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Neorhizobium tomejilense sp. nov., first non-symbiotic *Neorhizobium* species isolated from a dryland agricultural soil in southern Spain

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ABSTRACT

We describe for the first time a non-symbiotic species of the recently described genus *Neorhizobium*, lacking nodulation or nitrogen fixation genes. The strains were isolated from a dryland agricultural soil in southern Spain where no record of legume cultivation is available, thus we propose the name *Neorhizobium tomejilense* sp. nov. (type strain T17_20$^{\mathrm{T}}$, LMG 30623$^{\mathrm{T}}$ and CECT 9621$^{\mathrm{T}}$). *N. tomejilense* exhibit a clear distinct lineage from the other *Neorhizobium* species, *Neorhizobium galegae*, *Neorhizobium alkalisoli* and *Neorhizobium huautlense*, based on polyphasic evidence. Phylogenetic marker analysis of 16S rDNA, *atpD*, *glnII*, *recA*, *rpoB* and *thrC* genes and genomic identity data derived from the draft genomic sequences showed that *N. tomejilense* strains clearly separated from the other *Neorhizobium* species and that *N. galegae* represents the closest species, with Average Nucleotide Identities (ANIb) ranging from 90% (for type strain HAMBI 540$^{\mathrm{T}}$) to just under 95.0% (for two *N. galegae* sv. officinalis strains). Genomes from *N. galegae* and *N. tomejilense*, however, clearly differed in important traits, such as the number of rRNA operon copies or the number of tRNAs. Phenotypic characterisation of *N. tomejilense* also displayed differences with the other *Neorhizobium* species. Whole-cell matrix-assisted laser-desorption time-of-flight mass spectrometry (WC MALDI-TOF-MS) fingerprint analysis and the dendrogram derived from the fingerprint profiles, showed a clearly distinct group formed by the three *N. tomejilense* isolates (T17_20$^{\mathrm{T}}$, T20_22 and T11_12) from the other *Neorhizobium* especies.

Introduction

Rhizobia are a diverse group of soil α- and β-Proteobacteria able to establish endosymbioses with plants belonging to the *Fabaceae* (legumes) group. These symbioses, usually very specific, takes place in a specialised developed organ, the nodule, where rhizobia carry out the nitrogen fixation. Legume plants are able to feed from the nitrogen fixed by the rhizobia, which makes them independent from any external fixed nitrogen supply. The genus *Neorhizobium* is a recently described genus formed by three former *Rhizobium* species; *Neorhizobium alkalisoli*, *Neorhizobium galegae* and *Neorhizobium huautlense*, highly related to the *Rhizobium* and *Agrobacterium* genus [1]. *Neorhizobium* sp. members up to this work are, *N. galegae* isolated from root nodules of *Galega orientalis* but is also able to form nodules on *Galega officinalis* [2] comprising two symbiovars (sv) highly specific to their host in *Galega* plants. The type species of the genus, *N. galegae* HAMBI 540 was isolated from *G. orientalis*. *N. huautlense*, isolated from the root nodules of *Sesbania herbacea* in Mexico [3] and *N. alkalisoli* from root nodules of *Caragana intermedia* grown in saline-alkaline soils in the north of China [4]. We previously reported the isolation of non-symbiotic members of the genus *Neorhizobium* from a dryland agricultural soil in southern Spain. Genotypic and genomic characterization of these isolates suggested that they contained representatives of one, or perhaps two, new genomospecies within *Neorhizobium* [5]. In this work, a subset of strains from the most abundant of these novel genotypes was further characterized, including genome sequencing to draft level. Genomic, phylogenetic and phenotypic data obtained from these strains support the definition of a new *Neorhizobium* species, for which the name *Neorhizobium tomejilense* sp. nov. is proposed.

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Materials and methods

Isolation and identification

Neorhizobium isolation and identification was carried out as detailed in Soenens and Imperial [5]. Briefly, MNBP semi-selective medium [6] was used to enrich *Rhizobium-Neorhizobium* from soil followed by a selection of Luria-Broth (LB) [7] negative colonies. MNBP positive/Luria-Broth (LB) negative colonies were selected and screened for a rhizobium chromosomal marker, *fnrN*. *Neorhizobium* strains do not contain an *fnrN* gene. However, as described by Soenens and Imperial [5], a PCR band, unrelated to *fnrN*, could be amplified by PCR from the DNA of many *Neorhizobium* isolates. Positive colonies for *fnrN* were characterized taxonomically using 16S rDNA and *rpoB* gene and symbiotic potential was determined with the amplification and sequencing of the genes related to symbiosis *nodC* and *nifH.*

Phylogenetic analyses

For phylogenetical analyses, sequences were aligned with the ClustalW algorithm [8] in MEGA 6.0 [9]. Neighbour Joining (NJ), Maximum-likelihood (ML) and Maximum parsimony (ML) phylogenetical analyses were also done in MEGA 6.0 with bootstrap analyses based on 1000 subsets.

