Revision of the taxonomic status of type strains of *Mesorhizobium loti* and reclassification of strain USDA 3471<sup>T</sup> as the type strain of *Mesorhizobium erdmanii* sp. nov. and ATCC 33669<sup>T</sup> as the type strain of *Mesorhizobium jarvisii* sp. nov.

Pilar Martínez-Hidalgo,¹ Martha Helena Ramírez-Bahena,²,³ José David Flores-Félix,¹ Raúl Rivas,¹,³ José M. Igual,²,³ Pedro F. Mateos,¹,³ Eustoquio Martínez-Molina,¹,³ Milagros León-Barrios,⁴ Álvaro Peix²,³ and Encarna Velázquez¹,³

¹Departamento de Microbiología y Genética, Universidad de Salamanca, Salamanca, Spain
²IRNASA-CSIC, Salamanca, Spain
³Unidad Asociada Universidad de Salamanca-CSIC ‘Interacciones Planta-Microorganismo, Salamanca, Spain
⁴Departamento de Bioquímica, Microbiología, Biología Celular y Genética, Universidad de La Laguna, Tenerife, Spain

The species *Mesorhizobium loti* was isolated from nodules of *Lotus corniculatus* and its type strain deposited in several collections. Some of these type strains, such as those deposited in the USDA and ATCC collections before 1990, are not coincident with the original strain, NZP 2213<sup>T</sup>, deposited in the NZP culture collection. The analysis of the 16S rRNA gene showed that strains USDA 3471<sup>T</sup> and ATCC 33669<sup>T</sup> formed independent branches from that occupied by *Mesorhizobium loti* NZP 2213<sup>T</sup> and related to those occupied by *Mesorhizobium opportunistum* WSM2075<sup>T</sup> and *Mesorhizobium huakuii* IFO 15243<sup>T</sup>, respectively, with 99.9 % similarity in both cases. However, the analysis of concatenated *recA*, *atpD* and *glnII* genes with similarities lower than 96, 98 and 94 %, respectively, between strains USDA 3471<sup>T</sup> and *M. opportunistum* WSM2075<sup>T</sup> and between strains ATCC 33669<sup>T</sup> and *M. huakuii* IFO 15243<sup>T</sup>, indicated that the strains USDA 3471<sup>T</sup> and ATCC 33669<sup>T</sup> represent different species of the genus *Mesorhizobium*. These results were confirmed by DNA–DNA hybridization experiments and phenotypic characterization. Therefore, the two strains were reclassified as representatives of the two species *Mesorhizobium erdmanii* sp. nov. (type strain USDA 3471<sup>T</sup> = CECT 8631<sup>T</sup> = LMG 17826<sup>T</sup>) and *Mesorhizobium jarvisii* sp. nov. (type strain ATCC 33669<sup>T</sup> = CECT 8632<sup>T</sup> = LMG 28313<sup>T</sup>).
This situation was explained in 2001 when Willems et al. (2001b) reported that subcultures of the type strain of *M. loti* were different after 16S rRNA gene sequencing. According to Willems et al. (2001b), two colony types were detected in strain ATCC 33669T and named LMG 17826t1 and LMG 17826t2. Their 16S rRNA gene sequences showed that t1 was identical to that held in LMG under accession LMG 6125T, whereas t2 was identical to that held in IAM under accession IAM 13588T. The 5 nt differences found between accessions D14514 and X67229 are probably due to sequencing errors in the older accession (D14514), or this accession corresponds to a currently unavailable strain (Fig. 1).

Years later, the housekeeping genes *recA* and *atpD* were sequenced for type strains of *M. loti* deposited in the LMG and USDA collections. For strain LMG 6125T, two accessions are available in GenBank that correspond to two phylogenetically divergent lineages of *recA-atpD*, one of them (GenBank accessions EU039868 and EU039875 for *recA* and *atpD*, respectively) coincides with strain NZP 2213T and the other (GenBank accessions AM076365 and AM076372 for *recA* and *atpD*, respectively) with strain USDA 3471T. This could be in agreement with the results of Willems et al. (2001b) because LMG could have distributed the two different colonies (t1 and t2) present...
in ATCC cultures. Therefore, the results of the housekeeping analysis confirmed that of the 16S rRNA gene sequence analysis in suggesting that, at the time of writing, different species are named M. loti.

