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Phaseolus vulgaris is nodulated by the symbiovar viciae of several genospecies of *Rhizobium laguerreae* complex in a Spanish region where *Lens culinaris* is the traditionally cultivated legume

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ABSTRACT

Phaseolus vulgaris and *Lens culinaris* are two legumes with different distribution centers that were introduced in Spain at different times, but in some regions *L. culinaris* has been traditionally cultivated and *P. vulgaris* did not. Here we analysed the rhizobia isolated from nodules of these two legumes in one of these regions. MALDI-TOF MS analysis showed that all isolated strains matched with *Rhizobium laguerreae* and the phylogenetic analysis of *rrs*, *atpD* and *recA* genes confirmed these results. The phylogenetic analysis of these core genes allowed the differentiation of several groups within *R. laguerreae* and unexpectedly, strains with housekeeping genes identical to that of the type strain of *R. laguerreae* presented some differences in the *rrs* gene. In some strains this gene contains an intervening sequence (IVS) identical to that found in *Rhizobium* strains nodulating several legumes in different geographical locations. The *atpD*, *recA* and *nodC* genes of all isolated strains clustered with those of strains nodulating *L. culinaris* in its distribution centers, but not with those nodulating *P. vulgaris* in theirs. Therefore, all these strains belong to the symbiovar viciae, including those isolated from *P. vulgaris*, which in the studied region established effective symbiosis with the common endosymbiont of *L. culinaris*, instead to with its common endosymbiont, the symbiovar phaseoli. These results are particularly interesting for biogeography studies, because they showed that, due its high promiscuity degree, *P. vulgaris* is able to establish symbiosis with local symbiovars well established in the soil after centuries of cultivation with other legumes.

Introduction

Phaseolus vulgaris and *Lens culinaris* are two legumes indigenous to Central and South America [14] and to the Middle East [25], respectively. Both legumes coevolved in their respective distribution centers with fast-growing rhizobial strains belonging to genus *Rhizobium*, which are able to nodulate and to fix atmospheric nitrogen in symbiosis with these legumes [42,43]. The symbiovar phaseoli is the main endosymbiont of *P. vulgaris* in its American distribution centers [3,4,8,12,15,40,44], whereas *L. culinaris* is nodulated by the symbiovar viciae in the Middle East distribution centers of this legume [30,31].

Both legumes, *P. vulgaris* and *L. culinaris*, currently worldwide cultivated, were introduced in other continents from their distribution centers at different times in history. In Spain, when *P. vulgaris* was introduced, *L. culinaris* was already cultivated for centuries in several regions, and in those with a dominance of rainfed fields it continues to be the traditionally cultivated legume whereas *P. vulgaris* was never introduced. In this work we selected one of these regions where rhizobia have not been analized to date in order to compare the species and symbiovars nodulating *P. vulgaris* and *L. culinaris*. This study is particularly interesting because two strains

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nodulating *P. vulgaris* in this region have been surprisingly classified into a phylogenetic group comprising *Rhizobium laguerreae* [11,16], a species containing strains of symbiovar viciae nodulating legumes from the cross inoculation group of *Vicia* [6,7,33,38], which has identical *rss* gene, but divergent *recA* and *atpD* genes, that *Rhizobium leguminosarum* [33].

Therefore, the first aim of this work was the identification of strains isolated from *P. vulgaris* and *L. culinaris* nodules in a Spanish region where *L. culinaris* has been traditionally cultivated using MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry). This methodology was shown to be an useful tool to differentiate among fast-growing rhizobial species [10], and its database has been actualized in this work with the addition of several recently described *Rhizobium* species nodulating *L. culinaris* and *P. vulgaris* in their distribution centers [42,43]. The second aim of this work was to investigate the phylogenetic relationships among the isolated strains and those nodulating the same hosts in their respective distribution centers through the analysis of the three core genes *rrs*, *recA* and *atpD* and the symbiotic gene *nodC*, which are commonly used in the phylogenetic analysis of rhizobial species and symbiovars, respectively [27].

Material and methods

Strains isolation and nodulation tests

All strains were isolated in the region named “La Armuña” (Salamanca, Spain) where *L. culinaris* is commonly cultivated. The rhizobia nodulating *L. culinaris* var. “rubia” were isolated from 10 plants recovered in two fields cultivated with this legume. The rhizobia nodulating *P. vulgaris* var. “pinta” were isolated using this legume as trap plant in soil samples collected in the same region because this legume is not cultivated here. All strains were isolated on YMA plates according to Vincent [46] and reinfection experiments were carried out on their host of origin as was previously described [29]. The N content in *P. vulgaris* shoots was measured as described by Mulas et al. [26]. Data were analyzed by one-way analysis of variance, and mean values compared by Fisher’s Protected LSD test (Least Significant Differences) ($P \leq 0.05$).

