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TAXONOMIC DESCRIPTION
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Mesorhizobium sanjuanii sp. nov., isolated from nodules of *Lotus tenuis* in the saline-alkaline lowlands of Flooding Pampa, Argentina

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Abstract

Two rhizobial strains, BSA136^T and BSA150, related to the genus *Mesorhizobium* were isolated from root nodules of *Lotus tenuis* grown in saline-alkaline lowlands soil from Argentina. These strains showed different repetitive element palindromic PCR fingerprinting patterns but shared more than 99 % sequence similarity for both 16S rRNA and *recA* genes. Despite the symbiotic *nodC* gene sequences of our strains being related to the canonical *Lotus* biovar species comprising *Mesorhizobium loti* and *Mesorhizobium japonicum*, the 16S rRNA phylogenetic marker suggests that their taxonomical identities are closely related to *Mesorhizobium helmanticense*, *Mesorhizobium metallidurans*, *Mesorhizobium thianshanense*, *Mesorhizobium gobiense* and *Mesorhizobium tarimense*. Multilocus sequence analysis performed with seven housekeeping genes confirmed that BSA136^T belongs to a separate clade within the genus *Mesorhizobium*. The results of comparisons for *in silico* DNA–DNA hybridization and average nucleotide identity indexes between the genomes of BSA136^T and closest-related *Mesorhizobium* species were below the threshold for species delineation. Phenotypic features differentiated BSA136^T from its closest-related species. On the basis of our results, BSA136^T and BSA150 can be considered to represent a novel species of the genus *Mesorhizobium*, for which the name *Mesorhizobium sanjuanii* sp. nov. is hereby proposed. The type strain of this species is BSA136^T (=CECT 9305^T=LMG 30060^T), for which the draft genome sequence is available.

Lotus is a genus of legume plants with some species being used as pasture-forage in North and South America, Europe and New Zealand [1–3]. Its wide forage potential is partly due to its high N_2-fixing capacity in association with rhizobia. *Mesorhizobium loti* has traditionally been recognized as the typical species establishing effective symbiosis with a group of *Lotus* species, which include *Lotus tenuis*, *Lotus corniculatus*, *Lotus japonicus* and *Lotus filicaulis* [4, 5]. Particularly, *L. tenuis* grows in diverse environments, even under stressed soil conditions such as flooding, drought, salinity and alkalinity [6].

During a survey of rhizobial strains nodulating *L. tenuis* growing in three types of soils from Argentina, 77 unique isolates mostly associated with the genus *Mesorhizobium* were obtained. A total of 24 strains isolated from saline-alkaline soils were classified in four ribogroups after 16S rRNA restriction fragment length polymorphism (RFLP) analysis [7]. Interestingly, none of them appeared to be taxonomically related to the canonical *L. tenuis* symbiont, *M. loti*. Strains BSA136^T and BSA150 belong to the most abundant RFLP ribogroup of isolates from high salt and alkali soil conditions (Chascomús, Buenos Aires, Argentina; 35° 35′ S, 58° 00′ W; 2004).

DNA from the two isolates was extracted and purified using an AccuPrep Genomic DNA Extraction Kit (Bioneer) according to the manufacturer's guidelines. Repetitive element palindromic PCR (rep-PCR) fingerprint profiling and 16S rRNA and *recA* gene sequencing was carried out

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Keywords: *Mesorhizobium*; *Lotus tenuis*; new species.
Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; HSP, high-scoring segment pair; rep-PCR, repetitive element palindromic PCR; RFLP, restriction fragment length polymorphism; TY, triptone–yeast.
The draft genome of *Mesorhizobium sanjuanii* sp. nov. strain BSA136^T is available under the accession number: NWQG00000000.1. GenBank accession numbers for the 16S rRNA gene sequences for *M. sanjuanii* BSA136^T and BSA150 strains are MF979853 and MF979852, respectively. Those for *recA* gene sequences are MF991136 and MF991135, respectively, and for the partial *nodC* gene sequences are MF991138 and MF991137, respectively.
One supplementary table and two supplementary figures are available with the online version of this article.