Taking into account that the 16S rRNA gene sequences of strains deposited in LMG, IAM and ATCC were obtained before 1991 and we received the type strain ATCC 33669T in 1993, we also sequenced the 16S rRNA gene for strain ATCC 33669T received in our lab (we obtained it from one original lyophilized vial conserved in our lab since 1993, so no contamination due to subcultures was possible). Surprisingly, the sequence of our strain ATCC 33669T did not coincide with any sequences deposited for the type strains of the species M. loti; some of them corresponding to strain ATCC 33669T (see Fig. 1). Therefore, in 1993 the ATCC collection distributed a strain ATCC 33669T that was different to those previously available (accession numbers D14514, AJ315351 and AJ315352 previously mentioned).

This situation led us to analyse the sequences of several genes for the strains currently available in the NZP, USDA and LMG collections as well as our own strain, which we had received from ATCC in 1993 (the IAM collection is no longer available and so we were unable to include the type strain from this collection in this study). The results, based on phylogenetic and phenotypic studies, showed that strain NZP 2213T and the strain currently deposited under accession number LMG 6125T belong to the same species (M. loti), but strain USDA 3471T and the strain received from ATCC in 1993 (number ATCC 33669T) belong to two different species. This result was confirmed by DNA–DNA hybridization and phenotypic characterization and, as strain NZP 2213T has priority in the naming, according to the official description of R. loti (Jarvis et al., 1982), we propose the reclassification of strain USDA 3471T to a novel species named Mesorhizobium erdmanni sp. nov. and the strain received from ATCC in 1993, ATCC 33669T, to a novel species named Mesorhizobium jarvisi sp. nov.

The 16S rRNA gene sequences of strains NZP 2213T, USDA 3471T and ATCC 33669T were obtained according to Rivas et al. (2007a), those of the recA and atpD genes as described by Gaunt et al. (2001), that of the glnII gene as described by Vinuesa et al. (2005) and that of the nodC gene as described by Rivas et al. (2007b). All these sequences were aligned with those of all species of the genus Mesorhizobium using the CLUSTAL W program (Thompson et al., 1997). The distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). The phylogenetic trees were inferred using the neighbour-joining (NJ) and maximum-likelihood (ML) models (Saitou & Nei, 1987; Rogers & Swofford, 1998), which yielded similar results; only the results of NJ analysis are shown (Fig. 1). For all the phylogenetic analyses, MEGA 5.0 (Tamura et al., 2011) was used.

The results of 16S rRNA gene sequence analysis showed that the 16S rRNA gene sequence of strain NZP 2213T obtained in this study was identical to that of LMG 6125T [the sequence with accession number NR_025837 corresponding to strain NZP 2213T (= ATCC 33669T) has been curated by NCBI staff from the sequence with accession number D14514; this, as previously mentioned, could have errors or it could correspond to a strain that is currently unavailable] but that of strains USDA 3471T and ATCC 33669T occupied different branches in the phylogenetic trees obtained after NJ and ML analyses (Fig. 1). These branches formed a cluster with the type strains of Mesorhizobium opportunism WSM2075T and Mesorhizobium huakuii IFO 15243T with internal similarities near to 99.9 %.

High similarity between 16S rRNA gene sequences is a common finding among the species of the genus Mesorhizobium, which are clearly distinguishable on the basis of housekeeping gene analysis (Fig. 2). The concatenated sequences of the recA, atpD and glnII genes of strains NZP 2213T, USDA 3471T and ATCC 33669T obtained in this study were analysed, confirming that strains USDA 3471T and ATCC 33669T formed independent branches related to those occupied by M. opportunism WSM2075T and M. huakuii IFO 15243T, respectively (Fig. 2). Strain USDA 3471T showed 95.9, 97.5 and 93 % similarity, respectively, to the most closely related species, M. opportunism WSM2075T, and strain ATCC 33669T showed 95.9, 97.2 and 91 % similarity, respectively, with respect to its most closely related species, M. huakuii IFO 15243T. Such distances are commonly found among different species of the genus Mesorhizobium, suggesting that strains USDA 3471T and ATCC 33669T, previously designated as type strains of M. loti, actually each represent a novel species within this genus (see Fig. 2).

This was confirmed by DNA–DNA hybridization experiments carried out following the method of Ezaki et al. (1989) with the recommendations of Willems et al. (2001a). Strain USDA 3471T was hybridized with M. opportunism WSM2075T and strain ATCC 33669T with M. huakuii IFO 15243T and showed 50 % (± 9 %) and 54 % (± 6 %) DNA–DNA relatedness, respectively. Both values are lower than the threshold value of 70 % DNA–DNA relatedness for delineation of bacterial species (Wayne et al., 1987), indicating that strains USDA 3471T and ATCC 33669T each belong to a separate novel species of the genus Mesorhizobium.

