 3051T and B. canariense BTA-1T of 36% (±3%), which is lower than 70%, the threshold value of DNA–DNA similarity for definition of bacterial species (Wayne et al., 1987).

DNA analysis for DNA base composition was prepared according to Chun & Goodfellow (1995). The mol% G+C content of DNA was determined using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G+C content of strain USDA 3051T was 65.9%.

The cellular fatty acids were analysed by using the Microbial Identification System (MIDI) Sherlock 6.1 and the library RTSBA according to the technical instructions provided by this system (Sasser, 1990). The strains were cultured aerobically on YMA medium at 28°C and cells were collected during the late-exponential phase of growth. The major fatty acids of Bradyrhizobium group I (Menna et al., 2009) are summed feature 8 (C18:1ω6c/C18:1ω7c) and C16:0 (Table 1) with slight differences in their percentages showing that fatty acid analysis is not a useful technique for the differentiation of these species. The strains B. lupini comb. nov. USDA 3051T and B. canariense BTA-1T differed slightly in the percentage of C16:0 and in the fatty acids from summed feature 3 (C16:1ω7c/C16:1ω6c) (Table 1).
Phenotypic characterization was performed as previously described for species of the genus Bradyrhizobium (Ramírez-Bahena et al., 2009; Chahboune et al., 2011; Wang et al., 2013; Guerrouj et al., 2013). API 20NE galleries (BioMérieux) and Biolog GN2 MicroPlates were inoculated according to the manufacturers’ instructions. The galleries were incubated for 7 days at 28 °C. Assimilation of several compounds as carbon (D-glucose, D-galactose, L-arabinose, sucrose, raffinose, trehalose, mannitol and L-proline) or nitrogen (L-glutamate and L-aspartate) sources was analysed using yeast nitrogen base (YNB) and yeast carbon base (YCB) (Becton Dickinson) as basal media, respectively. The pH of the basal medium was adjusted with 1 M NaOH to pH 7 and 0.05 g bromothymol blue was added before sterilization by autoclaving. The carbon and nitrogen sources were sterilized by filtration using filters with a pore diameter of 0.22 μm (Millipore) and added to the sterilized basal media at concentrations of 0.7% and 0.1% respectively. The tubes, each containing 5 ml of liquid medium, were incubated for 4 weeks at 28 °C. Growth temperature range was determined by incubating cultures in YMA (Vincent, 1970) at 4, 15, 28, 37 and 45 °C. Growth pH range was determined in the same medium with final pH 4.5, 6, 7, 8, 9 and 10. PCA buffer (0.4 M Na₂HPO₄ and 0.2 M citric acid) was used to adjust the medium to pH 4 and 6, phosphate buffer (0.2 M Na₂HPO₄ and 0.2 M NaH₂PO₄) was used for pH 7 and 0.2 M Tris/EDTA buffer was used for pH 8, 9 and 10. Salt tolerance was tested in the same medium containing 0.5, 1, 1.5, 2 and 2.5% (w/v) NaCl. For testing the natural antibiotic resistance the disc diffusion method on YMA was used. The discs contained the following antibiotics: ampicillin (2 μg), erythromycin (2 μg), ciprofloxacin (5 μg), penicillin (10 IU), polymyxin B (300 IU), cloxacillin (1 μg), oxytetracycline (30 μg), gentamicin (10 μg), cefuroxime (30 μg), or neomycin (5 μg), (Becton Dickinson, BBL). Phenotypic characteristics of the novel species are reported below in the species description and the differences with respect to the most closely related species of the genus Bradyrhizobium are recorded in Table 2. Although symbiotic genes do not offer taxonomic information because they are located in easily interchangeable elements (plasmids or symbiotic islands), the analysis of

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**Fig. 2.** Neighbour-joining phylogenetic tree based on concatenated recA and glnII gene sequences showing the position of strain USDA 3051T within the genus *Bradyrhizobium*. Bootstrap values (percentages) calculated for 1000 replications are indicated. Bar, 1 nucleotide substitution per 100 nt.

**Table 1.** Cellular fatty acid composition of strain USDA 3051T and type strains of related species from the genus *Bradyrhizobium* according to both 16S rRNA and housekeeping gene analyses