Genome sequencing and ANI values

Draft genome sequences for strains T20_22, T17_10^{T} and T11_12 and for type *Neorhizobium* strains not available in public databases, *N. huautlense* DSM 21817^{T} and *N. alkalisoli* DSM 21817^{T}, were obtained in this work. To this end, bacteria were grown in Tryptone Yeast (TY) [10] and the pellet obtained was used to extract total DNA using the CTAB method [11]. DNA quantification and quality were measured in Nanodrop (NanoDrop Technologies, Wilmington, USA) and Qubit (Invitrogen by Life Technologies Holdings Pte. Ltd, Singapore) instruments, and integrity and purity were checked by electrophoresis through a 0.8% agarose gel. Bacterial strains were sequenced externally (MicrobesNG, Birmingham, UK) with Illumina MiSeq 2 × 250 bp, and reads were assembly using SPAdes [12] and annotated with Prokka [13]. For Average Nucleotide Identity (ANI) calculation, ANI-MUMmer (ANIm, data not shown) and ANI-blast (ANIb) were calculated using JSpeciesWS [14].

Phenotypic characterization

The GEN III MicroPlates^{TM} (Biolog, Inc.) system was used for phenotypic characterization of carbon and nitrogen sources utilization and sensitivity to some chemicals, following the manufacturer's instructions except for the following modifications. Strains were grown on TY plates, cultures were resuspended in IF-A inoculation fluid (GEN III MicroPlates^{TM}, Biolog, Inc.) containing $MgSO_4$ (5 mM; A. Peix, personal communication), and adjusted to an O.D. $_{600}$ of 0.2. Portions of 0.1 ml were added to each well, and plates were incubated at 28 °C at 150 rpm on a rotary shaking (Infors HT Multitron) for 48 h. For growth at different pH, Yeast Mannitol (YM) medium was adjusted to pH 8–9.6 with 50 mM Tris and cultures were grown simultaneously at 28 °C in YM medium at pH 6.8, 8 or 9.6 for up to four days. To test for growth at different temperatures, cultures were incubated in YM medium (pH 6.8), again simultaneously, at 28 °C, 30 °C or 37 °C for up to four days.

Analysis of cellular fatty acids

Cellular fatty acid methyl esters (FAMEs) were prepared and separated using the method described by Sasser [15] and identified with the MIDI Sherlock Microbial Identification System (version 6.1), using the TSBA6 database at the Spanish Type Culture Collection (CECT, Burjassot, Valencia). *Neorhizobium tomejilense* T20_22, T17_20^{T} and T11_12 and the *Neorhizobium* sp. type strains were grown in TY for 48 h at 28 °C and harvested in late exponential growth phase.

MALDI-TOF analysis

To determine the whole-cell protein profile, a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis was carried out at the Scientific-Technical Services (University of Balearic Islands, Spain). MALDI-TOF MS characterization of freshly-grown colonies was carried as described by Sanchez et al. [16]. Strains were cultured on YMB plates at 30 °C for 72–96 h. One microlitre of the extract (these extracts were obtained based on the ethanol/formic acid extraction procedure of Bruker Daltonics) was placed onto a spot of a ground steel plate. Each sample was overlaid with 1 μL of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid) and air dried at room temperature. Measurements were performed on an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany). Spectra were recorded in the linear, positive mode in the mass range 2–20 kDa (IS1 = 20 kV, IS2 = 18.7 kV, lens = 6.50 kV, and PIE = 200 ns). Spectra were obtained for all strains analysed twice each with three replicates. The profiles obtained for each strain was analysed and compared, and the corresponding dendrogram was constructed with the MALDI BioTyper software (version 1.0; Bruker Daltonics).

Gene and genome accession numbers

Genbank accessions for genes and genomes used in this study are listed in Supplementary Table 1.

Results and discussion

In a previous study [5] we reported for the first time the isolation from soil of novel, non-symbiotic members of the *Neorhizobium* genus. Phylogenetic analysis of representative genotypes from fifty-three of these non-symbiotic isolates using 16S rDNA and concatenated *atpD*, *glnII*, *recA*, *rpoB* and *thrC* gene sequences suggested that the genotype represented by strain T20_22, that includes twelve isolates, constitutes a phylogenetically independent group. Draft genome sequencing analysis of T20_22 [5] and of two additional strains, T17_20^{T} and T11_12 (this work) further supports the phylogenetic coherence of this independent group.