DNA for analysis of 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 G+C contents of the DNA of strains USDA 3471T and ATCC 33669T were 61.5 and 62.7 mol%, respectively.

Phenotypic characterization was performed using API 20NE and API 32GN galleries (bioMérieux) inoculated according to the manufacturer’s instructions with sterile MgSO4.7H2O added to the supplied medium up to a concentration of 0.2 g l−1 with the aid of a disposable Pasteur pipette. We also used API 50CH galleries (bioMérieux) inoculated with suspensions of each strain in a basal medium containing 0.2 g l−1 NaH2PO4, 0.2 g l−1 MgSO4, 0.1 g l−1 NaCl, 0.8 g l−1 yeast extract and 0.04 g l−1 bromocresol purple, adjusted to
pH 7 (Ramírez-Bahena et al., 2012). The results were read after 2 weeks of incubation at 28 °C for API 50CH and 7 days for API 20NE and API ID32GN. The temperature range for growth was determined by incubating cultures in yeast mannitol agar (YMA) medium at 4, 15, 28, 37 and 45 °C. The pH range for growth was determined in the same medium with final pH values of pH 4, 6, 7, 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. To test natural antibiotic resistance, the disc diffusion method on YMA medium was used. The discs contained the following antibiotics: ampicillin (2 μg), erythromycin (2 μg), ciprofloxacin (5 μg), penicillin (10 IU), polymyxin (300 IU), clavulanic (1 μg), oxteracycline (30 μg), gentamicin (10 μg), cephaloxine (30 μg) or neomycin (5 μg) (Becton Dickinson, BBL). The type strains M. opportunristum WSM2075T and M. huakuii IFO 15243T were included in the phenotypic study as reference strains. 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 Mesorhizobium are recorded in Table 1.

Although symbiotic genes do not offer taxonomic information because they are located in easily interchangeable elements (plasmids or symbiotic islands), the analysis of the nodC gene allowed the identification of strains at the symbiovar level (Peix et al., 2015). The analysis of this gene confirmed that strains USDA 3471T and ATCC 33669T are closely related to the type strain M. loti NZP 2213T, which belongs to the same symbiovar (Fig. S1, available in the online Supplementary Material).

Fig. 2. Neighbour-joining phylogenetic tree based on concatenated recA and glnD1 gene sequences showing the position of USDA 3471T and ATCC 33669T within the genus Mesorhizobium. Bootstrap values calculated for 1000 replications are indicated at nodes. Bar, 1 nucleotide substitution per 100 nt.

The results from this study showed that strains USDA 3471T and ATCC 33669T each represent a novel species for which we propose the names Mesorhizobium erdmanii sp. nov., and Mesorhizobium jarvisii sp. nov., respectively.


The characteristics of this species are as described by Jarvis et al. (1982) and Jarvis et al. (1997), but those of the strain deposited in the ATCC collection, ATCC 33669, included...
Table 1. Phenotypic differences between USDA 3471T, ATCC 33669T and their most closely related species, *M. opportunistum* WSM2075T and *M. huakuii* IFO 15243T, and the type strain of the type species of the genus, *M. loti* NZP 2213T.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 37 °C</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth in 2% (w/v) NaCl</td>
<td>–</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Assimilation of (API 20NE):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gluconate</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Malate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Assimilation of (API ID32GN):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valerate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Alanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acid from (API 50CH):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
<td>w</td>
<td>w</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adonitol</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Galactose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Mannose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Mannitol</td>
<td>w</td>
<td>w</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>L-Arabitol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Resistance to:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Penicillin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

in the original description (Jarvis et al., 1982) no longer conform to the species description.

Description of *Mesorhizobium erdmanii* sp. nov.

*Mesorhizobium erdmanii* [erd.man’i.i. N.L. masc. gen. n. *erdmanii* named after L. W. Erdman (deceased), who made valuable contributions to rhizobia research].

