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The high diversity of *Lotus corniculatus* endosymbionts in soils of northwest Spain

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Abstract The diversity of rhizobia that establish symbiosis with *Lotus corniculatus* has scarcely been studied. Several species of *Mesorhizobium* are endosymbionts of this legume, including *Mesorhizobium loti*, the type species of this genus. We analysed the genetic diversity of strains nodulating *Lotus corniculatus* in Northwest Spain and ten different RAPD patterns were identified among 22 isolates. The phylogenetic analysis of the 16S rRNA gene showed that the isolated strains belong to four divergent phylogenetic groups within the genus *Mesorhizobium*. These phylogenetic groups are widely distributed worldwide and the strains nodulate *L. corniculatus* in several countries of Europe, America and Asia. Three of the groups include the currently described *Mesorhizobium* species *M. loti*, *M. erdmanii* and *M. jarvisii* which are *L. corniculatus* endosymbionts. An analysis of the *recA* and *atpD* genes showed that our strains belong to several clusters, one of them very closely related to *M. jarvisii* and the remanining ones phylogenetically divergent from all currently described *Mesorhizobium* species. Some of these clusters include *L. corniculatus* nodulating strains isolated in Europe, America and Asia, although the *recA* and *atpD* genes have been sequenced in only a few *L. corniculatus* endosymbionts. The results of this study revealed great phylogenetic diversity of strains nodulating *L. corniculatus*, allowing us to predict that even more diversity will be discovered as further ecosystems are investigated.

Keywords *Lotus corniculatus* · *Mesorhizobium* · Symbiosis · Nodulation · Spain

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1 Introduction

The genus *Lotus*, which belongs to the tribe Loteae, is distributed worldwide being able to adapt to different environmental stresses. It has been introduced into non-native areas by human activities (Escaray et al. 2012). *Lotus corniculatus* is a perennial legume used for pasture and silage production in many temperate countries (Grant and Small 1996). It has a natural distribution in Western Europe and North Africa (http://www.fao.org/Ag/agp/agpc/doc/Gbase/DATA/pf000344.htm) where it establishes a nitrogen-fixing symbiosis with rhizobia that have been scarcely studied despite of the value of *L. corniculatus* as fodder for animals (Ramírez-Restrepo and Barry 2005). The endosymbiont of *L. corniculatus* was initially named *Rhizobium loti* (Jarvis et al. 1982) but was later reclassified as *Mesorhizobium loti* (Jarvis et al. 1997). Recently it has been shown that the type strains of this species held in different culture collections represent three species, *Mesorhizobium loti*, *Mesorhizobium erdmanii* and *Mesorhizobium jarvisii* having phylogenetically divergent 16S rRNA and housekeeping genes (Martínez-Hidalgo et al. 2015).

Although there are only a few studies of the *L. corniculatus* endosymbionts, they show high phylogenetic diversity among

strains isolated in Europe (Ampomah and Huss-Danell 2011; Lorite et al. 2010; De Meyer et al. 2011; Gossmann et al. 2012) and South America (Binde et al. 2009; Sotelo et al. 2011). They form different clusters within the genus *Mesorhizobium*. Several endosymbionts of *L. corniculatus* have also been isolated from a province of South Spain and found to comprise two different phylogenetic lineages clustering with *Mesorhizobium albiziae* and with *Mesorhizobium alhagi*, on the basis of the analyses of the core genes 16S rRNA, *atpD* and *recA* (Lorite et al. 2010). In Spain, *L. corniculatus* is widely distributed in many regions as part of the natural plant cover. Nevertheless, there are no studies about the endosymbionts of this legume in most Spanish soils.

Therefore, the aims of the present study were to examine the genetic diversity of strains isolated from *L. corniculatus* in Salamanca (Northwest Spain), using RAPD fingerprinting and phylogenetic analysis of their 16S rRNA, *recA* and *atpD* genes, and then to compare the results with those from *L. corniculatus* endosymbiont strains isolated in other geographic locations.

2 Materials and methods

2.1 Bacterial strains and nodulation experiments

The strains were isolated from 35 effective nodules (pink colour) of three *L. corniculatus* plants in flowering stage collected in an alfisol soil from Carbajosa de la Sagrada (a Mediterranean region from Salamanca, NW Spain, latitude 40° 55′59″N and 5° 39′05′′O, altitude 789 mamsl) using the standard method of Vincent (1970) on YMA plates at 28 °C. Re-infection experiments in *L. corniculatus* were performed using the previously published conditions (Robledo et al. 2008). Seeds were scarified with sulfuric acid for 12 min and then rinsed several times with sterile distilled water. After that, seeds were surface-disinfected with 2.5 % sodium hypochlorite for 10 min and then, rinsed five times with sterile distilled water. Seeds were germinated in 1 % (*w*/*v*) agar plates for 24 h in dark. One day-old seedlings were transferred to tubes and inoculated with 1 ml of a suspension containing approximately $1x10^8$ UFC/ml. Tubes were placed in a growth chamber at 24 °C, 20 °C day-night cycle, 16 h photoperiod, and 60 % relative humidity. N-free and N-fed uninoculated treatments were performed in the same conditions. Roots were examined and the number of nodules was counted 30 days after inoculation.