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as previously described [7]. BSA136^T and BSA150 showed different rep-PCR fingerprinting patterns (Fig. S1, available in the online version of this article) but shared 100 and 99.53 % sequence similarity to each other for both 16S rRNA and *recA* genes, respectively. To establish the phylogenetic position of the strains within the genus

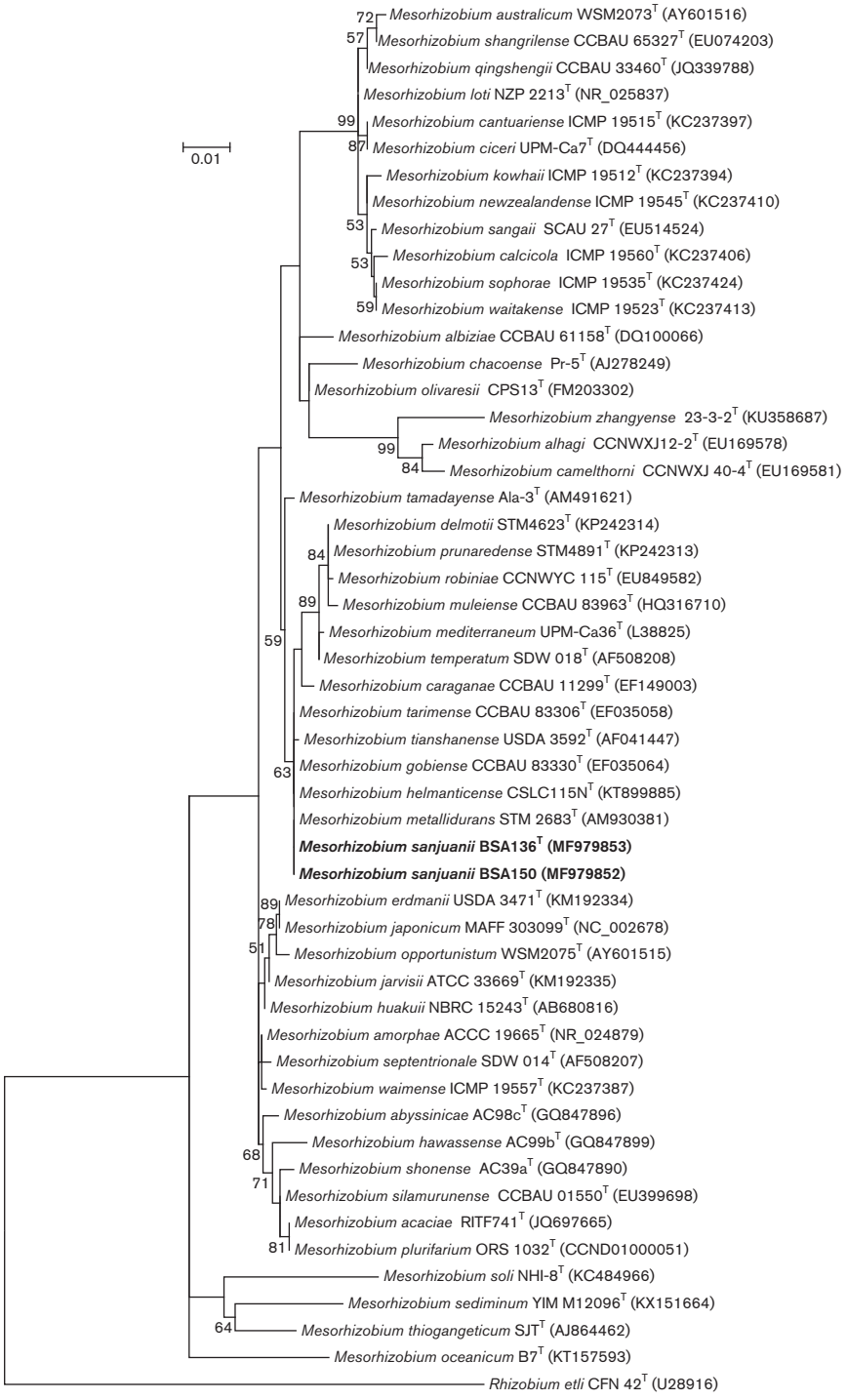

Fig. 1. Maximum-likelihood phylogenetic tree based on *Mesorhizobium* type strains. 16S rRNA gene sequences (1270 nucleotides) showing the position of *Mesorhizobium sanjuanii* sp. nov. used in this study (indicated in bold). The tree was reconstructed using Kimura's two-parameter (G+I) model. Bootstrap values (above 50 %) calculated for 1000 replications are indicated at the nodes. GenBank accession numbers are in parentheses. Bar, 1 substitution per 100 nucleotide positions.