MALDI-TOF MS performing and data analysis

The sample preparation and the MALDI-TOF MS analysis were carried out as was previously published [10] using a matrix of saturated solution of α-HCCA (Bruker Daltonics, Germany) in 50% acetonitrile and 2.5% trifluoracetic acid. We used amounts of biomass between 5 to 100 mg to obtain the spectra as indicate the manufacturer. The calibration mass were the Bruker Bacterial Test Standards (BTS) which were as follows (masses as averages): RL36, 4365.3 Da; RS22, 5096.8 Da; RL34, 5381.4 Da; RL33meth, 6255.4 Da; RL29, 7274.5 Da; RS19, 10300.1 Da; RNase A, 13683.2 Da and myoglobin, 16952.3 Da.

The score values proposed by the manufacturer are the following: a score value between 2.3 and 3.00 indicates highly probable species identification; a score value between 2.0 and 2.299 indicates secure genus identification and probable species identification, a score value between 1.7 and 1.999 indicates probable genus identification, and a score value <1.7 indicates no reliable identification.

The type strains of recently described species nodulating *P. vulgaris* or *L. culinaris* such as *Rhizobium esperanzae*, *Rhizobium acidisoli*, *Rhizobium hidalgonense*, *Rhizobium ecuadorense*, *Rhizobium lentis*, *Rhizobium binae*, *Rhizobium bangladeshense* and *R. laguerreae* FB206^T were added to our database [10]. To add the strains to the reference library, 36 independent spectra were recorded for each strain (three independent measurements at twelve different spots each). Manual/visual estimation of the mass spectra was performed using Flex Analysis 3.0 (Bruker Daltonics GmbH, Germany) performing smoothing and baseline substraction. Checking existence of flatlines, outliers or single spectra with remarkable peaks differing from the other spectra was done, taking into account that mass deviation within the spectra set shall not be more than 500 ppm. Finally, 20 spectra were selected, removing questionable spectra from the collection. To create peak lists of the spectra, the BioTyper software (Bruker Daltonics GmbH, Germany) was used as described above. The 20 independent peak lists of a strain were used for automated “main spectrum” generation with default settings of the Biotyper software. Thereby, for each library entry a reference peak list (main spectrum) which contains information about averaged masses, averaged intensities, and relative abundances in the 20 measurements for all characteristic peaks of a given strain was created, so a main spectrum displayed the most reproducible peaks typical for a certain bacterial strain.

Cluster analysis was performed based on comparison of strain-specific main spectra created as described above. The dendrogram was constructed by the statistical toolbox of Matlab 7.1 (MathWorks Inc., USA) integrated in the MALDI Biotyper 3.0 software. The parameter settings were: ‘Distance Measure = Correlation’ and ‘Linkage = Average’. The linkage function is normalized according to the distance between 0 (perfect match) and 1000 (no match).

Phylogenetic analyses of rrs, atpD, recA and nodC genes

The amplification and sequencing of *rrs*, *recA* and *atpD*, and *nodC* genes were carried out as indicated by Rivas et al. [32], Gaunt et al. [13] and Laguerre et al. [19], respectively. The sequences obtained were compared with those from the GenBank using the BLASTN program [1]. The obtained sequences and those of related bacteria retrieved from GenBank were aligned using the Clustal W program [39]. The phylogenetic distances were calculated according to Kimura´s two-parameter model [17]. The phylogenetic trees were inferred using the neighbour joining model [34] and MEGA 7.09 [18] was used for all the phylogenetic analyses.

RNA extraction and C-DNA synthesis

Total RNA was extracted from bacterial cells using the MasterPureTM RNA Purification Kit (Epicentre) following manufacturer’s instructions. Starting from 1 μg of total RNA, first-strand cDNA was synthesized by reverse transcription with random hexamers using the ImProm-II^{TM} Reverse Transcription System (Promega). Second-strand synthesis was performed by strand displacement with *Escherichia coli* ligase, DNA polymerase I and RNAse H (all from New England Biolabs). Reverse-transcription negative PCR control reactions (without reverse transcriptase) were run to check for DNA contamination.

Results and discussion

MALDI-TOF MS analysis

MALDI-TOF MS is a reliable method for bacterial identification originally conceived for the identification of pathogenic bacteria [2,41]. Nevertheless, this methodology has proven to be useful for the identification of other bacterial such as those from family *Rhizobiaceae* [10]. The results obtained by MALDI-TOF MS analysis showed that all strains isolated from both *P. vulgaris* and *L. culinaris* nodules matched with the type strain of *R. laguerreae* FB206^T with score values higher than 2.0, including the two strains previously isolated from *P. vulgaris* in the same region, PEPV16 and PEPV40 [11,16] (Table 1). The mathematical analysis of the spectra from the strains of this study, showed that they are divided into seven

Table 1
Results of MALDI-TOF MS analysis of strains analysed in this study.