<table>
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<th>Fatty acid</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tr>
<td>C16:0</td>
<td>15.9</td>
<td>18.8</td>
<td>14.5</td>
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<td>13.4</td>
<td>11.8</td>
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<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
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<td>1.2</td>
<td>1.6</td>
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<tr>
<td>Summed feature 3</td>
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<td>3.5</td>
<td>2.1</td>
<td>2.2</td>
<td>1.1</td>
<td>0.5</td>
<td>0.8</td>
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<td>(C_{16:1}ω7c/C_{16:1}ω6c)</td>
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<tr>
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<td>3.6</td>
<td>3.9</td>
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<td>1.1</td>
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<tr>
<td>C17:1ω8c</td>
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<td>0.4</td>
<td>0.4</td>
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<td>0.5</td>
<td>0.5</td>
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<td>Summed feature 8</td>
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<td>79.9</td>
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<tr>
<td>(C_{18:1}ω7c/C_{18:1}ω6c)</td>
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<tr>
<td>C18:1ω7c 11-methyl</td>
<td>ND</td>
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<td>0.2</td>
<td>5.2</td>
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<td>0.2</td>
<td>0.4</td>
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</table>

Data are percentages of total fatty acids; those present in amounts lower than 1% are not shown. ND, Not detected. Data are from this study except for those for *B. ganzhouense* that are from Lu et al. (2014).
Table 2. Differential phenotypic characteristics of USDA 3051T and type strains of related species from the genus Bradyrhizobium according to both 16S rRNA and housekeeping gene analyses

<table>
<thead>
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<th>Characteristic</th>
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<tr>
<td>Nitrate reduction</td>
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<td>Urease</td>
<td>w</td>
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<td>Growth at/with:</td>
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<td>pH 4.5</td>
<td>-</td>
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<td>37°C</td>
<td>-</td>
<td>w</td>
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<td>+</td>
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<tr>
<td>1% NaCl</td>
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<td>w</td>
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<td>Assimilation as carbon source of:</td>
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<td>D-Mannose</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
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<tr>
<td>Gluconate</td>
<td>-</td>
<td>+</td>
<td>w</td>
<td>+</td>
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<td>+</td>
<td>w</td>
<td>w</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Adipate</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>w</td>
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</table>

Acid production from rhamnose, arabinose and xylose as carbon source is positive, whereas alkaline reaction is observed with galactose as carbon source. Causes nodule formation on Lupinus spp. The following additional characteristics were determined from the present study. Strictly aerobic. Growth at pH 4.5 is negative and the optimum pH for growth is pH 7. Growth is negative at 4°C and at 37°C with optimum growth at 28°C. Growth is negative in presence of 1% NaCl or higher. Nitrate reduction is negative. Arginine dihydrolase and β-galactosidase production is negative. Urease and aesculin hydrolysis are weakly positive. In the API 20NE system the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol and adipate is positive, that of citrate is weakly positive and that of N-acetylglucosamine, maltose, gluconate, malate, caprate and phenylacetate is negative. In media containing YNB as nitrogen source the assimilation of D-glucose, L-arabinose and D-mannitol is positive, that of D-galactose is weakly positive and that of sucrose, raffinose, trehalose and L-proline is negative. In media containing YCB as carbon source the use as nitrogen source of L-aspartate and L-glutamate is positive. In Biolog GN2 microplates oxidation of L-arabinose, D-arabitol, L-fucose, D-galactose, α-D-glucose, D-mannitol, D-mannose, D-sorbitol, methylpyruvate, cis-aconitate, citric acid, gluconic acid, β-hydroxybutyric, DL-lactate, propionate, quinate, d-succarate, succinamate, glucuronamide, d-alanine, l-aspartic acid, l-glutamic acid, l-leucine, l-pyroglutamic acid and D-serine as carbon source is positive. Oxidation of α-cyclodextrin, dextrin, glycogen, N-acetylglucosamine, adonitol, cellobiose, d-erythritol, gentiobiose, α-lactose, lactulose, maltose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, sucrose, trehalose, turanose, xylitol, monomethyl-succinate, acetic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, sebacic acid, bromosuccinic.
acid, L-alanimamide, L-alanine, L-alanylglycine, glycyl-L-aspartic, glycyl-L-glutamic, L-histidine, hydroxy-L-proline, L-ornithine, L-proline, L-serine, L-threonine, DL-carnitine, γ-amino butyric acid, urocanic acid, asparagine and phenylalanine is weakly positive. Oxidation of Tween 40, Tween 80, 2,3-butanediol, glycerol, DL-lactic acid, aspartic, glycyl-L-glutamic, L-histidine, hydroxy-L-proline, p-hydroxyphenylacetic acid, α-ketoglutaric acid, malonic acid, succinic acid, L-asparagine and L-phenylalanine is weakly positive. Resistant to ampicillin, penicillin, cloxacillin, polymyxin B, ciprofloxacin, gentamicin and erythromycin and sensitive to tetracycline, cefuroxime and neomycin. Nodulates *Cytisus villosus* nov. isolated from effective nodules of *Cytisus villosus* grown in the Moroccan Rif.

The type strain is USDA 3051T (=CECT 8630T=LMG 28514T). The DNA G+C content of strain USDA 3051T is 65.9 mol%.

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