Mesorhizobium, the 16S rRNA and *recA* gene sequences of the two strains were aligned with the corresponding sequences of rhizobia obtained through the Ribosomal Database Project Release 11.5 [8] and the National Center for Biotechnology Information (NCBI) Genbank Database using the CLUSTAL module implemented by the MEGA software version 7.0 [9]. Distances were calculated by using a pairwise-deletion procedure and the maximum-likelihood method was used to reconstruct phylogenetic trees by means of the MEGA7 software. The robustness of the tree topologies was evaluated by bootstrap analysis (1000 replicates). The *L. tenuis*-nodulating strains BSA136^T and BSA150 were found to cluster together with *Mesorhizobium metallidurans*, *Mesorhizobium helmanticense*, *Mesorhizobium thianshanense*, *Mesorhizobium gobiense* and *Mesorhizobium tarimense* (Fig. 1), confirming that these strains are not related to the *Lotus* canonical microsymbionts, *M. loti* and *Mesorhizobium japonicum*, as previously suggested by the 16S rRNA RFLP results [7]. Moreover, comparison of 16S rRNA sequences revealed similarity values of 99.8 % to *M. metallidurans* STM 2683^T, *M. helmanticense* CSLC 115N^T and *M. gobiense* CCBAU 83330^T, 99.7 % with *M. tarimense* CCBAU 83306^T and 99.6 % with *M. thianshanense*

