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Analysis of rhizobial endosymbionts of *Vicia, Lathyrus* and *Trifolium* species used to maintain mountain firewalls in Sierra Nevada National Park (South Spain)

Pablo J. Villadas[a,1], Ana V. Lasa[a,1], Pilar Martínez-Hidalgo[b,c], José David Flores-Félix[b], Eustoquio Martínez-Molina[b], Nicolás Toro[a], Encarna Velázquez[b,*], Manuel Fernández-López[a]

[a] *Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain*
[b] *Departamento de Microbiología y Genética, Universidad de Salamanca, Salamanca, Spain*
[c] *Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, USA*

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ABSTRACT

Forest fires lead to the annual disappearance of many natural formations that require the creation of firewall areas. They can be maintained by enriching their pastures with attractive plants for grazing livestock, mainly legumes, which have a high protein content and low dependence on N fertilizers due to their ability to establish nitrogen-fixing symbiosis with rhizobia. In this study, the rhizobia isolated from the nodules of six legumes from the genera *Vicia*, *Lathyrus* and *Trifolium* were analysed in a firewall zone established in Lanjarón (Granada) close to the Sierra Nevada National Park (Spain). The results showed a high genetic diversity of the isolated strains that had 3, 16, 14 and 13 different types of *rrs*, *recA*, *atpD* and *glnII* genes, respectively. All strains were phylogenetically close to the species from the *Rhizobium leguminosarum* group, although they were not identified as any of them. The isolated strains belonged to the symbiovars viciae and trifolii but high phylogenetic diversity was found within both symbiovars, since there were 16 and 14 *nodC* gene types, respectively. Some of these strains clustered with strains isolated in other countries and continents, but others formed *atpD*, *recA*, *glnII* and *nodC* clusters and lineages only found to date in this study.

Introduction

Forest fires are a serious problem that cause the annual loss of many natural formations. This is particularly serious when they affect national parks where endemisms or threatened plants are located, as occurs in the case of Sierra Nevada National Park in Southeast Spain. This park is one of the nine Biosphere Reserves in the UNESCO GLOCHAMOST (Global and Climate Change in Mountain Sites) project (http://www.unesco.org/new/en/natural-sciences/environment/ecological-sciences/specific-ecosystems/mountains/glochamost). It constitutes a unique refuge for biodiversity where 2100 plant species have been catalogued as endemics, of which 116 are threatened [7]. Therefore, fires affecting this ecosystem can lead to the disappearance of unrecoverable species, and fire prevention is one of the main objectives of park management. One of the best known preventive practices against fire is the creation and maintenance of firewall areas that can be attained by mechanical means or by livestock grazing [28,47]. However, the Sierra Nevada National Park is a pioneer in Spain for the establishment of firewalls maintained by grazing goats and sheep without mechanical intervention. This requires the enrichment of firewall pastures with plants attractive enough for grazing livestock in order to clean the grass from the area ready for the summer. Within these plants, legumes have advantages over grasses or cereals because they are mainly based on low dependence of N fertilizers and a high protein content [31]. Therefore, it is good practice to sow the pastures with autochthonous legumes whose endosymbionts are already present in the soil and, if necessary, inoculate seeds before sowing in order to ensure their persistence. Since fast growing rhizobia are the easiest candidates

* Corresponding author at: Departamento de Microbiología y Genética, Lab. 209, Edificio Departamental de Biología, Campus Miguel de Unamuno, 37007 Salamanca, Spain. Fax: +34 923 224876.
E-mail address: evp@usal.es (E. Velázquez).
[1] These authors contributed equally to this work.

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to handle for preparing inoculants, the most adequate legumes for sowing firewalls are those that establish symbiosis with these types of rhizobia. Within them, the species of the genus *Rhizobium* establish symbiosis with *Vicia* and *Trifolium*, two legumes that are present in European pastures [22].

Vicia and other legumes from the same cross inoculation group, such as *Pisum*, *Lens* and *Lathyrus*, are nodulated by the symbiovar viciae of several species, such as *Rhizobium leguminosarum*, *Rhizobium pisi*, *Rhizobium fabae*, *Rhizobium laguerreae*, *Rhizobium bangladeshense*, *Rhizobium lentis*, *Rhizobium binae* and *Rhizobium anhuiense* [2,4,8,12–14,21,32,33,35–38,44–46,48,50,51,54–58,61,62,64], whereas *Trifolium* species are nodulated by the symbiovar trifolii of *R. leguminosarum*, *R. pisi* and *R. aegyptiacum* [23,25,34,41,42,44,46,52,63]. Some of these strains have been isolated in North Spain [2,34] and in Central Spain [44–46], but there are no data concerning the rhizobial strains nodulating these hosts in South Spain.

Therefore, the aims of this study were the identification of the rhizobial species and symbiovars nodulating several species of the genera *Vicia*, *Lathyrus* and *Trifolium* naturally present in the Lanjarón region (Granada, South Spain) through the phylogenetic analysis of chromosomal and symbiotic genes, as well as the analysis of their relationships with strains nodulating these legume hosts in other geographical locations.

Materials and methods

Isolation of rhizobial strains and nodulation tests

Sampling was carried out in March 2013 at Lanjarón (36° 56′ N, 3° 29′ W) in an experimental plot close to a firewall area in order to isolate rhizobia establishing symbiosis with different grassland legumes naturally present in this mountainous area. The herbaceous part of the soil was removed and the soil samples were conserved at 4 °C. Legume seeds of *Lathyrus cicera*, *Lathyrus intricatus* (*Lathyrus hygrophilus*), *Vicia sativa* subsp. *angustifolia* (*Vicia amphicarpa*), *Vicia disperma*, *Trifolium cherleri* and *Trifolium glomeratum* were collected in the same area. The seeds of these legumes were sterilized by immersion in 1% sodium hypochlorite for 10 min, and subsequently washed five times with sterile distilled water before soaking in water for 3 h. Subsequently, the seeds were washed again with sterile distilled water and transferred into sterile petri dishes containing sterile filter paper. The seeds were germinated in these plates for 2–4 days at 20 °C in darkness, and a minimum of 1 mL sterile water was maintained on each plate during this time.

The germinated seeds were transferred to 12-cm diameter pots (four seedlings per pot) using six pots for each plant species. These pots were prepared with a 3:1 ratio of washed sterile sand and a mixture of the soils sampled from the firewall area. The sand and the pots were autoclaved once a day for three consecutive days to avoid proliferation of strains with resistant forms (spores). After sterilization, the sand was mixed with the soil and poured into the pots. To avoid moisture loss and fungal contamination, the surface of each pot was covered with sterile perlite. The pots were maintained for four weeks in a growth chamber with a day/night cycle of 16/8 h and 22/16 °C, and periodic watering with sterilized water [7]. After this time, the plants were removed from the pots, the roots were washed with sterile water, and the nodules showing a pink colour, indicating they were effective for nitrogen fixation, were collected individually. Isolation of rhizobia was carried out on tryptone-yeast (TY) medium according to Beringer [6].

The nodulation experiments were carried out on the same legumes from which the rhizobial strains were isolated, as previously described [34].