Phylogenetic analysis of 16S rDNA from T20_22, T17_20^{T} and T11_12 confirmed their placement within the *Neorhizobium* genus. A ML tree derived from full 16S rDNA alignments is shown in Fig. 1; similar results were obtained using MP and NJ methods (data not shown). Since the genus *Neorhizobium* has been defined from a number of earlier described *Rhizobium* species [1], sequences from type strains of close Rhizobiaceae species were included to support their phylogenetic differences. Tomejil isolates clustered together (in fact, they have identical 16S rDNA gene sequences), very close to several *N. galegae* sequences, including the type strain HAMBI 540^{T}, and clearly separated from the other two *Neorhizobium* species, *N. huautlense* DSM 21817^{T} and *N. alkalisoli* DSM 21826^{T}, and from the genus *Rhizobium* and other genera within the Rhizobiaceae, with a very high bootstrap support.

An in-depth evaluation at species level was performed with a multilocus sequence analyses of the full-length, concatenated genes *atpD*, *glnII*, *recA*, *rpoB* and *thrC*, used to describe the *Neorhizobium* genus [1]. NJ and MP methods (data not shown) gave

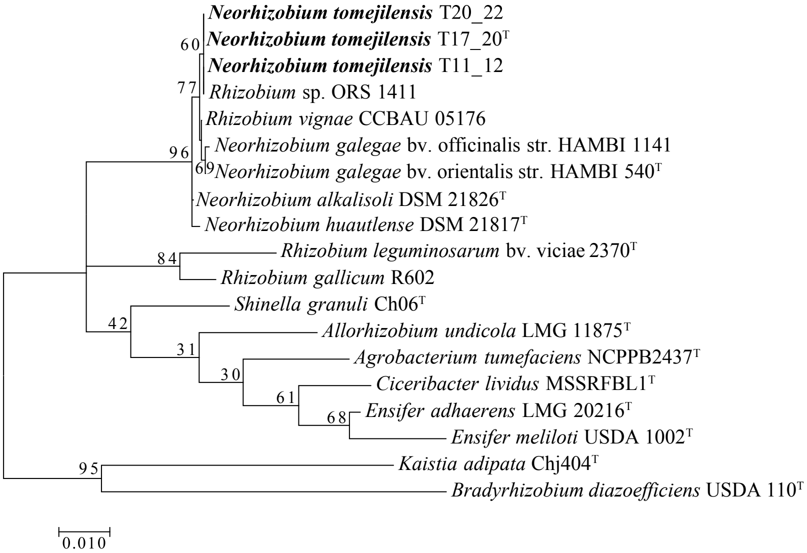

Fig. 1. Maximum likelihood phylogenetic tree based on 16S rDNA sequences (1,476 bp) of *Neorhizobium tomejilense* sp. nov, *Neorhizobium* type strains and representative *Rhizobium* type strains. Bootstrap values (1000 replications) are shown at the branching points. Bar represents the number of substitutions per base.

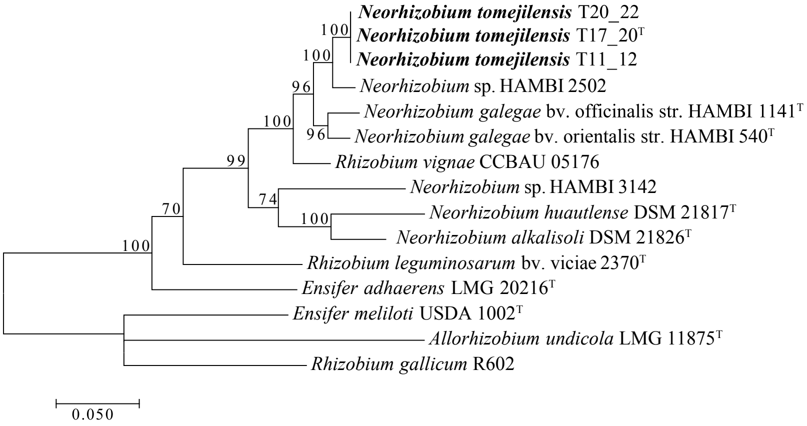

Fig. 2. Maximum likelihood phylogenetic tree based on full *atpD*, *glnII*, *recA*, *rpoB* and *thrC* concatenated sequences (8386 nt) of *Neorhizobium tomejilense* sp. nov, and relevant sequences from *Neorhizobium* strains and from members of the *Rhizobium-Allorhizbium-Agrobacterium* clade used to define the *Neorhizobium* genus [1]. Bootstrap values (1000 replications) are shown at the branching points. Bar represents the number of substitutions per base.