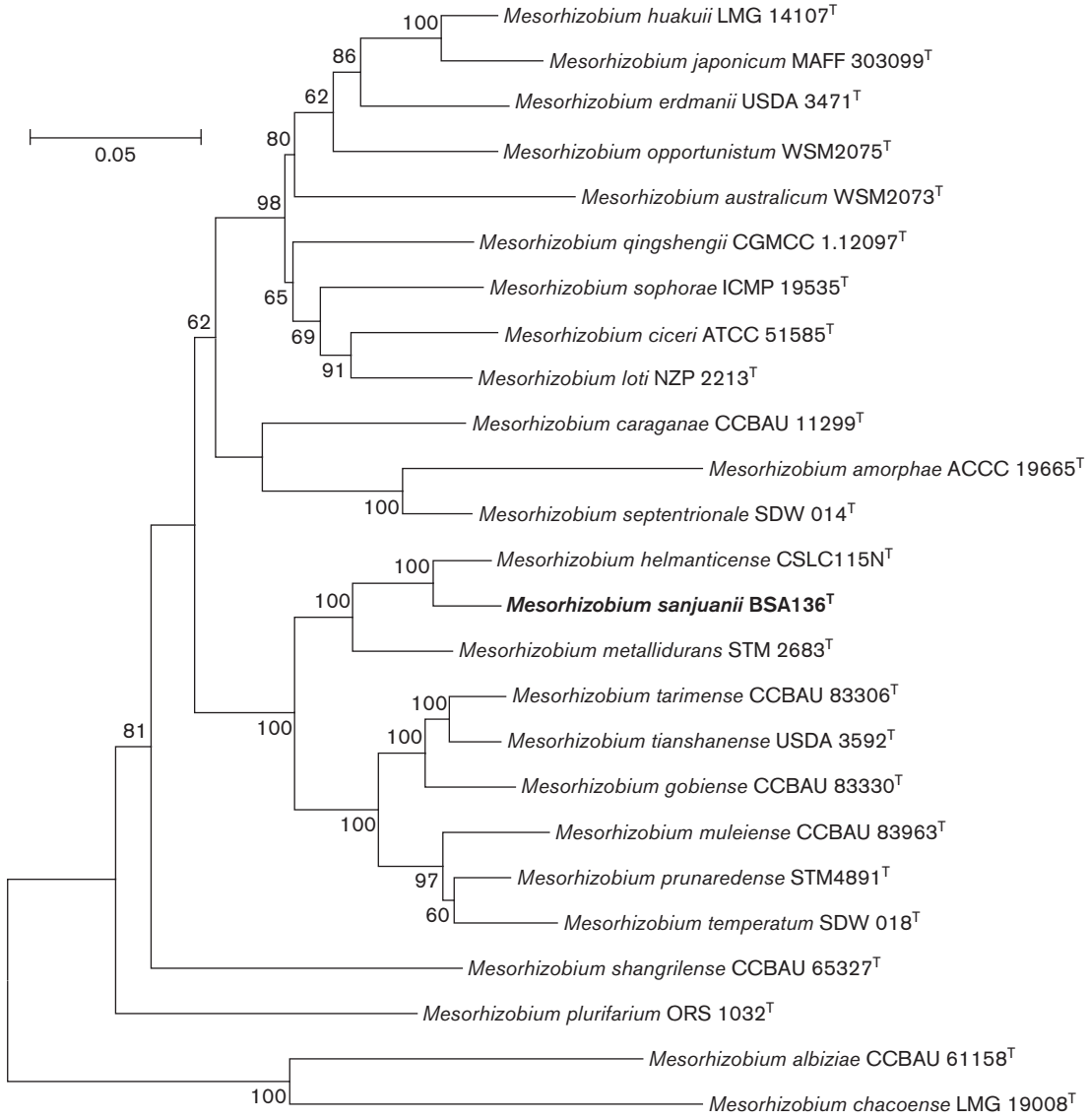

Fig. 2. Maximum-likelihood phylogenetic tree based on concatenated *dnaK* (223 nt), *glnII* (405 nt), *gyrB* (578 nt), *recA* (298 nt), *rpoB* (441 nt), *thrA* (668 nt) and *truA* (332 nt) gene sequences showing the position of *Mesorhizobium sanjuanii* sp. nov. used in this study (indicated in bold) within the genus *Mesorhizobium*. Genbank accession numbers are detailed in Table S1. The tree was reconstructed using the general time reversible (G+I) model. Bootstrap values (above 50 %) calculated for 1000 replications are indicated at the nodes. Bar, 5 % sequence divergence.

USDA 3592^T. In turn, the *recA* phylogenetic analysis groups strains BSA136^T and BSA150 in a separate cluster (Fig. S2), with 96.37 and 95.15 % sequence similarity to *M. helmanticense* CSLC 115N^T and *M. metallidurans* STM 2683^T, respectively. These levels of similarity are below the proposed threshold range for the species boundary, 98.2–99.0 % [10–12].

Among the two newly isolated strains, BSA136^T was designated as a representative for an extended characterization. A polyphasic taxonomic approach, including *in silico* whole genome comparisons, analysis of housekeeping and nodulation genes, morphological/phenotypic features and major fatty acids profiles, was followed according to the suggested recommendations regarding the description of new species [13]. For genomic comparisons, the genome of BSA136^T strain was sequenced. For total DNA extraction, the strain was cultured in triptone–yeast (TY) extract broth to early stationary phase, harvested by centrifugation and DNA was obtained using an AccuPrep Genomic DNA Extraction Kit (Bioneer). An Illumina 300 bp insert standard shotgun library was reconstructed and sequenced using the Illumina HiSeq 2000-1 TB platform. The resulting reads were quality trimmed [14] and the draft genome was assembled using gsAssembler (version 2.8). The obtained genome size was 6.4 Mbp, the DNA G+C ratio was 62.51, the number of contigs was 343, N50 was 64.3 and the sequencing depth of coverage was 14.66. The reference genome sequences used for whole genome comparisons were retrieved from NCBI GenBank.

Phylogenetic trees are commonly used to elucidate systematic relationships between different species. Although the 16S rRNA gene is widely used as a taxonomic marker, it often cannot appropriately support species delineation, such the case of the rhizobial species [15]. For this reason, multilocus sequence analysis based on concatenated housekeeping genes is preferred to assign and identify rhizobial species. The selection and use of housekeeping genes for taxonomic purposes is a critical step forward and the *ad hoc* committee for re-evaluation of species definition has suggested the use of a minimum of five housekeeping genes [13]. Seven protein-coding genes, *recA*, *glnII*, *dnaK*, *rpoB*, *gyrB*, *truA* and *thrA*, were previously shown to produce a robust phylogeny of the genus *Mesorhizobium* [16]. Rhizobial reference sequences for all the housekeeping genes were collected from the NCBI Genbank database and aligned with the corresponding sequences of BSA136^T. Sequences for each gene in a given species were concatenated and the MEGA7 package was used to infer the molecular phylogeny by using the maximum-likelihood method based on a matrix with the distance correction calculated by the general time reversible model with 1000 resamplings in the bootstrap analysis. The phylogenetic tree based on the concatenated sequences revealed that the novel strain BSA136^T belongs to a monophyletic cluster with 100 % bootstrap support (Fig. 2). Sequence similarities between BSA136^T and most closely phylogenetically related species, *M. helmanticense* CSLC 115N^T and *M. metallidurans* STM 2683^T, have been calculated for the seven housekeeping genes (Table 1) and values below the threshold for species delineation (98.2–93.1 % similarity) have been observed.