2.2 DNA extraction and RAPD fingerprinting

Total genomic DNA from the isolates was extracted according to Rivas et al. (2001). Briefly, strains were grown for 48 h in TY medium (0.4 % tryptone, 0.3 % yeast extract and 0.09 % Ca2Cl) and cells were collected by centrifugation at room temperature in a microspin centrifuge at 5000 x*g* and then washed with 100 μl of an aqueous solution of 0.1 % (*w*/*v*) sarkosyl. The DNA was extracted with 100 μl of 0.05 M NaOH (DNA-free) heating at 100°C for 4 min. Samples were then placed in an ice bath and 900 μl of water was added to each microtube and mixed thoroughly. After an additional centrifugation at 5000 x*g*, 700 μl of the supernatants were harvested and frozen at −20 °C. The RAPD profiles were obtained as was previously described (Rivas et al. 2006) using the M13 primer (5′-CAGGGTGGCGGTTCT-3′) and an AmpliTaq reagent kit (Perkin-Elmer Biosystems, California, USA). PCR conditions were as follows: preheating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 45 °C for 1 min and extension at 75 °C for 2 min, and a final extension at 72 °C for 7 min. A total of 17 μl of each PCR amplification product were electrophoresed in 1.5 % agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) during 2 h at 6 V/cm and stained with ethidium bromide (0.5 μg ml^{-1}) and visualized using the Molecular Imager Chemidoc XRS System (Biorad, U.S.A). The RAPD patterns were obtained in the same electrophoretic conditions and using the same molecular weigth standard as size marker (Standard VI; Hofmann-La Roche, Switzerland).

2.3 Phylogenetic analyses

The amplification and sequencing of 16S rRNA gene were carried out according to Rivas et al. (2007) and *atpD* and *recA* gene sequences were obtained according to Gaunt et al. (2001). PCR amplifications were performed with a REDExtract-N-Amp™ PCR Kit (Sigma Co., USA) following the manufacturer's instructions. Bands corresponding to the different genes were purified directly from the gel by room temperature centrifugation using a DNA gel extraction device (Millipore Co., USA) for 10 min at 5.000 x*g*, according to the manufacturer's instructions. Sequencing reactions were performed on an ABI PRISM® 3100 sequencer using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., USA), as supplied by the manufacturer. The obtained sequences were compared with those from EzTaxon-e server (Kim et al. 2012). Sequences were aligned using the Clustal X software (Thompson et al. 1997). The distances were calculated according to Kimura's two-parameter model (Kimura 1980). A phylogenetic tree was inferred using the neighbor-joining analysis (Saitou and Nei 1987). MEGA5 software (Tamura et al. 2011) was used for all analyses.

3 Results and discussion

3.1 Genetic biodiversity of strains

The 22 strains isolated from *L. corniculatus* nodules in Salamanca were able to induce nodules in this host (Table 1). Their genetic diversity was analysed by RAPD fingerprinting that allowed differentiation among strains from *Mesorhizobium* (Rivas et al. 2006; Armas-Capote et al. 2014). The strains isolated in this study displayed 10 different RAPD patterns (Fig. 1, Table 1) that showed genetic diversity allowing us to select a representative strain from each RAPD type for the analysis of the 16S rRNA gene.

3.2 Phylogenetic analysis of the 16S rRNA gene

The complete 16S rRNA gene sequences were obtained for the 10 strains representing the different RAPD types and compared with those from the EzTaxon-e database, which contains the type strains from all described species. The results obtained showed that the strains isolated in this study were closely related to different species of the genus *Mesorhizobium* with similarity values higher than 99 % in all cases (Table 1).

Considering the closeness of several *Mesorhizobium* species, in the phylogenetic analysis of the 16S rRNA gene we only included the strains isolated from *L. corniculatus* in other studies when the available sequences have more than 1200 nt. The strains isolated by De Meyer et al. (2011) from Belgium were not included because their sequences were too short. Some species that do not have identical 16S rRNA gene sequences, appear to be identical in the phylogenetic tree due to the lack of the initial nucleotides in the available 16S rRNA gene sequences. This is the case, for example in *M. gobiense*, *M. metallidurans* and *M. tarimense*, where the differences are located in the first 70 bp that are absent in the available 16S rRNA gene sequences. For this reason, differences observed after the analysis of the EzTaxon-e database, which performed pairwise analyses, are not in some cases observed in the phylogenetic trees.