RAPD fingerprinting

For DNA extraction, the cells incubated for 24 h in liquid TY medium [6] at 28 °C were centrifuged at 10,000 × *g* for 30 s and washed with 0.1% Sarkosyl in TE buffer (Tris–HCl, 10 mM; EDTA, 1 mM; pH 8) in order to facilitate cellular lysis. DNA was extracted with the RealPure genomic DNA extraction kit (Durviz, S.L.U., Valencia, Spain) following the manufacturer's instructions. Briefly, pelleted cells were washed with 0.1% L-laurylsarcosine in TE buffer at pH 8. The cells were lysed by incubation for 5 min at 80 °C and then subjected to RNAse treatment at 37 °C for 60 min. The proteins were removed, and the DNA was precipitated with 2-propanol. RAPD patterns were obtained as previously described [40] using the primer M13 (5′-GAGGGTGGCGGTTCT-3′). PCR conditions were: 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 10 μL of each PCR product was electrophoresed on 1.5% agarose gel in TAE buffer (40 mM Tris, 0.1142% acetic acid, 0.2 mM EDTA, pH 8.5) at 6 V/cm, stained in a solution containing 100 μL L^{-1} GelRed™ (Biotium Inc., Hayward, CA), and photographed under UV light. The standard used as a size marker was Φ29 digested with *Hind*III. A dendrogram was constructed based on the matrix generated using the UPGMA method and the Pearson coefficient with Bionumerics version 4.0 software (Applied Maths, Austin, TX).

Sequence analysis of rrs, atpD, recA, glnII *and* nodC *genes*

The *rrs* gene was amplified and sequenced according to Rivas et al. [39], the *atpD* and *recA* genes as described by Gaunt et al. [11], the *glnII* gene as described by Vinuesa et al. [59], and the *nodC* gene as described by Laguerre et al. [20]. PCR amplifications were performed with the Master *Taq* Kit® (5PRIME, Germany) following the manufacturer's instructions. The bands corresponding to the different genes were purified directly from the gel by elution of the excised band and filtration through Silica gel columns using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) following the manufacturer's instructions. The sequence reaction was performed on an ABI PRISM 3130xl Genetic Analyzer using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., USA) as supplied by the manufacturer. The sequences obtained were compared to those held in GenBank by using the BLASTN program [1]. They were aligned by using Clustal W software [54]. Distances calculated according to Kimura's two-parameter model [18] were used to infer phylogenetic trees with the neighbour-joining method [49] and MEGA5 software [53]. Confidence values for nodes in the trees were generated by bootstrap analysis using 1000 permutations of the data sets. The accession numbers of the sequences obtained in this study are shown in Table S1.

Results and discussion

Isolated strains and RAPD fingerprinting

A total of 43 strains were isolated from *V. amphicarpa*, 38 strains from *V. disperma*, 36 strains from *L. intricatus*, 40 strains from *L. cicera*, 42 strains from *T. cherleri* and six strains from *T. glomeratum*. They were all able to reinfect the host from which they were isolated and formed effective (pink) nodules. The genetic diversity of the isolated strains was analysed by RAPD fingerprinting, a technique commonly applied to the genetic diversity analysis

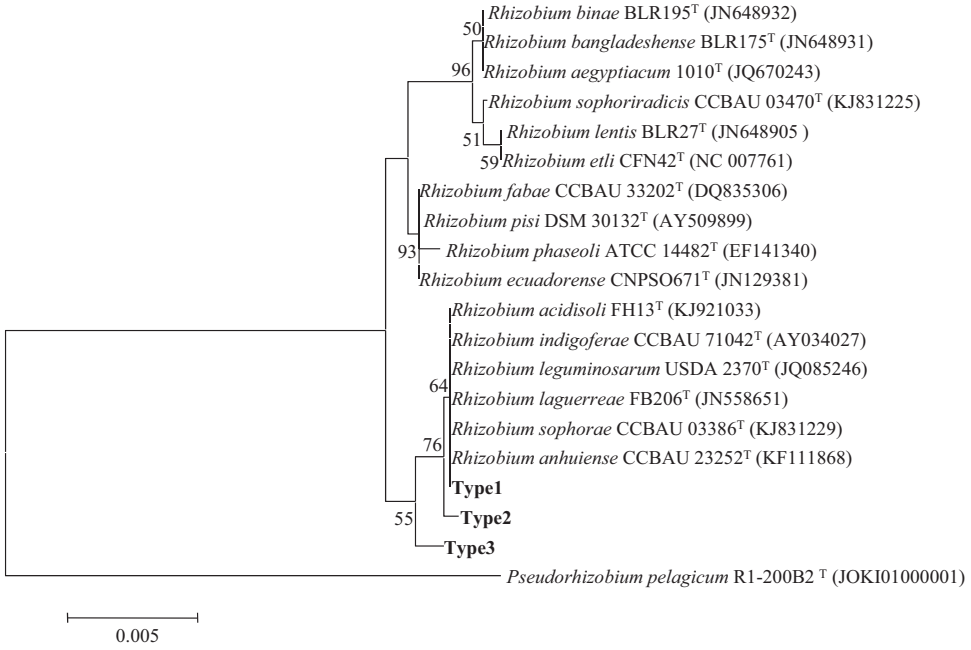

Fig. 1. Neighbour-joining phylogenetic rooted tree based on *rrs* gene sequences showing the position of the studied strains and the closest related type strains of the validly described *Rhizobium* species. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitutions per 1000 nt. Strains corresponding to the different *rrs* types are given in Table S3 and the accession numbers in Table S1.