Table 1. Housekeeping genes sequence similarities (%) between *M. sanjuanii* BSA136^T and closest phylogenetically related species

Housekeeping gene	*M. helmanticense* CSLC115N^T*	*M. metallidurans* STM2683^T†
dnaK	98.12	96.97
glnII	94.13	93.10
gyrB	97.45	95.15
recA	96.37	96.90
rpoB	98.19	97.29
thrA	96.72	94.52
truA	95.69	95.69

*GenBank accession number: CAUM00000000.1.

†GenBank accession number: PZJX00000000.1.

Average nucleotide identity (ANI) has recently been accepted as an alternative to the traditional DNA–DNA hybridization (DDH) method and similarity values >95–96 % are considered as the threshold to determine if two bacterial strains belong to the same species [17]. ANI calculations between strain BSA136^T and its closest type strains, *M. helmanticense* CSLC 115N^T [18] and *M. metallidurans* STM 2683^T [19], were performed using the JSpeciesWS (http://jspecies.ribohost.com/jspecies ws) web server [20] based on pairwise alignment of the genomes using the BLAST+ (ANIb) or MUMmer (ANIm) tools. ANIb of 93.92 and 90.26 % and ANIm values of 94.90 and 92.02 % resulted from comparison with *M. helmanticense* CSLC 115N^T and *M. metallidurans* STM 2683^T genomes, respectively. Therefore, values obtained for the two indexes were consistent and below the proposed cut-off for species boundary [17]. *In silico* DDH values were determined online using the Genome-to-Genome Distance Calculation (GGDC 2.1) service (http://ggdc.dsmz.de/distcalc2.php) as described by Meier-Kolthoff *et al.* [21] using the recommended BLAST+ method. Results of the GGDC are based on the recommended formula 2 [sum of all identities found in high-scoring segment pairs (HSPs) divided by overall HSP length], which is independent of genome length and is thus robust against the use of incomplete draft genomes [21, 22]. *In silico* DDH values were 58.8 and 44.8 % for comparisons between the BSA136^T strain and the type strains of the *M. helmanticense* and *M. metallidurans* genomes, respectively; values that are well below the threshold of 70 % for species delineation. Taken together, these results confirm that the newly isolated strains belong to a new species in the genus *Mesorhizobium*.

Although housekeeping genes are useful for the establishment of the rhizobial taxonomic status, they do not offer information regarding the bacterial symbiotic behaviour in terms of the legume hosts. The analysis of the symbiotic