results similar to those shown in the ML tree in Fig. 2. In this case, and due to the higher resolution obtained, T20_22, T17_20^T and T11_12 also kept closely related to *N. galegae* HAMBI 540^T, and separated from other members of the *Rhizobium-Allorhizobium-Agrobacterium* clade [1], but were clearly grouped in a different branch with very high bootstrap support. These results therefore suggest that this group of Tomejil soil strains belong to a new *Neorhizobium* species differentiated from all other described *Neorhizobium*.

In order to provide support for the classification of these Tomejil isolates as a novel species, Average Nucleotide Identity (ANI) was used to estimate homologies at the genomic level including all the *Neorhizobium* species described. Draft genomes were obtained from T20_22, T17_20^T, T11_12 and type strains for two of the *Neorhizobium* species, *N. huautlense* DSM 21817^T and *N. alkalisoli* DSM 21826^T, whose genomic sequences were not available in public databases. Calculated ANIb values are indicated in Table 1, where, in addition to the genome of the type strain for the third *Neorhizobium* species, *N. galegae* HAMBI 540^T [18], nine other *N. galegae* genomes were included, four belonging to symbiovar orientalis (HAMBI 2427, 2566, 2605, and 2610 [17]), and five to symbiovar officinalis (HAMBI 1141 [18] and HAMBI 490, 1146, 1145 and 1189 [17]). Pairwise ANI genomic comparisons between T20_22, T17_20^T and T11_12 were above 97%, over the ≈95-96% boundary set for species definition [19]. Within type strains of previously described *Neorhizobium* species, *N. galegae* HAMBI 540^T showed the highest (90.2%) ANI value with T20_22, T17_20^T and T11_12, while these were quite distant from *N. huautlense* DSM 21817^T and *N. alkalisoli* DSM 21826^T. These results confirm what had been observed with the 16S rDNA and concatenated *atpD*, *glnII*, *recA*, *rpoB* and *thrC* phylogenetical analyses.

The availability of genome sequences for strains belonging to all the described *Neorhizobium* species allowed a general genomic characterization of the genus (Table 2). *N. tomejilense* and *N. galegae* shared similar genome sizes and GC (%) composition, as opposed to *N. alkalisoli* and *N.huautlense*, which shared a smaller genome size and a lower GC (%). All the sequenced strains contained between one and three plasmids of the *repABC* type, some of them quite large [18]. In symbiotic strains, one of these plasmids carries symbiotic genes (notably *nod* and *nif* genes) [17,18]. *N. tomejilense* strains do not harbour symbiotic genes, as concluded from the PCR screening with *nifH* and *nodC* universal primers, and also from BLAST genomic searches (Table 2) and thus probably lack the symbiotic plasmid. However, they contained one (T11_12 and T20_22) or two (T17_20^T)

plasmids. Differences were also observed in the numbers of rRNA operons and tRNAs. *N. galegae* strains contain three rRNA operons [17,18], while strains from the other species appeared to contain four or five copies. Similarly, *N. galegae* contained 40-41 tRNAs (plus 9 associated to the rRNA operons), while the other species contained 45-46 (plus 9-15 associated to the rRNA operons).

The incorporation to Table 1 of a total of ten *N. galegae* strains from different geographic origins [17,18] and belonging to the two symbiovars described for *N. galegae* [17,21], allowed a more detailed appraisal of genomic diversity within the genus *Neorhizobium* through the use of ANI. Up to five different genomospecies were observed within *N. galegae*, two within symbiovar orientalis and three within symbiovar officinalis, grouping two, three, one, two, and three strains, respectively. One of the putative genomospecies (HAMBI 1145 and 1189) displayed much higher pairwise ANIb values with *N. tomejilense* strains (94.4-95.0%), just below the species boundary, an observation that should warrant further characterization of these strains. Some of the *Galega*-nodulating genomospecies had a coherent geographical origin (eg. HAMBI 2566, 2605, 2610, all from Caucasus [17]), while others did not (eg. HAMBI 490 and 1146, from Finland and New Zealand, respectively [17]). These results suggest that a substantial diversity exists even within the *Galega*-nodulating strains that, however, display a conserved set of symbiotic genes [17]. Given that these symbiotic genes are present in plasmids [18], it is possible that they can be acquired by diverse non-symbiotic *Neorhizobium* populations resident in soil, such as those we previously described [5] and from which the *N. tomejilense* strains described in this work derive. In this respect, it is noteworthy that the five putative *Galega*-nodulating genomospecies include strains of just one symbiovar, suggesting a certain specificity regarding the acquisition and maintenance of symbiotic plasmids.