Strains	MALDI-TOF MS group	Organism (best match)	Score value
Isolated from *P. vulgaris*			
PEPV03	I	*Rhizobium laguerreae* FB206^T	2.131
PEPV04	II	*Rhizobium laguerreae* FB206^T	2.200
PEPV05	II	*Rhizobium laguerreae* FB206^T	2.215
PEPV07	VI	*Rhizobium laguerreae* FB206^T	2.131
PEPV08	II	*Rhizobium laguerreae* FB206^T	2.201
PEPV09	II	*Rhizobium laguerreae* FB206^T	2.244
PEPV10	II	*Rhizobium laguerreae* FB206^T	2.227
PEPV11	VI	*Rhizobium laguerreae* FB206^T	2.080
PEPV13	III	*Rhizobium laguerreae* FB206^T	2.272
PEPV14	II	*Rhizobium laguerreae* FB206^T	2.314
PEPV16	I	*Rhizobium laguerreae* FB206^T	2.181
PEPV18	VI	*Rhizobium laguerreae* FB206^T	2.069
PEPV19	I	*Rhizobium laguerreae* FB206^T	2.181
PEPV22	VI	*Rhizobium laguerreae* FB206^T	2.069
PEPV23	VI	*Rhizobium laguerreae* FB206^T	2.123
PEPV24	I	*Rhizobium laguerreae* FB206^T	2.083
PEPV25	III	*Rhizobium laguerreae* FB206^T	2.259
PEPV28	VII	*Rhizobium laguerreae* FB206^T	2.187
PEPV29	I	*Rhizobium laguerreae* FB206^T	2.050
PEPV30	I	*Rhizobium laguerreae* FB206^T	2.221
PEPV31	VII	*Rhizobium laguerreae* FB206^T	2.131
PEPV33	V	*Rhizobium laguerreae* FB206^T	2.297
PEPV36	III	*Rhizobium laguerreae* FB206^T	2.131
PEPV37	III	*Rhizobium laguerreae* FB206^T	2.136
PEPV38	III	*Rhizobium laguerreae* FB206^T	2.228
PEPV40	V	*Rhizobium laguerreae* FB206^T	2.317
PEPV41	I	*Rhizobium laguerreae* FB206^T	2.277
PEPV42	I	*Rhizobium laguerreae* FB206^T	2.157
Isolated from *L. culinaris*			
MLS01	IV	*Rhizobium laguerreae* FB206^T	2.268
MLS02	V	*Rhizobium laguerreae* FB206^T	2.317
MLS03	V	*Rhizobium laguerreae* FB206^T	2.382
MLS04	IV	*Rhizobium laguerreae* FB206^T	2.415
MLS05	II	*Rhizobium laguerreae* FB206^T	2.091
MLS07	IV	*Rhizobium laguerreae* FB206^T	2.351
MLS09	IV	*Rhizobium laguerreae* FB206^T	2.328
MLS11	VII	*Rhizobium laguerreae* FB206^T	2.151
MLS12	IV	*Rhizobium laguerreae* FB206^T	2.379
MLS15	VII	*Rhizobium laguerreae* FB206^T	2.173
MLS16	VII	*Rhizobium laguerreae* FB206^T	2.166
MLS17	IV	*Rhizobium laguerreae* FB206^T	2.454
MLS18	VII	*Rhizobium laguerreae* FB206^T	2.198
MLS19	IV	*Rhizobium laguerreae* FB206^T	2.397
MLS20	V	*Rhizobium laguerreae* FB206^T	2.259
MLS21	IV	*Rhizobium laguerreae* FB206^T	2.418
MLS22	IV	*Rhizobium laguerreae* FB206^T	2.300
MLSC02	V	*Rhizobium laguerreae* FB206^T	2.270
MLSC03	II	*Rhizobium laguerreae* FB206^T	2.071
MLSC04	VII	*Rhizobium laguerreae* FB206^T	2.186
MLSC07	V	*Rhizobium laguerreae* FB206^T	2.281
MLSC11	II	*Rhizobium laguerreae* FB206^T	2.151
MLSC12	V	*Rhizobium laguerreae* FB206^T	2.317
MLSC14	V	*Rhizobium laguerreae* FB206^T	2.189
MLSC15	II	*Rhizobium laguerreae* FB206^T	2.221
MLSC16	V	*Rhizobium laguerreae* FB206^T	2.266

groups with internal distance levels equal or lower than 0.8 (Fig. S1, Table I). Three of these groups contain strains nodulating *P. vulgaris* and *L. culinaris*, whereas the remaining groups only contain strains nodulating one of the two legumes (Fig. S1). Representative strains from these groups were selected for the phylogenetic analysis of core and symbiotic genes.