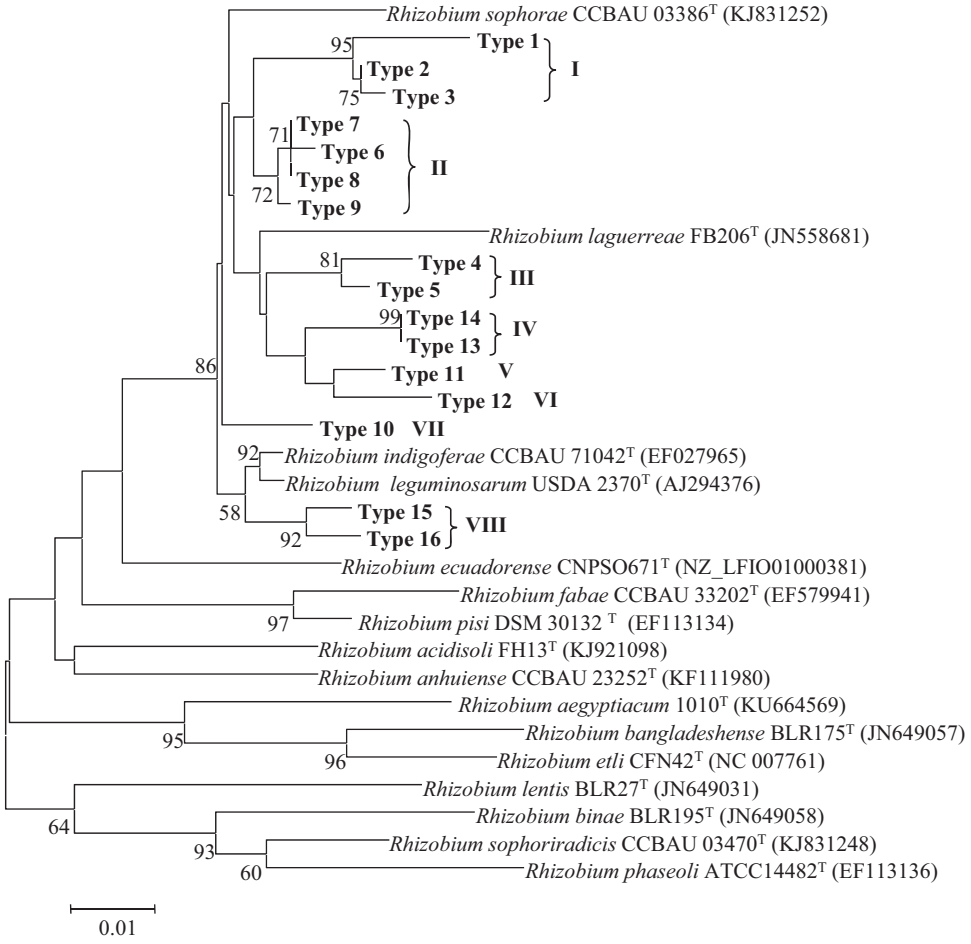

Fig. 2. Neighbour-joining phylogenetic tree based on the *recA* gene sequences showing the position of the studied strains and the closest type strains of the validly described *Rhizobium* species. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Strains corresponding to the different *recA* types are given in Table S3 and the accession numbers in Table S1.

of rhizobial strains isolated from *Vicia* and *Trifolium* [2,34,50,57]. The results of the RAPD analysis (Table S2, Fig. S1) showed a high diversity of the isolated strains and the existence of 54 groups or branches with a similarity lower than 70% from which representative strains of different hosts were selected for core and symbiotic gene analyses.

Analysis of the rrs *gene*

The *rrs* (16S rRNA) gene is the current basis of rhizobial classification within the family *Rhizobiaceae* [19] in which several new genera have recently been described and several others have been recovered following the analysis of this gene. Currently, this family includes the old genera *Rhizobium*, *Carbophilus*, *Chelatobacter*, *Ensifer*, *Sinorhizobium* (which contains remnant species not reclassified into the genus *Ensifer*) and *Shinella* [3,19], the recovered genera *Allorhizobium* and *Agrobacterium* [27], and the new genera *Ciceribacter* [16], *Neorhizobium* [26], *Pararhizobium* [27] and *Pseudorhizobium* [17]. Therefore, the analysis of the 16S rRNA gene is essential to classify legume nodule endosymbionts at the genus level.

All strains isolated in this study from *Vicia*, *Lathyrus* and *Trifolium* had closely related *rrs* genes and belonged to the phylogenetic group of *R. leguminosarum*, which is the type species of the genus *Rhizobium* (Table S3, Fig. 1). Most strains isolated in this study were *rrs* type 1 whose sequences were identical to those of the type strain of *R. leguminosarum*, the most common endosymbiont of *Vicia* and *Trifolium* in Spain [2,34], *Rhizobium indigoferae* nodulating several legumes in China [60], *R. laguerreae* nodulating *Vicia* in different continents [48], *R. anhuiense* nodulating *Vicia* and *Pisum* in China [64], *Rhizobium sophorae* nodulating *Sophora* in China [15], and *Rhizobium acidisoli* nodulating *Phaseolus* in Mexico [43]. The *rrs* gene of the strains showing *rrs* type 2 differed by one nucleotide compared to those with *rrs* type 1, and the strains with *rrs* type 3 differed by three nucleotides with respect to strains that had types 1 and 2 (Fig. 1). Considering the fact that several recently described species within the genus *Rhizobium* have identical *rrs* genes, the strains showing *rrs* types 2 and 3 could belong to undescribed species within this genus. Nevertheless, the high similarity of this gene in the species of the *R. leguminosarum* phylogenetic group makes the analysis of other genes necessary for the differentiation of these species. This was first observed in the revision of the taxonomic status of the species *R. leguminosarum* in which two housekeeping genes, *recA* and *atpD*, were used to differentiate this species from its closest relative *R. pisi* [33].

Analysis of recA, atpD and glnII genes

The *recA* and *atpD* genes were included in the most recent *Rhizobium* species descriptions, and particularly in those belonging to the *R. leguminosarum* phylogenetic group, which harboured identical *rrs* genes but divergent *recA* and *atpD* genes, such as *R. laguerreae* [48], *R. sophorae* [15], *R. anhuiense* [64] and *R. acidisoli* [43]. Only *R. indigoferae* [60] is probably a later synonym of *R. leguminosarum* considering the closeness of the *recA* and *atpD* genes between the type strains of these species [5,9]. The *glnII* gene has also been included in the description of recent new species of the *R. leguminosarum* group [15,43,64] and it is currently available for the type strains of several *Rhizobium* species, but not for all, and therefore the three genes were analysed separately in this study.

The analyses of these genes in the strains isolated in this work showed that they presented 16, 14 and 13 different types of *recA*, *atpD* and *glnII* genes, respectively (Table S3, Figs. S2–S4). Some strains with different *recA*, *atpD* and *glnII* types grouped with similarity values higher than 99%, but most of them formed groups with lower identity values or formed independent lineages (Figs. S2–S4).

Table 1
Types and clusters for the strains isolated in this study.

rrs types	*recA* types	*recA* clusters	*atpD* types	*atpD* clusters	*glnII* types	*glnII* clusters	*nodC* types within sv viciae	*nodC* clusters within sv viciae	*nodC* types within sv trifolii	*nodC* clusters within sv trifolii
1	1, 2, 3, 6, 7, 8, 9	I, II	1, 2, 3, 13, 14	I, II	1, 2, 3, 5, 6, 8	I, III, IV	1, 3, 4, 5, 7, 8, 9, 10, 11, 13, 14, 15, 16	I, II, III, IV, V, VI, VII, IX, X	1, 2. 3, 4, 7, 9, 11, 12, 13, 14	I, III, IV
2	15, 16	VIII	4, 5	VI	4	II	1, 8, 9, 12	I, VII, VIII, IX	8	II
3	4, 5, 10, 11, 12, 13, 14	III, IV, V, VI, VII	6, 7, 8 9, 10, 11, 12	III, IV, V	7, 9, 10, 11, 12, 13	IV, V	1, 2, 6	I, II	5, 6, 10	I, IV