The phylogenetic analysis of the 16S rRNA gene showed that the strains isolated in Northwest Spain clustered into four groups (fig. 2). The strains CSLC01N, CSLC28N, CSLC14N, CSLC36N and CSLC42N representing RAPD types I, II, IV, VIII and IX, respectively, belong to a wide cluster (cluster I) which includes the type strains of *M. huakuii* IAM 14158^T, *M. jarvisii* ATCC 33669^T, *M. amorphae* ACCT19665^T, *M. septentrionale* SDW014^T and *M. waimense* ICMP 19557^T. The strain ATCC 33669^T (before named *M. loti*) was also isolated from *L. corniculatus* nodules and it has been recently reclassified into a new species called *M. jarvisii* (Martínez-Hidalgo et al. 2015). Several strains isolated from *L. corniculatus* in European countries, for example Sweden (Ampomah and Huss-Danell 2011), Norway (Gossmann et al. 2012) and Spain (Lorite et al. 2010), and in American countries, such as Uruguay (Binde et al. 2009; Sotelo et al. 2011) and Brazil (Binde et al. 2009), belong to the same cluster. It should be noted that the strains SEMIA 848 and R6 isolated in Uruguay are incorrectly named as *M. amorphae* and *M. loti*, respectively. In the case of strain R6, this may be due to the fact that the strain ATCC 33669^T was named *M. loti* for decades before its reclassification as *M. jarvisii* (Martínez-Hidalgo et al. 2015).

The strain CSLC22N representing RAPD pattern type V clustered with the type strain of *M. erdmanii* USDA 3471^T (cluster III). This cluster also included strains isolated in Sweden (Ampomah and Huss-Danell 2011), Norway (Gossmann et al. 2012). Uruguay (Sotelo et al. 2011) and two strains nodulating *L. corniculatus*, R7A and MAFF 303099, isolated in New Zealand and whose genome has been sequenced (Kelly et al. 2014; Kaneko et al. 2000). The strains isolated in Sweden are also misnamed as *M. loti* because the strain USDA 3471^T was considered to be the type strain of this species before its reclassification as *M. erdmanii* (Martínez-Hidalgo et al. 2015).

The strains CSLC19N, CSLC37N and CSLC115N, representing RAPD types III, VI and X, respectively, clustered with the type strains of *M. caraganae* CCBAU 11299^T, *M. gobiense* CCBAU 83330^T, *M. metallidurans* STM 2683^T, *M. tarimense* CCBAU83306^T and *M. tianshanense* A1BS^T (cluster IV). This cluster also contained strains nodulating *L. corniculatus* isolated in Sweden (Ampomah and Huss-Danell 2011), Northern Mexico (Qian and Parker 2002) and Uruguay (Sotelo et al. 2011).

Finally, the strain CSLC30N representing RAPD group VII belongs to the 16S rRNA cluster VII, which included the type strain of *M. loti* NZP 2213^T, *M. ciceri* UPMCa7^T, *M. sangaii* SCAU27^T, *M. qinshensii* CCBAU 33460^T, *M. australicum* WSM2073^T, *M. shangrilense* CCBAU65327^T and *M. cantuariense* ICMP 19515^T. This cluster contains several strains isolated in Sweden, most of them incorrectly named *M. loti* (Ampomah and Huss-Danell 2011). This also includes two strains isolated in Norway (Gossmann et al. 2012) and two strains isolated in New Zealand, R88b and CJ3sym, whose genomes have been sequenced (Reeve et al. 2014).

Only cluster I contains strains isolated from both Northwest and South Spain (Lorite et al. 2010). The other strains isolated in South Spain belong to a cluster including the type strains of *M. alhagi* CCNWXJ12-2^T and *M. camelthorni* CCNWXJ40-4^T (cluster VI) and to an independent cluster formed by two strains only isolated in South Spain (cluster V).