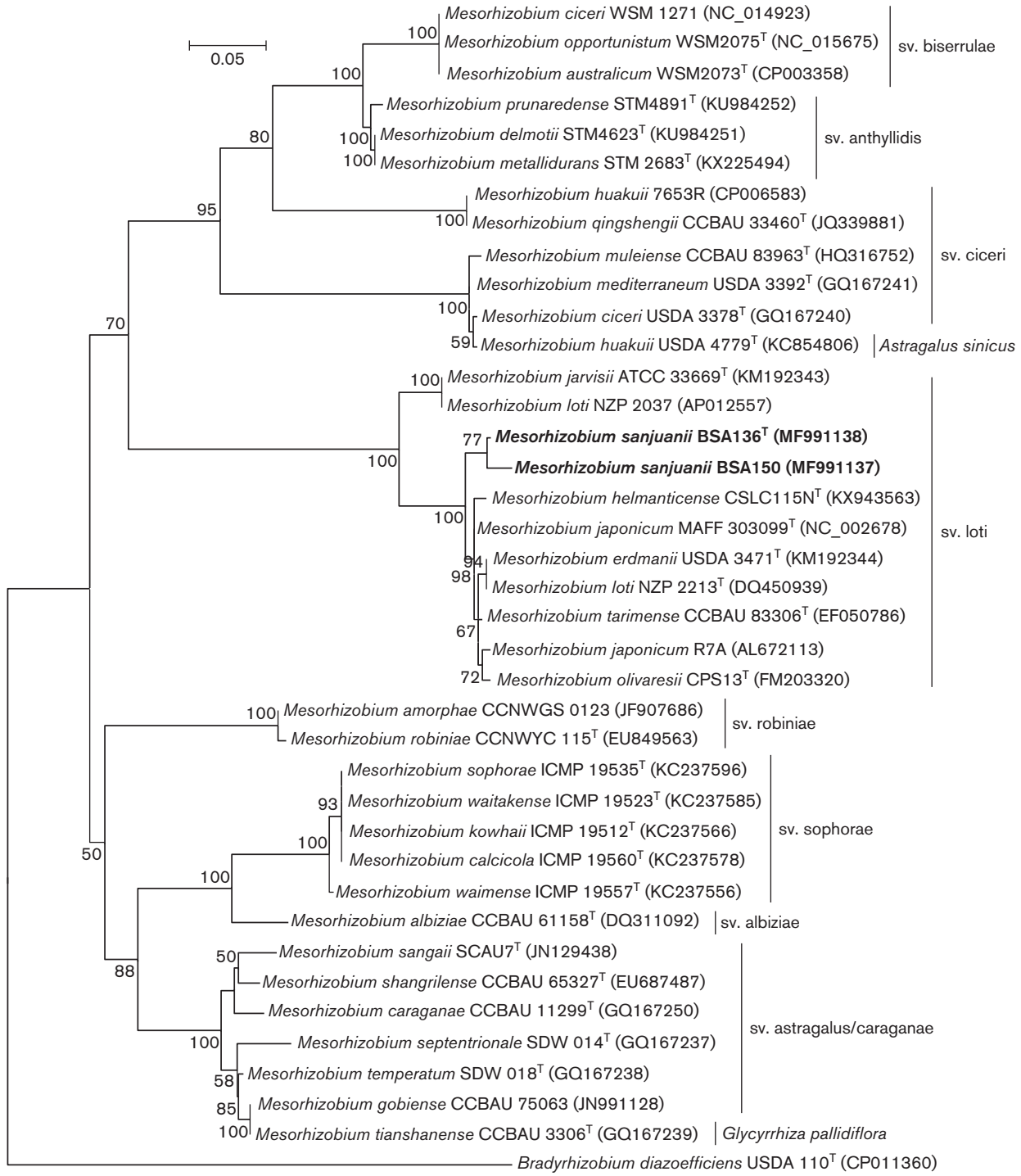

Fig. 3. Maximum-likelihood tree based on partial sequences of *nodC* gene (383 nucleotides) showing the relationships between *Mesorhizobium sanjuanii* sp. nov. (in bold) and other *Mesorhizobium* strains, indicating the different symbiovars (sv.) defined within the genus. The tree was reconstructed using the Tamura 3 (G+I) model. Bootstrap values (above 50 %) calculated for 1000 replications are indicated at the nodes. Bar, five substitutions per 100 nucleotides. Original host legumes are given for unknown symbiovars.

gene *nodC*, located in interchangeable elements (plasmids or symbiotic islands), has been proposed for the identification of strains at symbiovar level [15, 23, 24]. The *nodC* sequences for the strains BSA136^T and BSA150 were compared with other *Mesorhizobium nodC* genes in a maximum-likelihood tree using the Tamura 3 (G+I) model

(Fig. 3). Both strains, isolated from *L. tenuis* nodules, grouped with the reference strains belonging to the symbiovar loti, confirming the capacity of different *Mesorhizobium* species to share a same symbiovar [24–26]. Interestingly, BSA136^T branched separately (100 % bootstrap support) from the strains isolated from other *Lotus* species (such as *L. corniculatus*, *L. japonicus* and *Lotus divaricatus*), with similarity values in this cluster ranging from 97.7 % with *Mesorhizobium erdmanii* USDA 3471^T to 93.2 % to *M. loti* NZP 2037.

Phenotypic features of the strain BSA136^T were determined using the API 20NE (bioMérieux) and BIOLOG GENIII (Biolog) kits following the manufacturers' instructions. Distinctive features of BSA136^T compared with representative strains of the most phylogenetically related *Mesorhizobium* species are depicted in Table 2.