In view of the high ANI values within *N. tomejilense* (Table 1), and in order to avoid clonality of the isolates, we performed a Randomly Amplified Polymorphic DNA- Polymerase chain reaction (RAPD-PCR) using M13 primers. *N. tomejilense* T20_22, T17_20^T and T11_12 showed distinctive band profiles in the agarose gel. These profiles were also different to those from the *Neorhizobium* species type strains, in particular to *N. galegae* HAMBI 540^T, the closest species at phylogenetic and genomic level to *N. tomejilense* (Supplementary Fig. S1).

Differential phenotypic characteristics of the strains in the proposed new species and *Neorhizobium* species are indicated in Table 3. As previously reported, the *N. huautlense* type strain was able to grow at 37 °C [3], unlike all the other strains tested. All species were able to grow at pH 8, but not at pH 10 (data not shown). In the case of carbon and nitrogen sources, *N. tomejilense* was not able to use L-aspartic acid, in comparison to the other *Neorhizobium* species. In contrast, it was able to use pectin and sorbitol in comparison to its closest *Neorhizobium* species (*N. galegae* HAMBI 540^T). *N. tomejilense* strains were positive for many anti-bacterial and anti-fungal compounds, which was not the case for *N. galegae* HAMBI 540^T for example. They were also able to use many proton and electron donors, this was differential to *N. alkalisoli* DSM 21826^T. *N. tomejilense* and *N. huautlense* DSM 21817^{T}exhibit many differential characteristics, as *N. huautlense* DSM 21817^T showed to have a wide metabolic capacity, this was differential not only of *N. tomejilense* but of the other *Neorhizobium* species.

In addition to the classical phenotypic analysis a whole-cell matrix-assisted laser-desorption time-of-flight mass spectrometry (WC MALDI-TOF-MS) fingerprint analysis was also performed. The dendrogram (Fig. 3) representing the distances calculated from the fingerprint profiles among type strains from the *Neorhizobium* species and the Tomejil strains shows a clearly distinct group formed by the three Tomejil strains (T20_22, T17_20^T and T11_12) separated from all the *Neorhizobium* type species described.

Table 1
Percentage of average nucleotide identity based on the BLAST-algorithm (ANIb) among genomes of T17_20^T, T11_12, T20_22, type strains from the different *Neorhizobium* species, and available genomes from the two *N. galegae* symbiovars.

Strains	*N. tomejilense*			*N. galegae* sv. orientalis					*N. galegae* sv. officinalis					*N. alkalisoli*	*N. huautlense*
	T17_20^T	T11_12	T20_22	HAMBI 540^T	HAMBI 2427	HAMBI 2566	HAMBI 2605	HAMBI 2610	HAMBI 1141	HAMBI 490	HAMBI 1146	HAMBI 1145	HAMBI 1189	DSM 21826^T	DSM 21817^T
N. tomejilense T17_20^T	*	99.3	99.2	90.2	90.2	92.7	92.6	92.6	90.6	91.2	91.3	94.8	94.4	79.4	79.0
N. tomejilense T11_12	99.3	*	99.5	90.2	90.3	92.8	92.7	92.6	90.6	91.2	91.2	94.9	94.5	79.4	79.0
N. tomejilense T20_22	99.0	99.4	*	90.1	90.1	92.6	92.5	92.4	90.5	91.0	91.1	94.7	94.4	79.4	79.1
N. galegae sv. orientalis HAMBI 540	90.1	90.1	90.2	*	98.8	90.5	90.6	90.5	94.4	89.2	89.2	90.1	90.0	79.3	78.9
N. galegae sv. orientalis HAMBI 2427	90.1	90.1	90.1	98.8	*	90.6	90.4	90.4	94.2	89.0	89.0	90.1	89.9	79.2	78.9
N. galegae sv. orientalis HAMBI 2566	92.7	92.7	92.7	90.5	90.5	*	97.9	97.6	90.1	91.8	91.7	92.8	92.5	79.5	79.0
N. galegae sv. orientalis HAMBI 2605	92.5	92.5	92.5	90.4	90.3	97.9	*	97.4	90.1	91.6	91.6	92.6	92.3	79.5	79.0
N. galegae sv. orientalis HAMBI 2610	92.8	92.8	92.8	90.7	90.7	97.9	97.7	*	90.3	92.1	92.1	92.9	92.6	79.6	79.1
N.galegae sv. officinalis HAMBI 1141	90.6	90.6	90.6	94.5	94.4	90.3	90.4	90.2	*	89.3	89.3	90.6	90.5	79.3	79.0
N.galegae sv. officinalis HAMBI 490	91.2	91.2	91.2	89.3	89.2	91.9	91.8	91.9	89.4	*	97.9	91.5	91.2	79.4	79.2
N.galegae sv. officinalis HAMBI 1146	91.2	91.1	91.1	89.2	89.1	91.8	91.6	91.9	89.2	97.7	*	91.3	91.2	79.4	79.0
N.galegae sv. officinalis HAMBI 1145	95.0	95.0	95.0	90.4	90.3	93.0	93.0	93.0	90.7	91.6	91.6	*	95.3	79.5	79.2
N.galegae sv. officinalis HAMBI 1189	94.9	94.9	94.9	90.4	90.5	93.0	92.9	92.9	90.9	91.7	91.7	95.6	*	79.6	79.2
N. alkalisoli DSM 21826^T	79.5	79.6	79.5	79.5	79.4	79.7	79.7	79.8	79.4	79.7	79.7	79.6	79.6	*	87.4
N. huautlense DSM 21817^T	79.5	79.5	79.5	79.3	79.3	79.5	79.4	79.5	79.4	79.5	79.5	79.4	79.5	87.5	*