The strain N3 isolated in Uruguay (Sotelo et al. 2011) formed an independent lineage related to the cluster II, which also includes several type strains of *Mesorhizobium* species nodulating legumes other than *Lotus*. The cluster III contains the strain CSLC22N isolated in this study and several strains isolated from *L. corniculatus* nodules in Sweden (Ampomah

Table 1 Characteristics of strains nodulating *L. corniculatus* in Northwest Spain

Strain	RAPD type	Closest species in 16S rRNA gene (EzTaxon-e database)	Similarity (%)	Closest species in *recA* gene analysis	Similarity (%)	Closest species in *atpD* gene analysis	Similarity (%)	Nodules per plant (± SE)
CSLC01N	I	*M. huakuii* IAM 14158^T	100	*M. jarvisii* ATCC 33669^T	100	*M. jarvisii* ATCC 33669^T *M.erdmanii* USDA 3471^T	99.3 99.1	2 (±0.298)
CSLC03N, CSLC28N, CSLC35N, CSL C38N, CSLC116N	II	*M. huakuii* IAM 14158^T	100	*M. jarvisii* ATCC 33669^T	100	*M. jarvisii* ATCC 33669^T *M.erdmanii* USDA 3471^T	98.5 98.6	3.592 (±0.318)
CSLC06N, CSLC07N, CSLC15N, CSL C17N, CSLC18N, CSLC19N	III	*M. gobiense* CCBAU83330^T/ *M. metallidurans* STM2683^T	100	*M. metallidurans* STM2683^T	96.4	*M. metallidurans* STM2683^T	97.4	2.034 (±0.17)
CSLC09N, CSLC14N	IV	*M. huakuii* IAM 14158^T	100	*M. jarvisii* ATCC 33669^T	96.5	*M. caraganae* CCBAU 11299^T	92.5	2.591 (±0.376)
CSLC22N	V	*M. erdmanii* USDA 3471^T	99.9	*M. jarvisii* ATCC 33669^T	98.2	*M. jarvisii* ATCC 33669^T	97.9	1.545 (±0.207)
CSLC24N, CSLC31N, CSLC37N	VI	*M. gobiense* CCBAU83330^T/ *M. metallidurans* STM2683^T	100	*M. metallidurans* STM2683^T	96.4	*M. metallidurans* STM2683^T	97.4	2.154 (±0.227)
CSLC30N	VII	*M. ciceri* UPM Ca7^T	100	*M. ciceri* UPM Ca7^T	98,3	*M. ciceri* UPM Ca7^T	99.8	3 (±0.022)
CSLC36N	VIII	*M. huakuii* IAM 14158^T	100	*M. jarvisii* ATCC 33669^T	100	*M. jarvisii* ATCC 33669^T *M.erdmanii* USDA 3471^T	99.3 99.1	4.231 (±0.426)
CSLC42N	IX	*M. huakuii* IAM 14158^T	100	*M. jarvisii* ATCC 33669^T	99.2	*M. jarvisii* ATCC 33669^T	98.3	1.167 (±0.167)
CSLC115N	X	*M. gobiense* CCBAU83330^T/*M. metallidurans* STM2683^T	100	*M. metallidurans* STM2683^T	96.4	*M. metallidurans* STM2683^T	97.4	1.4 (±0.4)

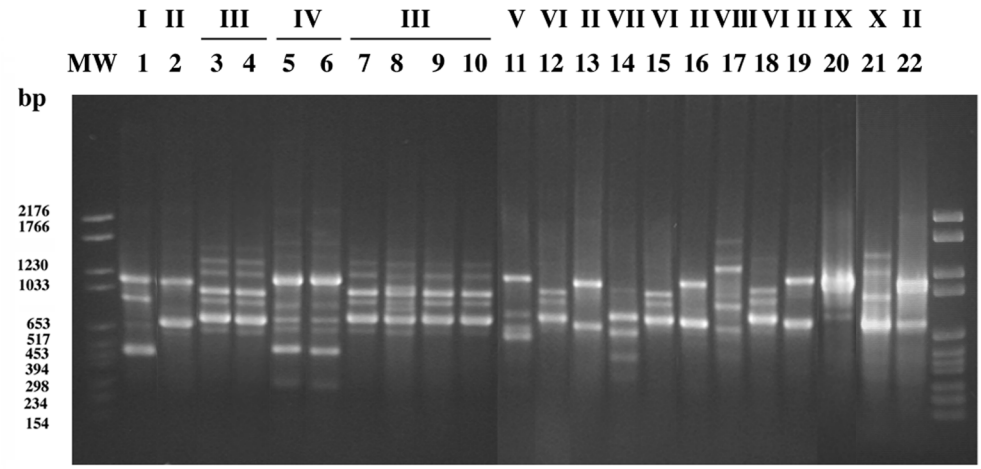

Fig. 1 RAPD profiles of strains isolated in this study CSLC01N (lane 1), CSLC03N (lane 2), CSLC06N (lane 3), CSLC07N (lane 4), CSLC09N (lane 5), CSLC14N (lane 6), CSLC15N (lane 7), CSLC17N (lane 8), CSLC18N (lane 9), CSLC19N (lane 10), CSLC22N (lane 11), CSLC24N (lane 12), CSLC28N (lane 13), CSLC30N (lane 14), CSLC31N (lane 15), CSLC35N (lane 16), CSLC36N (lane 17), CSLC37N (lane 18), CSLC38N (lane 19), CSLC42 N (lane 20), CSLC115N (lane 21), CSLC116N (lane 22). MW: molecular size markers with 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234 and 154 bp

and Huss-Danell 2011), the strain N33 in Uruguay (Sotelo et al. 2011) and the strains MAFF303099 and R7A isolated in New Zealand (Kaneko et al. 2000; Kelly et al. 2014). Moreover, this cluster includes the type strain *M. erdmanii* USDA 3471^T isolated from *L. corniculatus* nodules.