Chemotaxonomic characterization was performed on a BSA136^T culture grown aerobically on TY extract agar plates at 28 °C for 48 h. The cellular fatty acids were extracted and analysed according to the recommendations of the commercial Microbial Identification System (MIDI) and whole-cell fatty acid composition was determined by gas chromatography (Agilent Technologies 6890N) using the peak-naming table MIDI TSBA 5.0. The fatty acid profile of strain BSA136^T comprised $C_{18:1}\omega7c$ (37.6 %), $C_{12:0}$ (19.7 %), $C_{16:0}$ (11.1 %), $C_{16:1}$ ISO H (7.9 %), $C_{18:1}\omega7c$ 11-methyl (7.5 %), $C_{16:0}$ N OH (6.9 %), $C_{19:0}$ CYCLO $\omega8c$ (5.2 %) and $C_{17:0}$ ISO (4.1 %).

Table 2. Distinctive features of *Mesorhizobium sanjuanii* sp. nov. and the closest type species of the genus *Mesorhizobium*

Strains: 1, BSA136^T (this study); 2, *M. helmanticense* CSLC 115N^T (this study); 3, *M. metallidurans* LMG 24485^T [27]. +, Positive; −, negative; ±, weakly positive; ND, not determined.

Species	1	2	3
Assimilation of:			
Cellobiose	+	−	+
D-Fructose	+	−	+
D-Fucose	+	±	−
D-Galactose	+	−	+
Turanose	+	−	+
L-Aspartate	+	−	−
L-Fucose	+	±	−
L-Malate	+	−	+
L-Alanine	+	−	+
Lactate	±	+	−
Acetic acid	+	−	ND
Propionic acid	+	−	+
γ-Amino-butiric acid	+	−	+
D-Galacturonic acid	+	−	+
Growth conditions:			
pH 5	−	+	+
10 °C	−	+	+

The genotypic and phenotypic features described in this work reveal that the strain BSA136^T isolated from *L. tenuis* nodules represents a novel species within the *Mesorhizobium* clade for which the name *Mesorhizobium sanjuanii* sp. nov. is hereby proposed.

DESCRIPTION OF *MESORHIZOBIUM SANJUANII* SP. NOV.

Mesorhizobium sanjuanii (san.ju.a′ni.i N.L. gen. masc. n. *sanjuanii*, named after Dr Juan Sanjuán Pinilla of the Zaidin Experimental Station, Granada, Spain, for his valuable contribution to the development of rhizobial research in Spain and Latin America).

Gram-negative, aerobic, non-spore-forming rods. Colonies appearing on yeast extract–mannitol agar within 3–5 days incubation at 28 °C are circular, opaque, convex and cream-coloured. Generation time ranges between 5–7 h when grown in TY broth at 28 °C and 150 r.p.m.

The optimum temperature for aerobic growth is 28 °C, no growth is observed at 10 or 37 °C; all strains tolerate 1 % NaCl and grow over a pH range of pH 7–10. Strains are resistant to ampicillin, gentamicin and neomycin, susceptible to tetracyclin, cloramphenicol and kanamycin. Utilizes arabinose, D-fructose, D-fucose, D-fructose 6-phosphate, D-aspartic acid, maltose, trehalose, cellobiose, sucrose, lactose, methyl β-D-glucoside, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, *N*-acetyl-D-galactosamine, D-glucuronic acid, mucic acid, citric acid, α-D-glucose, D-mannose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glicerol, glycyl-L-proline, L-aspartic acid, L-alanine, L-glutamic acid, L-pyroglutamic acid, L-lactic acid, D-galacturonic acid, D-galactonic acid lactone, pectin, D-gluconic acid, quinic acid, α-ketobutyric acid, acetoacetic acid, propionic acid, acetic acid, formic acid, D-lactic acid methyl ester, α-ketoglutaric acid, L-malic acid, Br-succinic acid, gelatin and Tween 40 as asole carbon source. No growth is observed with dextrin, D-serine, L-serine, L-pyroglutamic acid, L-histidine, methyl piruvate, p-hydroxyphenylacetic acid, adipic acid, capric acid or potassium gluconate. No reduction of nitrates to nitrite or nitrates to nitrogen is observed. No indol production or arginine dihydrolase, gelatinase and β-galactosidase activities is detected. Urease activity and aesculin hydrolysis are positive. The most abundant fatty acids are $C_{18:1}\omega7c$, $C_{12:0}$ and $C_{16:0}$. The DNA G+C content of the type strain is 62.51 mol%.

The type strain, BSA136^T (=CECT 9305^T=LMG 30060^T), was isolated from nodules of *Lotus tenuis*.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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