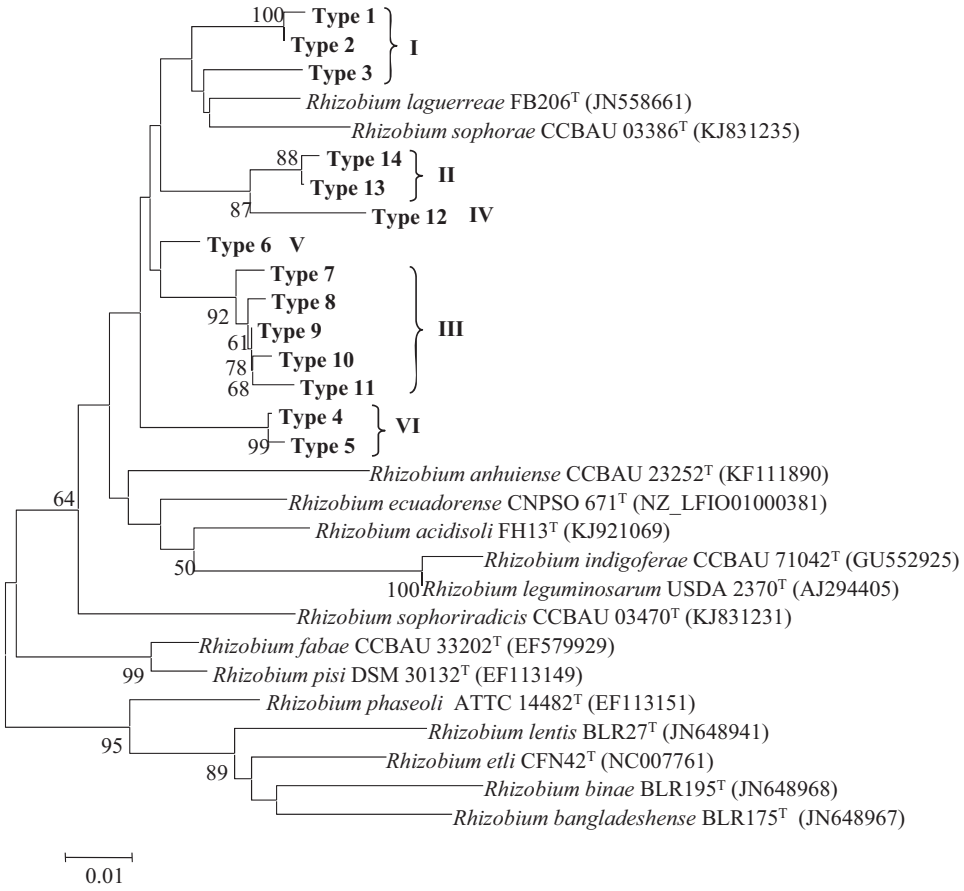

Fig. 3. Neighbour-joining phylogenetic tree based on the *atpD* gene sequences showing the position of the studied strains and the closest type strains of the validly described *Rhizobium* species. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Strains corresponding to the different *atpD* types are given in Table S3 and the accession numbers in Table S1.

The comparison of the *recA*, *atpD* and *glnII* types with the type strains of closely related *Rhizobium* species in the *rrs* gene analysis confirmed that all strains isolated in this study were related to the species of the *R. leguminosarum* phylogenetic group. Nevertheless, they could not be assigned to any of these species because the distances of the *recA*, *atpD* and *glnII* genes were similar or higher than those found between different species, which had similarity values for these genes lower than 97% (Figs. 2–4). The isolated strains were distributed into several clusters with internal similarities higher than 97% that included different strains depending on the analysed gene. In the case of the *recA* gene, eight phylogenetic groups or lineages were found, whereas only six and five phylogenetic groups or lineages were found after *atpD* and *glnII* gene analyses, respectively (Figs. 2–4).

Most of the strains showing *rrs* type 1 were included within cluster I in the *recA* and *atpD* gene analyses, and cluster I and lineage III in the *glnII* analysis (Tables 1 and S3). The closest species for strains of cluster I were *R. laguerreae*, with similarity values lower than 97% in the *atpD* gene, and *R. leguminosarum* with less than 98% similarity in the *recA* and *glnII* genes (Table S3, Figs. 2–4). In the case of strains from *glnII* lineage III, with only 95% similarity with respect to those from cluster I, the most closely related species was *R. laguerreae* with 95.8% similarity. Therefore, all these strains should be included in a single taxon, despite the phylogenetic divergence of strains from *glnII* lineage III, because several strains were distributed among the clusters and lineages for the three analysed genes. This taxon could not be assigned to any of the described species from the *R. leguminosarum* phylogenetic group when the distances commonly found among these species were considered.

The remaining strains with *rrs* type 1 belonged to cluster II in the *recA* and *atpD* gene analyses and corresponded to those from *glnII* cluster IV (Tables 1 and S3). The closest relatives of these strains were *R. leguminosarum* with less than 98% similarity in the *recA* gene and *R. laguerreae* with less than 96% and 95% in the *atpD* and *glnII* genes, respectively. Therefore, the strains from the mentioned clusters, with internal similarity values higher than 99% in the *recA*, *atpD* and *glnII* genes, could not be assigned to any described species of the genus *Rhizobium* and belonged to a different taxon phylogenetically divergent to that constituted by the remaining strains from *rrs* type 1 (Figs. 2–4).

The strains showing *rrs* type 2 were included in clusters VIII and VI in the phylogenetic trees of the *atpD* and *recA* genes, respectively, with internal similarities higher than 98%, and they corresponded to *glnII* lineage II (Tables 1 and S3). The closest related species was *R. leguminosarum* for both *recA* and *glnII* genes, with similarity values higher than 98%. However, in the case of the *atpD* gene the strains were phylogenetically divergent from all species, sharing similarities of lower than 96% with respect to *R. laguerreae*, which was the most closely related species (Figs. 2–4). Therefore, these strains could represent a novel species of the genus *Rhizobium* because, despite the fact that their *recA* and *glnII* genes were closely related to those of certain described species from the *R. leguminosarum* group, they had different *rrs* and *atpD* genes.

The strains with *rrs* type 3 belonged to *glnII* cluster V and lineage IV, *recA* clusters III and IV and lineages V–VII, and *atpD* cluster III and lineages IV and V (Tables 1 and S3). The similarities found among strains with *rrs* type 3 were higher than 94% in the case of the *atpD* gene, 96% in the case of the *recA* gene and 97% in the case