In summary, cluster I is the most widely distributed group due to its presence in Europe (South and Northwest Spain, Sweden and Norway), America (Brazil and Uruguay) and Asia (New Zealand), followed by the cluster III present in Europe (Northwest Spain and Sweden), America (Uruguay) and Asia (New Zealand). After this comes cluster IV, present in Europe (Northwest Spain and Sweden) and America (Northern Mexico and Uruguay) and cluster VII, present in Europe (Northwest Spain, Sweden and Norway) and Asia (New Zealand).

It is remarkable that some *L. corniculatus* strains are phylogenetically related with the type strains of the three *Mesorhizobium* species which are endosymbionts of this legume. These are now classified as *M. loti*, *M. erdmanii* and *M. jarvisii*, but were initially considered to be the same species. This highlights the need of a correct classification and naming of the type strains held in different culture collections in order to ensure that the type strains of a species is the same in all of them. This will avoid erroneous conclusions based on comparisons with type strains that are different but have been assigned to the same species.

3.3 Phylogenetic analysis of the *recA* and *atpD* genes

The 16S rRNA genes of several species from genus *Mesorhizobium* are very closely related as was showed above. However, they can be differentiated by their housekeeping genes, such as *recA* and *atpD* that are available for most of *Mesorhizobium* species, which allow the identification of new isolates. The *recA* gene has been analysed in the strains isolated from *L. corniculatus* in Belgium (De Meyer et al. 2011) and South Spain (Lorite et al. 2010) and the *atpD* gene was analysed in strains isolated in Spain and in Uruguay (Sotelo et al. 2011). The analysis of these two genes in our strains allowed us to know their identities and to analyse their phylogentic relationships with other *L. corniculatus* endosymbionts.

The analysis of *recA* gene showed that our strains belong to three different clusters within the genus *Mesorhizobium* (fig. 3). The strains from the 16S rRNA gene cluster I do not have the same *recA* gene sequences, with the exception of strains CSLC01N, CSLC28N and CSLC36N, but all of them formed a cluster including the type strains of the two species closely related in the 16S rRNA gene analysis, *M. jarvisii* ATCC 33669^T, isolated from *L. corniculatus* nodules, and *M. huakuii* IAM 14158^T. The strains CSLC01N, CSLC28N and CSLC36N with *recA* genes identical to those of *M. jarvisii* ATCC 33669^T and the strain CSLC42N with 99.2 % similarity in this gene, probably belong to *M. jarvisii*. However, the strain CSLC14N probably do not belong to this species since it has 96.5 % similarity with respect to *M. jarvisii* ATCC 33669^T (Table 1). This *recA* cluster also contains the strain CSLC22N belonging to the 16S rRNA gene cluster III, showing 98.2 % similarity with respect to *M. jarvisii* ATCC 33669^T. Since higher similarity values are presented by other species of the genus *Mesorhizobium*, such as *M. huakuii* and *M. qingsenghii*, we cannot assign the strain CSLC22 to the species *M. jarvisii*. This cluster also contains four strains that are phylogenetically divergent to our strains and to *M. jarvisii* ATCC 33669^T. These four strains, MAFF303099, R7A, R88b and CJ3sym, nodulate *L. corniculatus* and were isolated in

Fig. 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains isolated from *L. corniculatus* nodules in Northwest Spain and other geographical locations with respect to the type strains of the currently described species from genus *Mesorhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. The accession numbers for this gene in GenBank are: CSLC01N (KT899875), CSLC14N (KT899877), CSLC28N (KT899880), CSLC36N (KT899882), CSLC42N (KT899884), CSLC22N (KT899879), CSLC19N (KT899878), CSLC37N (KT899883), CSLC115N (KT899885) and CSLC30N (KT899881)