Analysis of the rrs gene

The *rrs* genes of the type strains from several species within the phylogenetic group of *R. leguminosarum*, including the species *R. laguerreae*, are identical (Fig. 1). Nevertheless, slight variations were found in the *rrs* genes of some strains belonging to this phylogenetic group isolated in Spain by Villadas et al. [45]. The results of the analysis of this gene in representative strains from MALDI-TOF MS groups also showed that they belong to five groups with some differences in their *rrs* genes (Fig. 1). The *rrs* gene sequence of strain PEPV11 was identical to those of the type strains of *R. laguerreae*, *R. leguminosarum*, *Rhizobium indigoferae*, *Rhizobium anhuiense*, *R. acidisoli*, *R. hidalgonense* and *Rhizobium sophorae* (cluster I) and that of the strain li10 representative of the *rrs* type 1 from Villadas et al. [45]. The *rrs* gene of strain PEPV16 was identical to those of strains presenting the *rrs* type 2 of Villadas et al. [45] represented in the tree by the strain lc10 (cluster II). These strains have a different nucleotide in their *rrs* genes with respect to the strains from cluster I (in the position 1070 of the *rrs* gene sequence of *R. laguerreae* FB206^T, accession number JN558651). The *rrs* type 3 from Villadas et al. [45], represented by the strain vd48, was not found in this study (lineage VI). The *rrs* genes of strains MLS17, PEPV31 and PEPV37 (cluster III) have 2 different nucleotides with

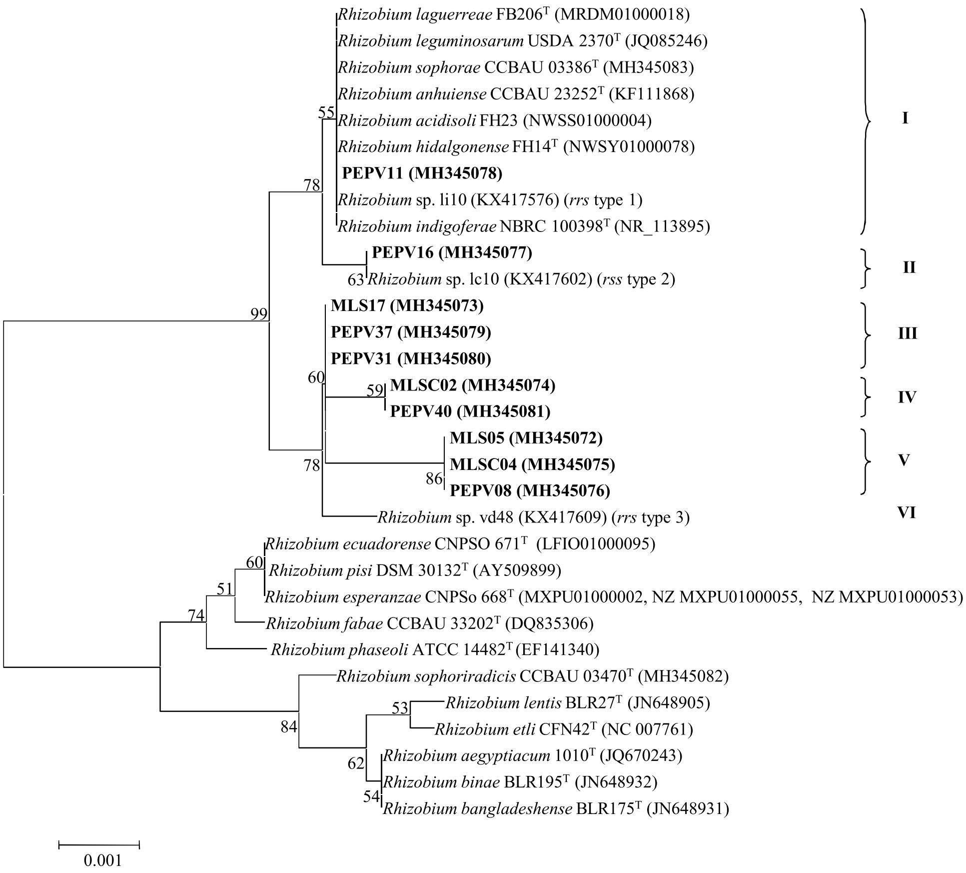

Fig. 1. Neighbour-joining phylogenetic rooted tree based on *rrs* gene sequences (1549 nt including the IVS fragment) showing the taxonomic location of representative strains from different groups of MALDI-TOF MS within the genus *Rhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 1000 nt. Accession numbers from Genbank are given in brackets.