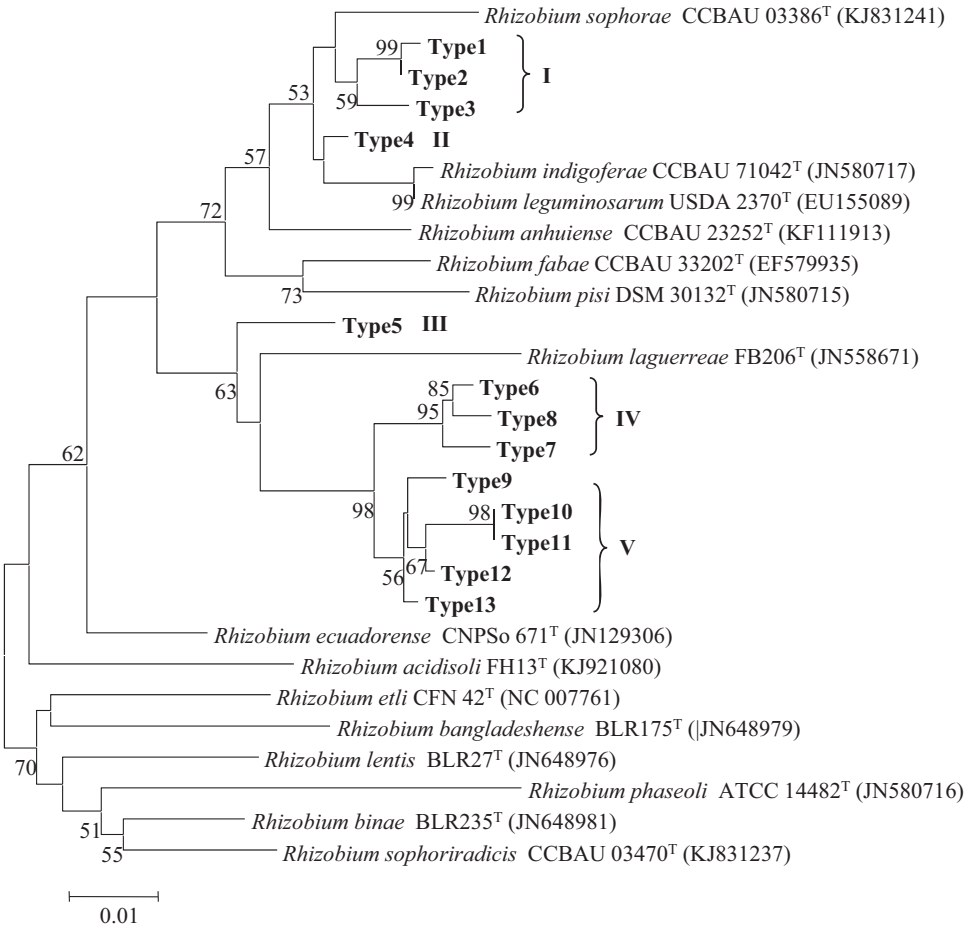

Fig. 4. Neighbour-joining phylogenetic tree based on the *glnII* gene sequences showing the position of the studied strains and the closest type strains of the validly described *Rhizobium* species. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Strains corresponding to the different *atpD* types are given in Table S3 and the accession numbers in Table S1.

of the *glnII* gene. Their closest related species were *R. sophorae* and *R. laguerreae* for the *atpD* and *glnII* genes, respectively, with less than 97% similarity in both cases, and *R. leguminosarum* in the *recA* gene with less than 98% similarity (Figs. 2–4). These values, which are within the current limits for *Rhizobium* species differentiation, and particularly the differences found in the *rrs* gene with respect to the species from the *R. leguminosarum* group, supported that the strains with *rrs* type 3 could belong to a novel species within the genus *Rhizobium*. Nevertheless, the delineation of this species was unclear since the strains included in *atpD* cluster III (internal similarities higher than 97%) had phylogenetically divergent *recA* and *glnII* genes, particularly the strains from *glnII* type 7, which were more related to the strains of cluster IV than those of cluster V in the phylogenetic analysis of this gene. Moreover, the strains showing *atpD* type 6 (lineage V) and *recA* type 10 (lineage VII) constituted phylogenetically divergent lineages.

Therefore, considering the similarity values of the *rrs*, *recA*, *atpD* and *glnII* genes commonly found among the described species of the *R. leguminosarum* group, the strains from this study could not be assigned to any of them and, although some gene sequences were at the limit for species differentiation, they could represent several new species within the genus *Rhizobium*.

A comparison with the *recA*, *atpD* and *glnII* genes of strains isolated from *Vicia*, *Pisum*, *Lens*, *Lathyrus* and *Trifolium* nodules in other geographical locations showed that some of them grouped with the strains from this study with similarity values higher than 98%. As occurred in the case of the type strains, particularly for the *glnII* gene, only one or two of these genes were available for certain strains isolated in other studies and they were also analysed separately (Figs. S5–S7).

The *recA* clusters I and II comprised strains isolated from *Trifolium*, *Vicia* and *Lathyrus* in this study and several European strains, such as those nodulating *Trifolium* in the Cyclades Islands (Greece), Belgium and North Spain, *Pisum* in Poland and Turkey, *Vicia* in Belgium and Northern Spain, *Lathyrus* in Belgium, *Lens* in Germany and *Cicer canariense* in North Spain, where surprisingly this host is nodulated by strains from the *R. leguminosarum* group [24]. Clusters IV–VII grouped European and African strains isolated from nodules of *Trifolium*, *Vicia* and *Lathyrus* in this current study, and from *Vicia* nodules in North Spain and Morocco. Cluster VIII comprised strains isolated from several hosts in our study, as well as others isolated in Europe and Asia, such as those nodulating *Vicia* in Belgium and *Lathyrus* in China. Cluster III, which grouped strains isolated from *Vicia* and *Trifolium* in this study, has only been found to date in Spain (see Fig. S5).

The *atpD* clusters I and VI were composed of strains isolated from all hosts in this study and several strains isolated in Asia and Europe, including strains nodulating *Lathyrus* in China, *Lens* in Germany, *Trifolium* in the Cyclades Islands (Greece) and *Trifolium* and *C. canariense* in North Spain. Cluster II contained strains only found to date in Europe isolated from nodules of *Pisum* in Poland, *Lens* in Germany and *Vicia* and *C. canariense* in North Spain. Cluster III and the lineages IV and V contained strains isolated from *Trifolium*, *Lathyrus* and *Vicia* nodules in our study and from *Vicia* nodules in North Spain, which have been found to date only in Spain (Fig. S6).

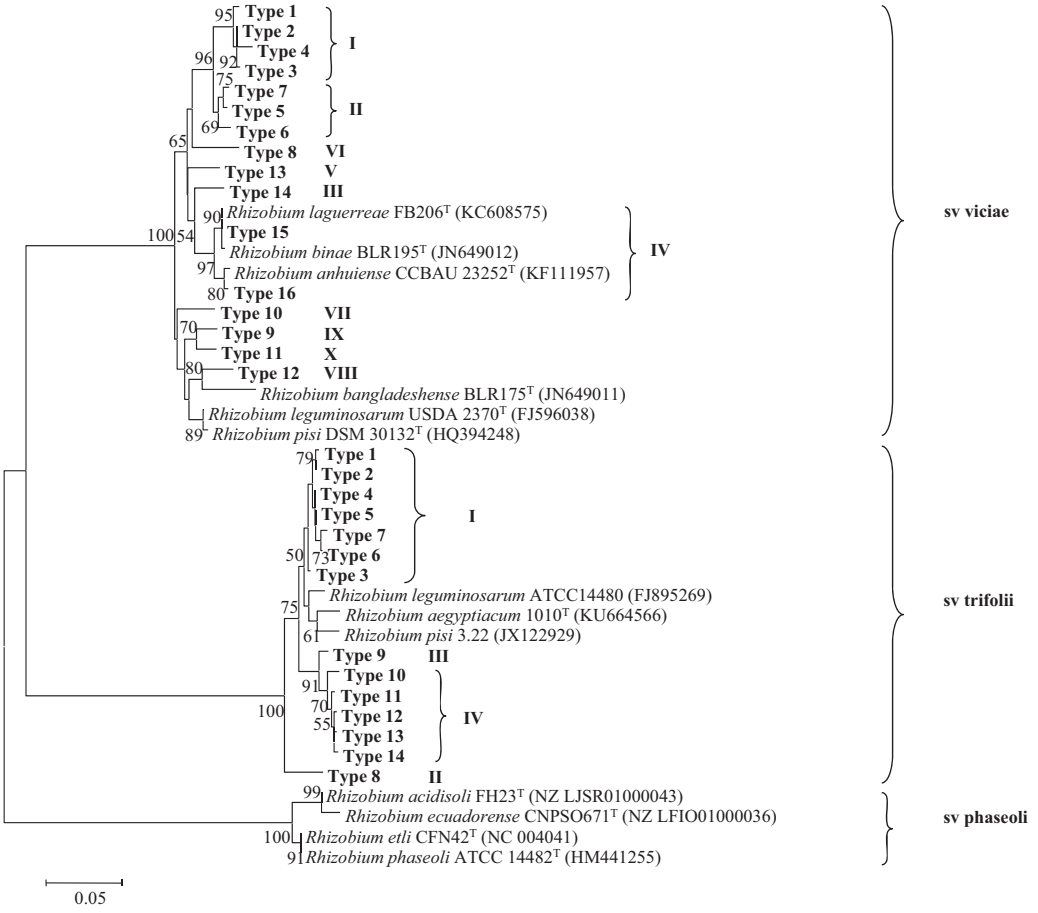

Fig. 5. Neighbour-joining phylogenetic tree based on *nodC* gene sequences showing the position of the studied strains within symbiovars viciae and trifolii. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitutions per 100 nt. Strains corresponding to the different *nodC* types are given in Table S3 and the accession numbers in Table S1.