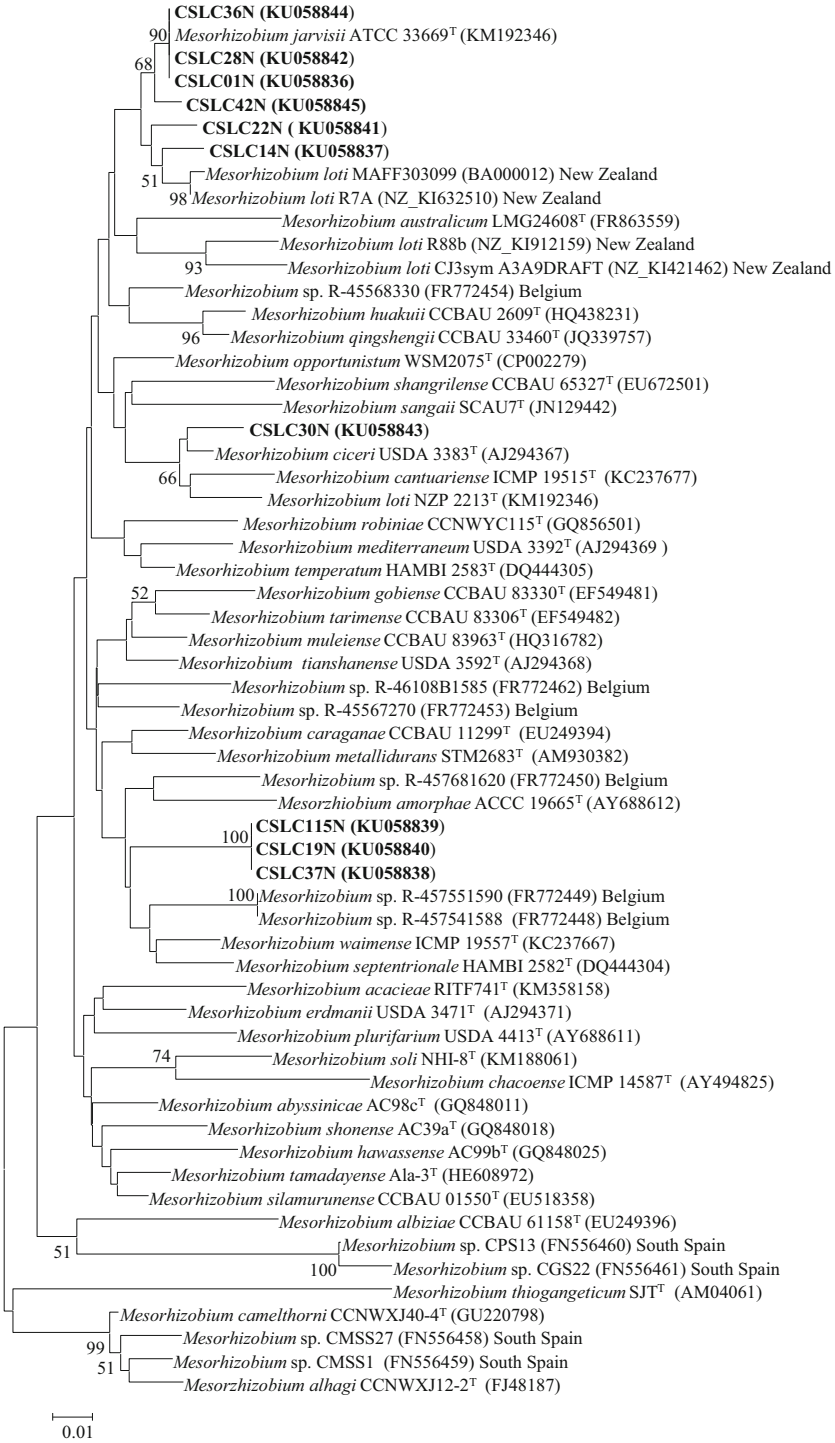

Fig. 3 Neighbour-joining phylogenetic tree based on partial *recA* gene sequences showing the position of strains isolated from *L. corniculatus* nodules in Northwest Spain and other geographical locations with respect to the type strains of the currently described species from genus *Mesorhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt

New Zealand (Kaneko et al. 2000; Kelly et al. 2014; Reeve et al. 2014). One *L. corniculatus* strain isolated in Belgium (De Meyer et al. 2011) is also related to this cluster but it was phylogenetically divergent to our strains. Since this strain has less than 96 % similarity with the type strains of *M. jarvisii* ATCC 33669^T and *M. huakuii* IAM 14158^T, it probably does not belong to these species nor to other species in this cluster, such as *M. australicum* and *M. qingshensii*.

The strains from the 16S rRNA gene cluster IV, CSLC19N, CSLC37N and CSLC115N, have identical *recA* gene sequences and occupy a phylogenetically divergent branch within the cluster that contains the type strains of *M. caraganae* and *M. metallidurans*. They were closely related in the 16S rRNA gene analysis, but this *recA* cluster also contains *M. amorphae*, *M. septentrionale* and *M. waimense*. The closest related species to the three strains from cluster IV

is *M. metallidurans* with 96.4 % similarity, suggesting that they do not belong to this species (Table 1). This cluster includes several strains isolated from *L. corniculatus* in Belgium (De Meyer et al. 2011), but they are phylogenetically divergent from eachother and from our strains.