respect to the strains from cluster I (in the positions 942 and 955). The *rrs* genes of strains MLSC02 and PEPV40 (cluster IV) presented 3 different nucleotides (in the positions 940, 942 and 955). Finally, the *rrs* genes of strains PEPV08, MLSC04 and MLS05 (cluster V) have 4 different nucleotides (in the positions 67, 69, 942 and 955) and they also contain an intervening sequence (IVS) of 73 bp located at the beginning of this gene (between the nucleotides 70 and 143). A similar IVS was reported by Willems and Collins [47] in the *rrs* gene of *Rhizobium leucaenae* CFN 299^T (previously named *Rhizobium tropici* IIA). The absence of this IVS in the transcribed rRNA of these four strains was demonstrated by comparing the amplicons corresponding to the *rrs* gene derived from DNA and RNA. All four isolates showed identical *rrs* gene sequences and sizes (data not shown) as was previously reported for other bacteria [28].

A search in Genbank showed that IVS are widely distributed in *rrs* genes of strains from the order *Rhizobiales*, classified into genera *Rhizobium*, *Sinorhizobium* (currently *Ensifer*) and *Agrobacterium* belonging to family *Rhizobiaceae*, *Mesorhizobium* from family *Phyllobacteriaceae* and *Ochrobactrum* from family *Brucellaceae* (Fig. S2A). The IVS of these strains are placed at the same position within the *rrs* gene and have identical sequences at the beginning (7 nt) and at the end (8 nt). Nevertheless, despite the low size of this fragment, their sequences are different in the strains analysed to date.

Analysing in parallel the IVS and the *rrs* genes of strains whose complete sequences (more than 1400 nt) are available in Genbank (Fig. S2A and S2B) we found that several of these strains that have been assigned to the genus *Rhizobium* should be included into the new genera *Neorhizobium*, *Pararhizobium* or *Allorhizobium* [23,24] (Fig. S2B). Some of these strains are also misclassified at species level, as occurs in the case of some strains from *Rhizobium galegae*, *Rhizobium huautlense*, *R. leguminosarum* and *R. tropici* (Fig. S2B). Concerning to the strains isolated from *L. culinaris* by Rashid et al. [30], they are not included in these phylogenetic analyses since the region where the IVS is located is absent in their *rrs* gene available sequences.

The IVS from *rrs* genes can be useful as a phylogenetic marker at species or strain levels [28] and in some groups it can be used to detect fecal pollution in water [9,35,37] being specific for faecal bacteria from different hosts [36]. In our case, the analysis of the IVS showed high variability among the members of order *Rhizobiales* and we did not found a clear relationship between the IVS and a taxonomic affiliation because strains from the same genus (i.e.: *Rhizobium*, *Pararhizobim* and *Neorhizobium*) showed divergent IVS types, whereas those from different genera (i.e.: *Mesorhizobium* and *Ochrobactrum*) showed the same IVS type (Fig. S2A). Nevertheless, the analysis of IVS could be useful for biogeography studies, since in some strains identical IVS are linked to identical *rrs* genes indicating a geographic dispersion of the same species/strains. The best examples are the strains of *R. leucaenae*, which have identical *rrs* genes, including IVS, with independence of their isolation site, America or Europe, suggesting a common origin for all of them probably due to the dispersion of these strains together with the seeds.

The most relevant finding from this work was the existence of strains with different *rrs* gene sequences, two of them identical to those of strains previously isolated in South Spain [45], but others found by first time in this study. Since all of them belong to the phylogenetic group of the type species of genus *Rhizobium*, *R. leguminosarum*, which contains several species with identical *rrs* gene