The *gnlII* clusters I and III contained strains isolated from nodules of all hosts from this study together with several strains isolated in European countries, such as those isolated from nodules of *Vicia* in Belgium and *Trifolium* in the Cyclades Islands (Greece). Cluster II contained strains isolated in this study from *Vicia*, *Lathyrus* and *Trifolium* nodules and the type strains of *R. leguminosarum* and *R. indigoferae*, which were isolated from *Pisum* in USA and *Kummerowia* in China, respectively. Clusters IV and V comprised strains isolated in this study from nodules of *Vicia*, *Lathyrus* and *Trifolium*, as well as several European and African strains isolated from nodules of *Vicia* in France, Belgium and Morocco (Fig. S7).

Collectively, the analyses of *recA*, *atpD* and *glnII* genes showed that most clusters grouped European strains together with those isolated in this current study, but also many clusters that included strains isolated in North Africa (Morocco), a country geographically close to Spain, whereas Asian strains were found only in a low number of clusters. It is remarkable that, despite the fact that legumes from the *Vicia* cross-inoculation group have been widely studied worldwide, several *recA*, *atpD* and *glnII* types have been found to date only in Granada (South Spain).

Considering the results of the core gene analyses, it could be concluded that the *R. leguminosarum* group was highly diverse from a phylogenetic point of view, since it was widely distributed in different countries and continents. From a taxonomic point of view, the results of these studies showed that it is necessary to be prudent with the description of new species within this group due to the high proportion of strains that belonged to different phylogenetic lineages depending on the analysed gene and to the fact that some of them were distributed among different clusters. These results, which suggested the existence of genetic recombination throughout the evolution of a specific ecosystem, make the classification of strains into a new or an already described species difficult in some cases within the phylogenetic group of *R. leguminosarum*. The taxonomic status of this group should be clarified in future studies with the description of new species or subspecies in order to include the phylogenetically divergent groups within the currently accepted species. Although subspecies is a non-existing taxonomic category in the rhizobia, the description of subspecies could clarify the taxonomic status of the multiple genospecies found in the *R. leguminosarum*-related group.

Analysis of the symbiotic nodC gene

The *nodC* gene is a phylogenetic marker commonly used to define symbiovars in *Rhizobium* [29,42]. This gene allows the differentiation of three symbiovars (sv.) within the species *R. leguminosarum* from which sv. phaseoli is the most common endosymbiont of *Phaseolus*, sv. viciae that of *Vicia*, *Pisum*, *Lens* and *Lathyrus*, and sv. trifolii that of *Trifolium* [10]. As expected, after the analysis of the *nodC* gene the strains isolated in our study from *Vicia* and *Lathyrus* belonged to sv. viciae and those isolated from *Trifolium* to sv. trifolii, whose *nodC* genes showed similarities lower than 90% (Fig. 5). Within both symbiovars viciae and trifolii, the similarity values were higher than 90%, but the genetic diversity of isolated strains was high with 16 and 14 *nodC* gene types, respectively (Table S3, Figs. S8 and S9).

The phylogenetic analysis of the *nodC* gene showed that the strains isolated in Granada were distributed into three phylogenetic clusters (similarity values higher than 97%) and seven lineages within sv. viciae, and into two phylogenetic clusters (similarity values higher than 98%) and two lineages within sv. trifolii (Fig. 5). Some strains were closely related to certain species from the *R. leguminosarum* phylogenetic group, as occurred in the case of the strain with *nodC* type 15 that grouped with the type strains of *R. binae* isolated in Asia (India) and *R. laguerreae* isolated in Africa (Tunisia). In addition, the strains with *nodC* type 16 grouped with the type strain of *R. anhuiense*. However, the *nodC* genes of the type strains from *R. pisi* and *R. leguminosarum* were phylogenetically distant to our strains (Fig. 5). Concerning the strains from sv. trifolii, the type strains from *R. pisi* and *R. aegyptiacum* were not closely related to the strains isolated in our study from *Trifolium* nodules (Fig. 5).

A comparison of the *nodC* genes carried by strains from sv. viciae isolated in this current study and in other geographical locations is shown in Fig. S10. Clusters I and II included strains isolated from nodules of *Lathyrus* and *Vicia* in our study, several European strains isolated from nodules of *Lens* in Turkey and *Vicia* in Central and North Spain, and several African strains isolated from *Vicia* nodules in Morocco. Cluster III grouped strains isolated from nodules of *Vicia* in this study and several strains isolated in Asia, America and Europe from nodules of different legumes, such as *Pisum* in India, *Lathyrus* in Central Spain and *Vicia* in South Korea, China, Peru, Belgium and Central Spain. Cluster IV grouped strains found in nodules of *Lathyrus* and *Vicia* in this study and several strains isolated in Asia, Africa, America and Europe from nodules of, for example, *Pisum* in the UK and China, *Lens* in Germany, Turkey and India, and *Vicia* in Japan, Tibet, China, Tunisia, Ethiopia, Morocco, Peru, Belgium and Central Spain. Cluster V comprised strains isolated in this study from *Lathyrus* nodules and in Morocco (Africa) from *Vicia* nodules. Clusters VI, VII, IX and X contained strains isolated from *Lathyrus* and *Vicia* nodules in this study and other European strains isolated from nodules of *Pisum* in Poland, *Lens* in Germany, and *Vicia* in Belgium and Central and North Spain. Lineage VIII found in this study in *Lathyrus* nodules has been found to date only in South Spain.

The strains from this study belonging to sv. trifolii also grouped with some strains isolated from *Trifolium* nodules in other countries and continents (Fig. S11). Clusters I and IV included European strains, which were isolated from nodules of different *Trifolium* species in this study, in the Cyclades and Naxos Islands (Greece), in Belgium and in Central Spain. The independent lineages II and III have been found to date only in South Spain (Granada). Curiously, the strains from sv. trifolii isolated in North Spain from *Trifolium* and *C. canariense* nodules (RTP05, RCCHU01 and RCCHU06) that belonged to certain *atpD* and *recA* clusters containing the strains from Granada were phylogenetically divergent to those strains in the *nodC* gene analysis (therefore they were not included in Figs. S10 and S11).