Possible genomospecies (pairwise ANIb values above 95%) are indicated with shaded boxes.

Table 2
Genomic properties of *Neorhizobium* species.

	N. tomejilense	*N. galegae*[a]	*N. alkalisoli*	*N. huautlense*
No. strains	3	10	1	1
Assembly quality	Draft	Complete (2)Draft (8)	Draft	Draft
Total size (Mb)	6.52 – 6.62	6.00–6.86	5.87	5.73
GC (%)	61.5	61.2–61.6	60.3	60.0
Plasmids[b]	1-2	1–3	3	3
rRNA operons[c]	4-5	3	4	4
tRNAs[d]	45 (+12–15)[e]	40-41 (+9)	46 (+12)[e]	45 (+12)[e]
nod/nif symbiotic genes[f]	–	+	+	+

[a] Data taken from [17].
[b] In draft sequences, this was estimated as the number of unique BLAST hits against the *N. galegae* HAMBI 540^{T} plasmid replication *repABC* genes.
[c] Since rRNA operon copies are identical or almost identical, in draft assemblies they collapse into a higher coverage consensus sequence. The number of copies was estimated from the excess read coverage of the assembled rRNA region over the average genome read coverage.
[d] Numbers of tRNAs associated to rRNA operons (three tRNAs per operon) are shown in brackets.
[e] Estimated by the tRNAscan-SE server [20].
[f] Presence or absence was estimated from results of BLAST hits against the *N. galegae* HAMBI 540^{T} symbiotic region.

Table 3
Differential characteristics among *N. tomejilense* strains T17_20^{T}, T11_12, T20_22 and type strains from *Neorhizobium* species.

Growth	1	2	3	4	5	6
37 °C	–	–	–	–	–	+
Carbon and nitrogen sources:						
D-Sorbitol	+	+	+	–	+	+
Pectin	+	+	+	–	–	+
Dextrin	–	–	–	w	–	+
D-Lactic acid methyl ester	–	–	–	–	–	+
Mucic Acid	–	–	–	–	+	w
D-Malic Acid	+	+	+	+	–	+
D-Serine	–	–	–	–	–	+
L-Serine	–	–	–	–	+	+
L-Aspartic acid	–	–	–	+	+	+
L-Histidine	–	–	–	–	–	+
D-Aspartic acid	–	–	–	–	–	+
L-Pyroglutamic acid	–	–	–	–	–	+
N-Acetyl Neuraminic acid	–	–	–	+	–	w
Propionic acid	+	+	+	+	–	+
Formic acid	+	+	+	–	–	+
Proton and electron donors:						
Sodium butyrate	+	+	+	–	–	+
Tetrazolium blue	+	+	+	+	–	+
Potassium tellurite	+	+	+	+	–	+
Antibiotic and antifungal resistance:						
Troleandomycin	+	+	+	+	–	+
Lincomycin	+	+	+	–	+	+
Vancomycin	+	+	+	–	+	+
Nalidixic Acid	+	+	+	–	+	+
Fusidic Acid	+	+	+	–	–	+

Species/Strains: 1, *N. tomejilense* T17_20^{T}; 2, *N. tomejilense* T11_12; 3, *N. tomejilense* T20_22; 4, *N. galegae* HAMBI 540^{T}; 5, *N. alkalisoli* DSM 21826^{T}, 6, *N. huautlense* DSM 21817^{T}. All data were obtained in this study. +, positive; –, negative; w, weak.