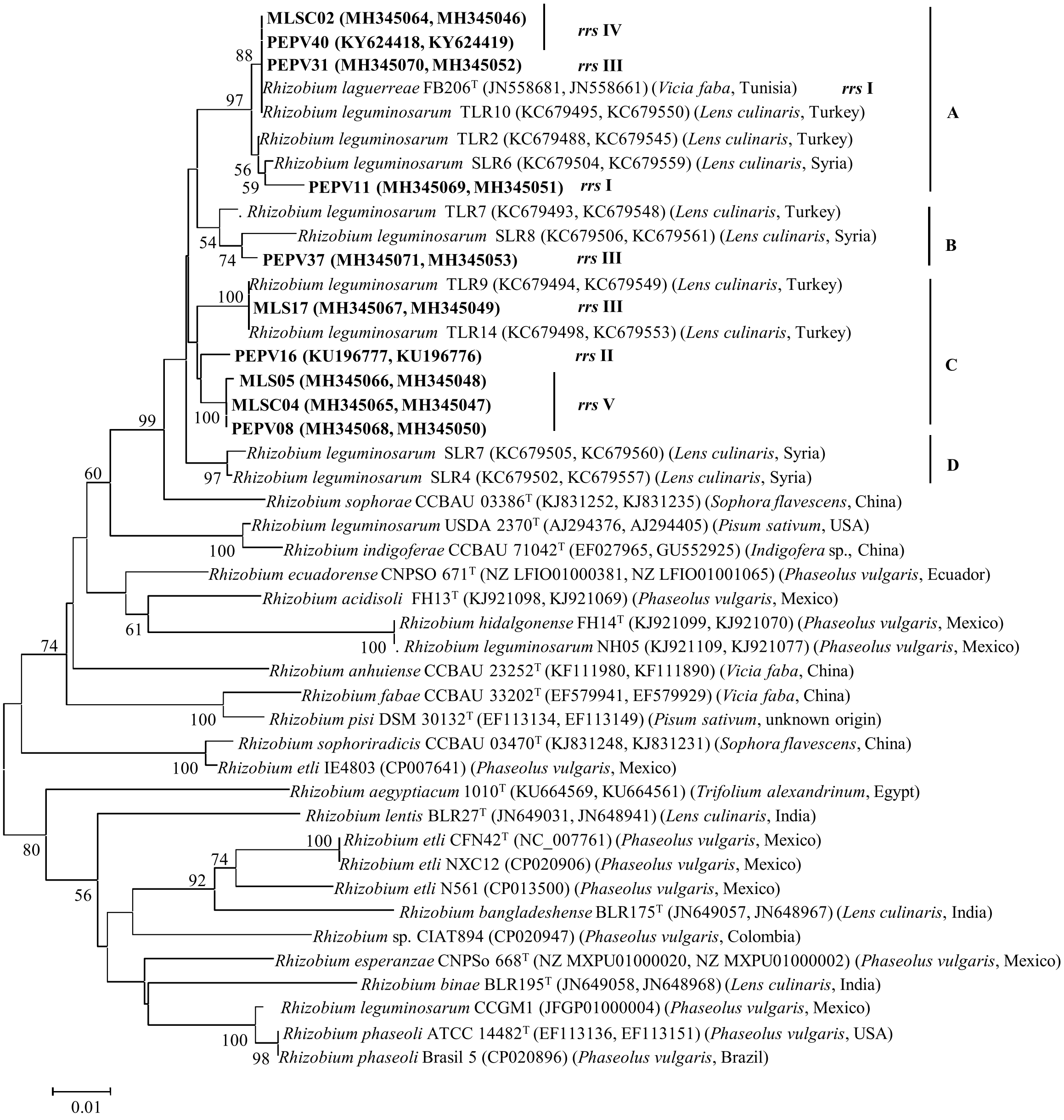

Fig. 2. Neighbour-joining phylogenetic tree based on *recA* and *atpD* concatenated gene sequences (728 nt) showing the position of representative strains from each group within genus *Rhizobium*. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

sequences but phylogenetically divergent *recA* and *atpD* genes, the taxonomic affiliation of the strains from this work should be clarified on the basis of the analysis of these genes.

Analysis of recA and atpD genes

The results of the phylogenetic analysis of *recA* and *atpD* genes showed that, with the exception of strains PEPV40 and MLSC02, which have *recA* and *atpD* genes identical to those of *R. laguerreae* FB206^T, the remaining strains showed similarity values lower than 96% and 99%, respectively. Nevertheless, considering both genes together, the closest related species to our strains is *R. laguerreae* in agreement with the results of MALDI-TOF MS.

As occurred with the type strains of the seven species included into the group of *R. laguerreae*, some strains isolated in this study with identical *rrs* genes carry divergent *recA* and *atpD* genes, as occurs in the case of the strains PEPV11 and *R. laguerreae* FB206^T (*rrs* cluster I) and in that of the strains MLS17, PEPV37 and PEPV31 (*rrs* cluster III). Conversely, the strains PEPV31 (*rrs* cluster III), PEPV40 and MLSC02 (*rrs* cluster IV) have different *rrs* genes, but identical *recA* and *atpD* genes that *R. laguerreae* FB206^T (Fig. 2). Finally, the strain PEPV16 (*rrs* cluster II) and the strains MLS05, MLSC04 and PEPV08 (*rrs* cluster IV) have different *rrs* and *recA* and *atpD* genes. All these strains are distributed into three clusters A, B and C in the phylogenetic tree of concatenated *recA* and *atpD* genes (Fig. 2). The strains from cluster A are closely related to *R. laguerreae* FB206^T, however the strains from clusters B and C have housekeeping genes divergent to those of this type strain and therefore they belong to two genospecies of *R. laguerreae*.