Therefore, the results of the *nodC* gene analysis showed that the strains isolated from nodules of legumes from the *Vicia* cross-inoculation group and from *Trifolium* species belonged to the symbiovars viciae and trifolii, respectively, which is consistent with their affiliation to tribes considered as restrictive hosts for nodulation [30]. Nevertheless, the strains isolated in this study formed several groups and lineages within both symbiovars, which showed the high phylogenetic diversity of the *nodC* genes carried by the strains, despite the fact that they were isolated from the same location. Although some strains isolated in this study belonged to groups containing strains from other continents, several of them have been found to date only in South Spain, which shows a high diversification degree of the *nodC* genes in different ecosystems.

In summary, our results showed that the strains isolated in Granada from *Vicia*, *Lathyrus* and *Trifolium* belonged to the *R. leguminosarum* phylogenetic group, but they could not be classified into any described species of this genus considering the current limits for species differentiation after core gene analyses. However, these strains did belong to symbiovars described within the genus *Rhizobium*, those nodulating *Vicia* and *Lathyrus* to sv. viciae, and those nodulating *Trifolium* to sv. trifolii. Although some of our strains were related to other strains isolated in other geographical locations in Spain, as well as in other countries and continents, several phylogenetic groups or lineages of *recA*, *atpD* and *nodC* genes have only been found to date in Granada (South Spain). The results of both core and symbiotic gene analyses showed, in agreement with those of other studies, the high phylogenetic diversity of strains nodulating *Trifolium*- and *Vicia*-related hosts. The existence of a high degree of diversification and genetic recombination in different ecosystems makes it difficult to delineate species in the *R. leguminosarum* phylogenetic group, which could be solved in some cases with the definition of subspecies instead of species in this group.

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2016.11.008.

References

[1] Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[2] Alvarez-Martínez, E.R., Valverde, A., Ramírez-Bahena, M.H., García-Fraile, P., Tejedor, C., Mateos, P.F., Santillana, N., Zúñiga, D., Peix, A., Velázquez, E. (2009) The analysis of core and symbiotic genes of rhizobia nodulating *Vicia* from different continents reveals their common phylogenetic origin and suggests the distribution of *Rhizobium leguminosarum* strains together with *Vicia* seeds. Arch. Microbiol. 191, 659–668.
[3] An, D.S., Im, W.T., Yang, H.C., Lee, S.T. (2006) *Shinella granuli* gen. nov., sp. nov., and proposal of the reclassification of Zoogloea ramigera ATCC 19623 as *Shinella zoogloeoides* sp. nov. Int. J. Syst. Evol. Microbiol. 56, 443–448.
[4] Aoki, S., Kondo, T., Prévost, D., Nakata, S., Kajita, T., Ito, M. (2010) Genotypic and phenotypic diversity of rhizobia isolated from *Lathyrus japonicus* indigenous to Japan. Syst. Appl. Microbiol. 33, 383–397.
[5] Aserse, A.A., Räsänen, L.A., Assefa, F., Hailemariam, A., Lindström, K. (2012) Phylogeny and genetic diversity of native rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in Ethiopia. Syst. Appl. Microbiol. 35, 120–131.
[6] Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84, 128–163.
[7] Cobo-Díaz, J.F., Martínez-Hidalgo, P., Fernández-González, A.J., Martínez-Molina, E., Toro, N., Velázquez, E., Fernández-López, M. (2014) The endemic *Genista versicolor* from Sierra Nevada National Park in Spain is nodulated by putative new *Bradyrhizobium* species and a novel symbiovar (sierranevadense). Syst. Appl. Microbiol. 37, 177–185.
[8] Degefu, T., Wolde-Meskel, E., Frostegård, Å. (2013) Phylogenetic diversity of *Rhizobium* strains nodulating diverse legume species growing in Ethiopia. Syst. Appl. Microbiol. 36, 272–280.
[9] Ferreira, L., Sánchez-Juanes, F., García-Fraile, P., Rivas, R., Mateos, P.F., Martínez-Molina, E., González-Buitrago, J.M., Velázquez, E. (2011) MALDI-TOF mass spectrometry is a fast and reliable platform for identification and ecological studies of species from family *Rhizobiaceae*. PLoS One 6, e20223.
[10] García-Fraile, P., Mulas-García, D., Peix, A., Rivas, R., González-Andrés, F., Velázquez, E. (2010) *Phaseolus vulgaris* is nodulated in northern Spain by *Rhizobium leguminosarum* strains harboring two nodC alleles present in American