Gram-stain-negative, aerobic rods. Colonies on YMA are white, circular and convex with a diameter of 1–2 mm within 4–5 days at 28 °C. Grows between 10 °C and 38 °C and optimally at 28 °C. The pH range for growth is pH 5 to 8 with optimum growth at pH 7. Grows with up to 1% (w/v) NaCl. In API 20NE, nitrate reduction, arginine dehydrodase and gelatinase are negative and urease and β-galactosidase are positive. Aesculin hydrolysis is positive. Assimilation of glucose, L-arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate and malate is positive. Assimilation of caprate, adipate, citrate and phenylacetate is negative. In API ID32GN, L-rhamnose, *N*-acetylglucosamine, D-ribose, inositol, sucrose, maltose, mannitol, glucose, melibiose, L-fucose, D-sorbitol, L-arabinose, L-alanine, L-histidine and L-proline are assimilated. Itaconate, suberate, malonate, propionate, caprate, valerate, citrate, 2- and 5-ketogluconate, glycerogen, 3- and 4-hydroxybenzoate, 3-hydroxybutyrate and L-serine are not assimilated. Salicin, acetate and DL-lactate are assimilated weakly. In API 50CH, acid production from D-arabinose, L-arabinose, D-ribose, D-xylose, L-xyllose, D-lyxose, D-fucose and L-fucose is positive. Acid from glycerol, D-adonitol, D-glucose, D-rhamnose, D-mannitol and *N*-acetylglucosamine is weakly positive. Acid production from erythritol, methyl L-d-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, inositol, D-sorbitol, dulcitol, methyl L-d-mannopyranoside, methyl L-d-glucopyranoside, amygdalin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xyitol, gentiobiose, turanose, D-tagatose, D-arabitol and L-arabitol is negative. In the same system, assimilation of esculin is positive and that of arbutin is weakly positive. Alkali production from potassium gluconate, and from 2- and 5-ketoglucconate is negative. Sensitive to ciprofloxacin, neomycin, ampicillin, gentamicin, cefuroxime, penicillin and tetracycline and resistant to cloxacillin, polymyxin B and erythromycin.

The type strain USDA 3471T (=CECT 8631T=LMG 17826T) was isolated from root nodules of *Lotus corniculatus*. The G+C content of the DNA of the type strain is 61.5 mol%.

Description of *Mesorhizobium jarvisii* sp. nov.

*Mesorhizobium jarvisii* (jar.vis’i.i. N.L. masc. gen. n. *jarvisii* named after B. D. W. Jarvis, who made valuable contributions in *Mesorhizobium* research).

Gram-stain-negative, aerobic rods. Colonies on YMA are white, circular and convex with a diameter of 1–2 mm within 4–5 days at 28 °C. Grows between 10 °C and 38 °C and optimally at 28 °C. The pH range for growth is pH 5 to 8 with optimum growth at pH 7. In API 20NE, nitrate reduction, arginine dehydrodase and gelatinase are negative and urease and β-galactosidase are positive. Aesculin hydrolysis is positive. Assimilation of glucose, L-arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate and malate is positive. Assimilation of caprate, adipate, citrate and phenylacetate is negative. In API ID32GN, L-rhamnose, *N*-acetylglucosamine, D-ribose, inositol, sucrose, maltose, mannitol, glucose, melibiose, L-fucose, D-sorbitol, L-arabinose, L-alanine, L-histidine, valerate and L-proline are assimilated. Itaconate, suberate, malonate, caprate, citrate, 2- and 5-ketoglucconate, glycerogen, 4-hydroxybenzoate and L-serine are not assimilated. Salicin, propionate, acetate, D-lactate, 3-hydroxybutyrate and 3-hydroxybenzoate are assimilated weakly. In API 50CH, acid production from glycerol, D-arabinose, L-arabinose, D-ribose, D-xyllose, L-xyllose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, L-d-xylose, D-fucose and L-fucose is positive. Acid production from D-sorbitol and *N*-acetylglucosamine is weakly positive. Acid from erythritol, methyl L-d-xylopyranoside, L-sorbose,
l-rhamnose, dulcitol, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, salicin, celllobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xyitol, gentiobiose, turanose, D-tagatose, D-arabitol and L-arabitol is negative. In the same assimilation of ascorbin is positive and that of arbutin is weakly positive. Alkali production from potassium gluconate, and from 2- and 5-ketogluconate is negative. Sensitive to ciprofloxacin, neomycin, ampicillin, gentamicin, cefuroxime, cloxacillin, polymyxin B, penicillin and tetracycline and resistant to erythromycin.

The type strain ATCC 33669T (\textsuperscript{1} = CECT 8632\textsuperscript{T}, LMG 28313\textsuperscript{T}) was isolated from root nodules of Lotus corniculatus. The G+C content of the DNA of the type strain is 62.7 mol%.

Acknowledgements

This work was supported by JCYL and MICINN (Spanish Governments) grants to E.V., E.M.-M. and R.R. and by a MAGRAMA grant to M.L.-B. J.D.-F.-F. is recipient of a pre-doctoral fellowship from Universidad de Salamanca. M.H. R.-B. is recipient of a JAE-Doc researcher contract from CSIC.

References