In the analysis of the concatenated *recA* and *atpD* genes we also included representative strains isolated in *L. culinaris* and *P. vulgaris* distribution centers (Fig. 2). The results showed that all strains isolated in this study were phylogenetically related to strains isolated from *L. culinaris* nodules in its distribution centers from Syria and

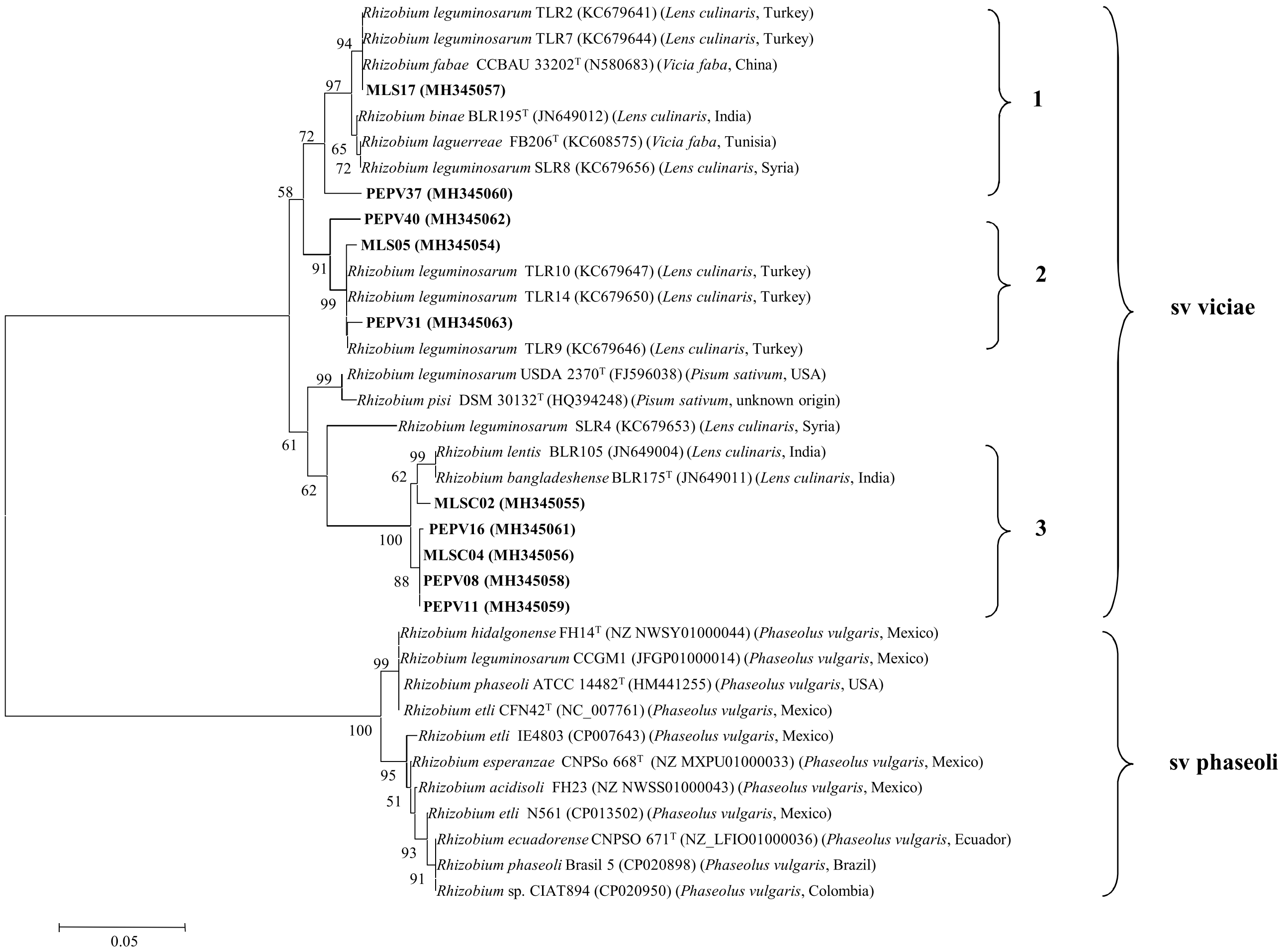

Fig. 3. Neighbour-joining phylogenetic tree based on *nodC* gene sequences (730 nt) showing the position of representative strains from each group within the symbiovars viciae and phaseoli. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