The strain CSLC30N from 16S rRNA gene cluster VII formed a *recA* gene branch belonging to a cluster containing its closest species in 16S rRNA gene analysis, *M. loti*, isolated from *L. corniculatus* nodules, *M. ciceri* and *M. cantuariense*, being the type strain of *M. ciceri* USDA 3383^T the most closely related with 98.3 % similarity (Table 1). This cluster does not contain other isolates of *L. corniculatus*. We do not know if the strains from this host isolated in Norway (Gossmann et al. 2012) and Sweden (Ampomah and Huss-Danell 2011), which belonged to the same cluster as the strain CSLC30N in the 16S rRNA gene analysis, belong to this *recA* gene cluster since this gene is not available in databases for these strains.

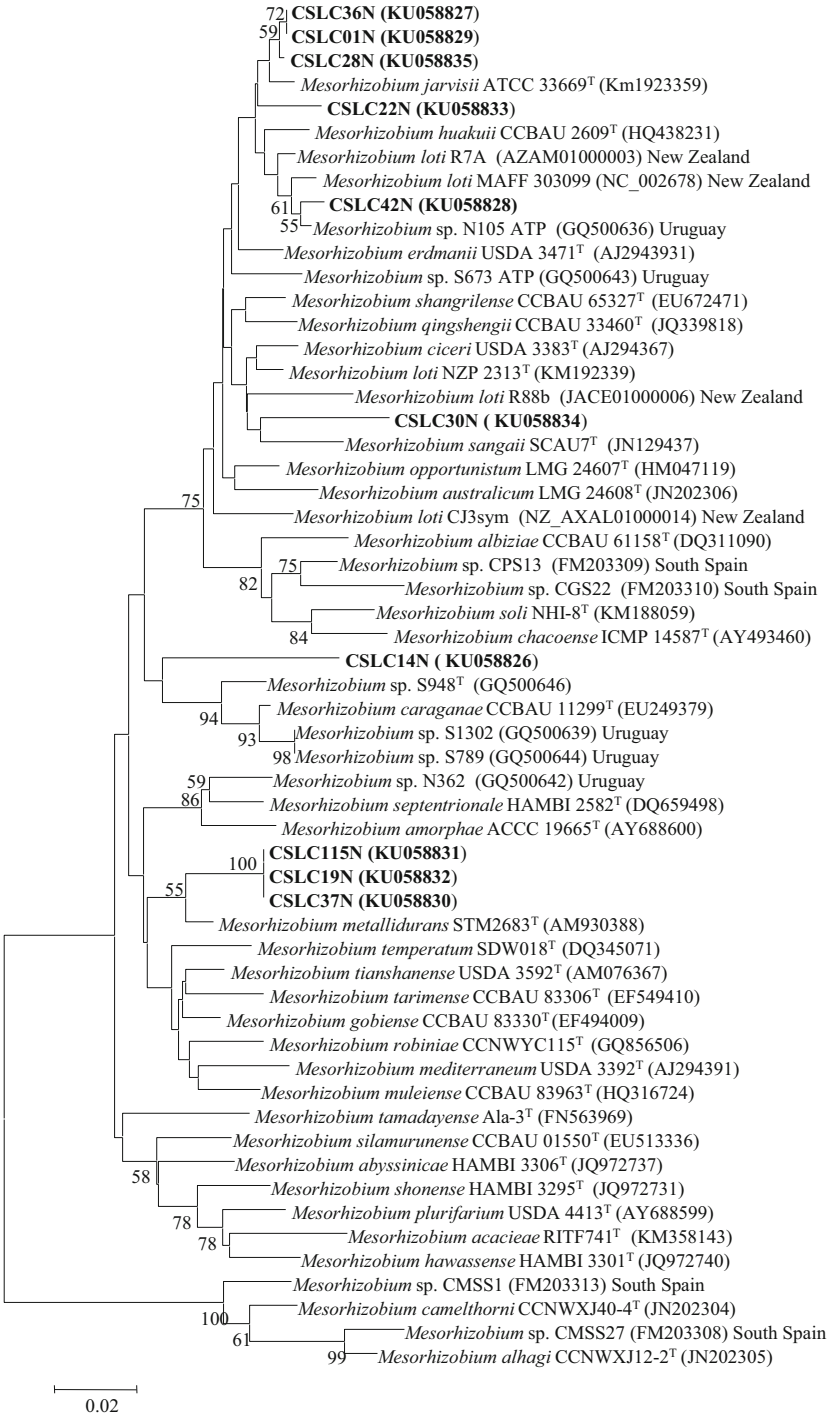

Fig. 4 Neighbour-joining phylogenetic tree based on partial *atpD* gene sequences showing the position of strains isolated from *L. corniculatus* nodules in Northwest Spain and other geographical locations with respect to the type strains of the currently described species from genus *Mesorhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt

The analysis of the *atpD* gene showed that our strains are divided into four clusters with some differences in their distribution with respect to that found after *recA* gene analysis (fig. 4). The strains from 16S rRNA gene cluster I, have different *atpD* gene sequences being particularly divergent in the *atpD* gene of the strain CSLC14N. This strain formed an independent lineage related to the type strain of *M. caraganae* CCBAU 11299^T with 92.5 % similarity. The remaining strains were phylogenetically related to the type strains of two species nodulating *L. corniculatus*, *M. jarvisii* ATCC 33669^T and *M. erdmanii* USDA 3471^T. The phylogenetic lineage formed by the strain CSLC14N belongs to a cluster that also included two strains, S1302 and S789 isolated from *L. corniculatus* nodules in Uruguay (Sotelo et al. 2011). The strains CSLC01N, CSLC36N and CSLC28N were equidistant between *M. jarvisii* ATCC 33669^T and *M. erdmanii* USDA 3471^T (similarity higher than 98.5 % in all cases) (Table 1). This makes difficult the identification of these strains as *M. jarvisii*, as suggested the *recA* gene analysis. The strain CSLC42N was also related to the type strain of *M. jarvisii* ATCC 33669^T with 98.3 % similarity, but in this case *M. erdmanii* USDA 3471^T was less closely related, with 96.7 % similarity (Table 1). This *atpD* gene cluster, also contains the strain CSLC22N from 16S rRNA gene cluster III, which present 97.8 % similarity with respect to its closest relative *M. jarvisii* ATCC 33669^T (Table 1). The *L. corniculatus* nodulating strains R7A and MAFF303099 isolated in New Zealand (Kaneko et al. 2000; Kelly et al. 2014) and the strain N105 isolated in Uruguay (Sotelo et al. 2011) also belong to this cluster. However, the strains R88b and CJ3sym, isolated from *L. corniculatus* nodules in New Zealand, formed two different lineages that are phylogenetically divergent.

The strains from the 16S rRNA cluster IV, CSLC19N, CSLC37N and CSLC115N, have identical *atpD* gene sequences. They occupied a phylogenetically divergent branch within a cluster that also contains the type strain of the species closest to these strains in the 16S rRNA gene analysis, i.e. *M. metallidurans* STM2683^T. This divergent branch does not include *M. caraganae* CCBAU11299^T or those of the species related to our strains in the analysis of the *recA* gene. The similarity with respect to the closest type strain of *M. metallidurans* STM 2683^T was 97.4 % (Table 1), which is a similarity value found for other *Mesorhizobium* species such as *M. ciceri* and *M. loti* or *M. shangrilense* and *M. qingshengii*. Therefore, in agreement with the results of the *recA* gene, the strains from the cluster IV probably belong to a new species of the genus *Mesorhizobium*. Related to this cluster is the strain N362 isolated in Uruguay (Sotelo et al. 2011) which belongs to a cluster containing the type strains of *M. septentrionale* and *M. amorphae*.

The strain CSLC30N from 16S rRNA gene cluster VII represents an *atpD* gene phylogenetic lineage clustering with the same strains than in 16S rRNA and *recA* clusters which were *M. sangaii*, *M. ciceri*, *M. loti*, *M.shangrilense* and *M. qingshensii*. From them, the closest related species to the strain CSLC30N is *M. loti* with 95.4 % similarity whereas in the *recA* gene analysis, the most closely related species was *M. ciceri*. As occurred in the case of *recA* gene, the *atpD* gene is not available for the strains isolated in Norway and Sweden, which belong to the same cluster as the strain CSLC30N in the 16S rRNA gene analysis, and so we are unsure if these strains belong to the same *atpD* gene cluster.

In conclusion, the results of the analysis of the *recA* and *atpD* genes revealed that some strains isolated in Belgium, Uruguay and New Zealand belong to clusters that also contain strains isolated in Northwest Spain. Nevertheless, the strains isolated in South Spain from *L. corniculatus* nodules, in agreement with the results found after 16S rRNA gene analysis, were found to belong to two different clusters that are phylogenetic divergent. The results of the 16S rRNA, *recA* and *atpD* gene analyses showed great phylogenetic diversity in strains nodulating *L. corniculatus* in different continents and countries. However, the endosymbionts of *L. corniculatus* have been analysed from rather few geographical locations and further studies of rhizobial strains nodulating this legume are needed to increase knowledge of this symbiosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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