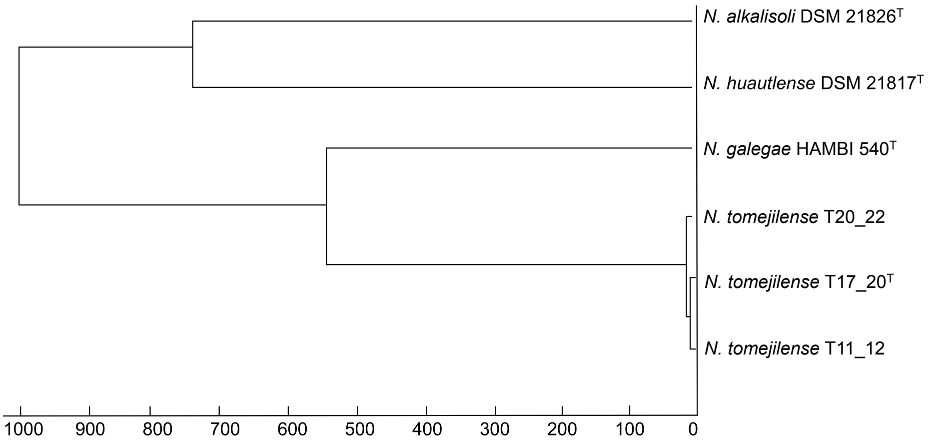

Fig. 3. Dendrogram constructed by applying average linkage clustering and Pearsońs distance correlation from a similarity matrix of identical mass peaks, computed from whole cell mass spectra of all *Neorhizobium* strains analysed. The dendrogram was generated by considering the average value of the duplicates for each strain. The bar indicates distance.

Table 4
Fatty acid patterns of *N. tomejilense* strains T17_20^{T}, T11_12, T20_22 and closest related type strains from *Neorhizobium* species.

	1	2	3	4	5	6
16:00	8.8	9.4	8.6	13.2	11.7	7.1
16:0 3OH	3.1	3.0	3.2	2.8	2.5	2.6
17:00	0.9	1.7	1.1	1.6	–	0.6
17:0 3OH	–	1.5	–	0.8	–	–
17:1 ω8c	0.7	1.3	0.7	0.8	–	–
18:00	1.4	0.8	1.8	0.7	0.8	2.6
18:0 3OH	0.8	1.3	1.2	0.8	0.6	1.4
19:0 cyclo ω8c	9.5	7.5	7.1	27.5	11.5	3.5
19:0 10-methyl	2.5	2.2	2.5	1.8	2.3	1.8
Summed Feature 2	5.7	5.0	5.4	4.7	4.8	5.1
Summed Feature 3	0.9	0.9	1.3	1.7	1.6	0.7
Summed Feature 8	65.7	65.4	67.2	41.1	63.9	73.4

Strains: 1, *N. tomejilense* T17_20^{T}; 2, *N. tomejilense* T11_12; 3, *N. tomejilense* T20_22; 4, *N. galegae* HAMBI 540^{T}; 5, *N. alkalisoli* DSM 21826^{T}; 6, *N. huautlense* DSM 21817^{T}. All data were obtained in this study. Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed Feature 2, 14:0 3OH/16:1 iso I; Summed Feature 3, 16:1 ω7c/16:1 ω6c; Summed Feature 8, 18:1 ω7c/18:1 ω6c. Only those fatty acids present in the novel strains are presented.

Fatty acid composition and abundance varied among all *Neorhizobium* species is indicated in Table 4. Fatty acid abundance did not change significantly among *N. tomejilense* strains, that had very similar fatty acid composition, except for 17:0 3OH fatty acid in strain T11_12. Fatty acid pattern and abundance in *N. tomejilense* strains was clearly different to those in the most similar strain within the genus, *N. galegae* HAMBI 540^{T}. *N. galegae* HAMBI 540^{T} has distinct fatty acids such as 17:0 cyclo, 20:2 ω6,9c, and 13:0