Turkey (Fig. 2). These strains are included in the three clusters, A, B and C, also containing the strains isolated in this study, and additionally they formed the fourth cluster D. Since all these strains are more closely related to *R. laguerreae* their current name *R. leguminosarum* should be changed (Fig. 2). Three of these strains isolated in Turkey, TLR9, TLR10 and TLR14, presented identical sequences of *recA* and *atpD* genes than some of Spanish strains indicating a dispersion of these strains from this distribution center to other geographical locations (Fig. 2). The remaining strains isolated in Syria and Turkey, although they are phylogenetically related with Spanish strains, formed several divergent lineages among them and with respect to these Spanish strains (Fig. 2). The clusters containing the strains isolated from *L. culinaris* and *P. vulgaris* nodules in this work do not contained strains isolated from *P. vulgaris* in its distribution centers from America, including two strains initially classified in the species *R. leguminosarum*, NH05 and CCGM1, which currently belong to *R. hidalgonense* and *Rhizobium phaseoli*, respectively (Fig. 2). The remaining strains isolated from *P. vulgaris* nodules in American countries are distributed in different clusters or lineages closely related to already described American species nodulating this legume.

Therefore, in agreement with the results of the phylogenetic analysis of *rrs* gene, those from that of *recA* and *atpD* genes support that several genospecies within *R. laguerreae* complex are the common endosymbionts of *L. culinaris* in several geographical locations, including its distribution centers, and also of promiscuous legumes, such as *P. vulgaris* when they are cultivated in soils where *L. culinaris* is the common cultivated legume.

nodC gene analysis

The *nodC* gene is currently the main phylogenetic marker for *Rhizobium* symbiovars [27] and the phylogenetic analysis of this gene allowed the differentiation of symbiovars phaseoli and viciae nodulating *P. vulgaris* and *L. culinaris*, respectively (Fig. 3). The results of the *nodC* gene analysis showed that all strains isolated in this study belong to the symbiovar viciae, despite their host of origin (Fig. 3). Nevertheless, these strains belong to three different clusters (1, 2 and 3), all of them encompassing strains isolated from both *L. culinaris* and *P. vulgaris* nodules in this work. The cluster 1 encompassed the type strains of *R. laguerreae*, *Rhizobium fabae* and *R. binae*, the strains PEPV37 and MLS17 and several strains isolated from *L. culinaris* in Syria and Turkey. The cluster 2 contains the strains PEPV31, PEPV40 and MLS05 and three strains isolated in Turkey from *L. culinaris*. The cluster 3 contains the strains PEPV08, PEPV11, PEPV16, MLSC02 and MLSC04 and reference strains for *R. lentis* and *R. bangladeshense*, two species originally isolated from *L. culinaris* nodules. Finally, a strain isolated in Syria from *L. culinaris* nodules formed an independent lineage phylogenetically related with the cluster 3 (Fig. 3). The strains which have housekeeping genes identical to those of strains TLR9, TLR10 and TLR14 do not carried identical *nodC* genes showing a high diversification degree of this gene in different geographical locations. The results of the *nodC* gene analysis demonstrated that the high promiscuity degree of *P. vulgaris* [21,22] allows its establishment of symbiosis with rhizobia nodulating local legumes in regions without history of its cultivation.

The symbiosis established with *P. vulgaris* by the strains from this study, which were able to reinfect their respective hosts (data not shown), was effective since the plants inoculated with representative strains from each *nodC* groups had significantly higher averages of shoot dry matter and nitrogen content that uninoculated controls. Nevertheless, these strains, all belonging to the symbiovar viciae, were significantly less effective that the type strain of *R. tropici* ATCC14482^T, which belongs to the symbiovar phaseoli (Table S1). These results confirmed those previously obtained for several rhizobial genera, which showed that different symbiovars nodulating the same host can have different symbiotic efficiency [5,20,40].

In summary, the obtained results showed that the American legume *P. vulgaris* establishes effective symbiosis with the symbiovar viciae of several genospecies of *R. laguerreae* in a region from Northwest Spain where *L. culinaris* is the traditionally cultivated legume. The phylogenetic analysis of three core genes allowed the differentiation of several genospecies within *R. laguerreae* complex and, unexpectedly, strains with housekeeping genes identical to that of the type strain of *R. laguerreae* presented some differences in the *rrs* gene. The *rrs* genes of some strains have an intervening sequence (IVS) identical to that found in *Rhizobium* strains nodulating several legumes in different geographical locations. The findings from this work showed the need of performing further studies of strains nodulating *P. vulgaris* in different European soils where other legumes than *P. vulgaris* are commonly cultivated in order to have a more complete picture of *Rhizobium*-legume symbiosis.

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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.2018.10.009.

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