Table 5
Description of *Neorhizobium tomejilense* sp. nov. according to Digital Protologue TA00537 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00537
Species name	*Neorhizobium tomejilense*
Genus name	*Neorhizobium*
Specific epithet	*tomejilense*
Species status	sp. nov.
Species etymology	to.me.jil.en'se. N.L. neut. adj. tomejilense, pertaining to Tomejil, the name of the experimental farm from which the type strain was isolated
Authors	Soenens A, Gomila M, Imperial J
Title	*Neorhizobium tomejilense* sp. nov., first non-symbiotic *Neorhizobium* species isolated from a dryland agricultural soil in southern Spain
Corresponding author.	Imperial J
E-mail of the corresponding author	juan.imperial@csic.es
Submitter	JUAN IMPERIAL
E-mail of the submitter	juan.imperial@csic.es
Designation of the type strain	T17_20
Strain collection numbers	LMG 30623 = CECT 9621
16S rRNA gene accession number	MH064335
Genome accession number [Refseq]	PVBG01
Genome status	Draft
Genome size	6559 kb
GC mol %	61.46
Country of origin	ESP
Region of origin	Andalucía, Sevilla, Carmona, Tomejil
Date of isolation	01/03/2014
Source of isolation	Soil
Sampling date	01/03/2014
Geographic location	Tomejil Experimental Station (IFAPA)
Latitude	37°24′33.10" N
Longitude	5°34′51.91" W
Altitude	77
Number of strains in study	3
Source of isolation of non-type strains	Soil
Growth medium, incubation conditions [temperature, pH, and further information] used for standard cultivation	YM (per liter: 1 g mannitol, 0.4 yeast extract, 0.1 g NaCl, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$) 28 °C, pH 6.8–7.2
Alternative medium 1	TY (per liter: Tryptone 5.0 g, yeast extract 3.0 g, $CaCl_2.H_2O$ 0.9 g)
Conditions of preservation	−80 °C with glycerol (20%) as cryoprotectant
Gram stain	Negative
Cell shape	Rod
Motility	Motile
If motile	Flagellar
Colony morphology	Circular, cream-coloured, semi-translucent and mucilaginous. Older colonies have a white centre.
Temperature range	12–30 °C
Lowest temperature for growth	12 °C (YMB, pH 6.8–7.0)
Highest temperature for growth	30 °C (YMB, pH 6.8–7.0)
Temperature optimum	28 °C
Lowest pH for growth	6 (YMB, 28 °C)
Highest pH for growth	8 (YMB, 28 °C)
pH optimum	7.0
pH category	Neutrophile

Table 5 (*Continued*)

Lowest NaCl concentration for growth	0 (YMB, 28 °C, pH 7.0)
Highest NaCl concentration for growth	1% (YMB, 28 °C, pH 7.0)
Salinity category	Nonhalophile (NaCl inhibitory at >1% NaCl)
Relationship to O_2	Aerobe
O_2 conditions for strain testing	Aerobiosis
Carbon source used [class of compounds]	Sugars, amino acids
Carbon source used [specific compounds]	D-glucose, D-maltose, D-fructose, L-fucose, D-galactose, D-trehalose, D-cellobiose, D-arabitol, D-mannitol, D-sorbitol, D-gluconic acid, L-lactic acid, methyl pyruvate, gamma-amino-butryric acid, L- and D-malic acid, propionic acid, acetic acid, formic acid, glycerol, D-salicin, pectin
Carbon source not used [specific compounds]	Dextrin, D-lactic acid methyl ester, mucic acid, D- or L-serine, D- or L-aspartic acid, L-histidine, L-pyroglutamic acid, *N*-acetyl neuraminic acid
Positive tests with BIOLOG	Butyrate, tetrazolium, tellurite. Troleandomycin, lincomycin, vancomycin, nalidixic acid and fusidic acid.
Nitrogen source	Ammonium, L-glutamic acid and glucuronamide
Terminal electron acceptor	Oxygen
Energy metabolism	Chemoorganotroph
Oxidase	Positive
Catalase	Positive
Major fatty acids	Summed feature 8 (18:1 ω7c/18:1 ω6c), 16:00 and 19:0 cyclo ω8c
Biosafety level	1
Habitat	Terrestrial, dryland agricultural soil
Biotic relationship	Free-living
Known pathogenicity	None

3OH/15:1 I summed feature 1 (not shown in Table 4), which differed from *N. tomejilense*, as well as from *N. huautlense* DSM 21817$^{\mathrm{T}}$ and *N. alkalisoli* DSM 21826$^{\mathrm{T}}$.

The formal proposal of the new species “Neorhizobium tomejilense sp. nov.” is given in Table 5 with the Taxonumber TA00537.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.09.001.

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