Rhizobium etli strains: biogeographical and evolutionary implications. Can. J. Microbiol. 56, 657–666.
[11] Gaunt, M.W., Turner, S.L., Rigottier-Gois, L., Lloyd-Macgilp, S.A., Young, J.W.P. (2001) Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. Int. J. Syst. Evol. Microbiol. 51, 2037–2048.
[12] Gurkanli, C.T., Ozkoc, I., Gunduz, I. (2014) Genetic diversity of *Vicia faba* L. and *Pisum sativum* L. nodulating rhizobia in the central Black Sea region of Turkey. Ann. Microbiol. 64, 99–112.
[13] Hassan, M.M., Fahmi, A.I., Eissa, R.A., Nagaty, H.H. (2015) Diversity of rhizobia nodulating faba bean (*Vicia faba*) growing in Egypt. J. Microb. Biochem. Technol. 07, 152–159.
[14] Hou, B.C., Wang, E.T., Li, Y., Jia, R.Z., Chen, W.F., Man, C.X., Sui, X.H., Chen, W.X. (2009) Rhizobial resource associated with epidemic legumes in Tibet. Microb. Ecol. 57, 69–81.
[15] Jiao, Y.S., Yan, H., Ji, Z.J., Liu, Y.H., Sui, X.H., Wang, E.T., Guo, B.L., Chen, W.X., Chen, W.F. (2015) *Rhizobium sophorae* sp. nov. and *Rhizobium sophoriradicis* sp. nov., nitrogen-fixing rhizobial symbionts of the medicinal legume *Sophora flavescens*. Int. J. Syst. Evol. Microbiol. 65, 497–503.
[16] Kathiravan, R., Jegan, S., Ganga, V., Prabavathy, V.R., Tushar, L., Sasikala, Ch., Ramana, Ch.V. (2013) *Ciceribacter lividus* gen. nov., sp. nov., isolated from rhizosphere soil of chick pea (*Cicer arietinum* L.). Int. J. Syst. Evol. Microbiol. 63, 4484–4488.
[17] Kimes, N.E., López-Pérez, M., Flores-Félix, J.D., Ramírez-Bahena, M.H., Igual, J.M., Peix, A., Rodriguez-Valera, F., Velázquez, E. (2015) *Pseudorhizobium pelagicum* gen. nov., sp. nov. isolated from a pelagic Mediterranean zone. Syst. Appl. Microbiol. 38, 293–299.
[18] Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
[19] Kuykendall, L.D. (2005) Family I. *Rhizobiaceae*, in: Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M. (Eds.), The Alpha-, Beta-, Delta- and Epsilonproteobacteria, the Proteobacteria, Part C, Bergey's Manual of Systematic Bacteriology, vol. 2, 2nd ed., Springer, New York, pp. 324–362.
[20] Laguerre, G., Nour, S.M., Macheret, V., Sanjuan, J., Drouin, P., Amarger, N. (2001) Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiology 147, 981–993.
[21] Lei, X., Wang, E.T., Chen, W.F., Sui, X.H., Chen, W.X. (2008) Diverse bacteria isolated from root nodules of wild *Vicia* species grown in temperate region of China. Arch. Microbiol. 190, 657–671.
[22] Lüscher, A., Mueller-Harvey, I., Soussana, J.F., Rees, R.M., Peyraud, J.L. (2014) Potential of legume-based grassland-livestock systems in Europe: a review. Grass Forage Sci. 69, 206–228.
[23] Marek-Kozaczuk, M., Leszcz, A., Wielbo, J., Wdowiak-Wróbela, S., Skorupska, A. (2013) *Rhizobium pisi* sv. trifolii K3.22 harboring nod genes of the *Rhizobium leguminosarum* sv. trifolii cluster. Syst. Appl. Microbiol. 36, 252–258.
[24] Martínez-Hidalgo, P., Flores-Félix, J.D., Menéndez, E., Rivas, R., Carro, L., Mateos, P.F., Martínez-Molina, E., León-Barrios, M., Velázquez, E. (2015) *Cicer canariense*, an endemic legume to the Canary Islands, is nodulated in mainland Spain by fast-growing strains from symbiovar trifolii phylogenetically related to *Rhizobium leguminosarum*. Syst. Appl. Microbiol. 38, 346–350.
[25] Mauchline, T.H., Hayat, R., Roberts, R., Powers, S.J., Hirsch, P.R. (2014) Assessment of core and accessory genetic variation in *Rhizobium leguminosarum* symbiovar trifolii strains from diverse locations and host plants using PCR-based methods. Lett. Appl. Microbiol. 59, 238–246.
[26] Mousavi, S.A., Österman, J., Wahlberg, N., Nesme, X., Lavire, C., Vial, L., Paulin, L., de Lajudie, P., Lindström, K. (2014) Phylogeny of the *Rhizobium-Allorhizobium-Agrobacterium* clade supports the delineation of *Neorhizobium* gen. nov. Syst. Appl. Microbiol. 37, 208–215.
[27] Mousavi, S.A., Willems, A., Nesme, X., de Lajudie, P., Lindström, K. (2015) Revised phylogeny of *Rhizobiaceae*: proposal of the delineation of *Pararhizobium* gen. nov., and 13 new species combinations. Syst. Appl. Microbiol. 38, 84–90.
[28] Pardini, A., Natali, F., Tallarico, R. (2007) Horse grazing in firebreaks sown with *Trifolium brachycalycinum* (Katznl. & Morley) and *Cynodon dactylon* (L.) Pers. Agrofor. Syst. 70, 17–24.
[29] Peix, A., Ramírez-Bahena, M.H., Velázquez, E., Bedmard, E.J. (2015) Bacterial associations with legumes. Crit. Rev. Plant Sci. 34, 17–42.
[30] Perret, X., Staehelin, C., Broughton, W.J. (2000) Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. 64, 180–201.
[31] Phelan, P., Moloney, A.P., McGeough, E.J., Humphreys, J., Bertilsson, J., O'Riordan, E.G., O'Kiely, P. (2015) Forage legumes for grazing and conserving in ruminant production systems. Crit. Rev. Plant Sci. 34, 281–326.
[32] Rahi, P., Kapoor, R., Young, J.P., Gulati, A. (2012) A genetic discontinuity in root-nodulating bacteria of cultivated pea in the Indian trans-Himalayas. Mol. Ecol. 21, 145–159.
[33] Ramírez-Bahena, M.H., García-Fraile, P., Peix, A., Valverde, A., Rivas, R., Igual, J.M., Mateos, P.F., Martínez-Molina, E., Velázquez, E. (2008) Revision of the taxonomic status of the species *Rhizobium leguminosarum* (Frank 1879) Frank 1889AL, *Rhizobium phaseoli* Dangeard 1926AL and *Rhizobium trifolii* Dangeard 1926AL. *R. trifolii* is a later synonym of *R. leguminosarum*. Reclassification of the strain *R. leguminosarum* DSM 30132 (=NCIMB 11478) as *Rhizobium pisi* sp. nov. Int. J. Syst. Evol. Microbiol. 58, 2484–2490.
[34] Ramírez-Bahena, M.H., Velázquez, E., Fernández-Santos, F., Peix, A., Martínez-Molina, E., Mateos, P.F. (2009) Phenotypic, genotypic, and symbiotic diversities in strains nodulating clover in different soils in Spain. Can. J. Microbiol. 55, 1207–1216.
[35] Rashid, M.H., Gonzalez, J., Young, J.P.W., Wink, M. (2014) *Rhizobium leguminosarum* is the symbiont of lentils in the Middle East and Europe but not in Bangladesh. FEMS Microbiol. Ecol. 87, 64–77.
[36] Rashid, M.H., Schäfer, H., Gonzalez, J., Wink, M. (2012) Genetic diversity of rhizobia nodulating lentil (*Lens culinaris*) in Bangladesh. Syst. Appl. Microbiol. 35, 98–109.
[37] Rashid, M.H., Young, J.P., Everall, I., Clercx, P., Willems, A., Braun, M.S., Wink, M. (2015) Average nucleotide identity of genome sequences supports the description of *Rhizobium lentis* sp. nov., *Rhizobium bangladeshense* sp. nov. and *Rhizobium binae* sp. nov. from lentil (*Lens culinaris*) nodules. Int. J. Syst. Evol. Microbiol. 65, 3037–3045.
[38] Riah, N., Béna, G., Djekoun, A., Heulin, K., de Lajudie, P., Laguerre, G. (2014) Genotypic and symbiotic diversity of *Rhizobium* populations associated with cultivated lentil and pea in sub-humid and semi-arid regions of Eastern Algeria. Syst. Appl. Microbiol. 37, 368–375.
[39] Rivas, R., García-Fraile, P., Mateos, P.F., Martínez-Molina, E., Velázquez, E. (2007) Characterization of xylanolytic bacteria present in the bract phyllosphere of the date palm *Phoenix dactylifera*. Lett. Appl. Microbiol. 44, 181–187.
[40] Rivas, R., Peix, A., Mateos, P.F., Trujillo, M.E., Martinez-Molina, E., Velázquez, E. (2006) Biodiversity of populations of phosphate solubilizing rhizobia that nodulate chickpea in different Spanish soils. Plant Soil 287, 23–33.
[41] Robledo, M., Velázquez, E., Ramírez-Bahena, M.H., García-Fraile, P., Pérez-Alonso, A., Rivas, R., Martínez-Molina, E., Mateos, P.F. (2011) The *celC* gene, a new phylogenetic marker useful for taxonomic studies in *Rhizobium*. Syst. Appl. Microbiol. 34, 393–399.
[42] Rogel, M.A., Ormeño-Orrillo, E., Martinez Romero, E. (2011) Symbiovars in rhizobia reflect bacterial adaptation to legumes. Syst. Appl. Microbiol. 34, 96–104.
[43] Román-Ponce, B., Zhang, Y.J., Vásquez-Murrieta, M.S., Sui, X.H., Chen, W.F., Padilla, C.A.J., Guo, X.W., Gao, J.L., Yan, J., Wei, G.H., Wang, E.T. (2016) *Rhizobium acidisoli* sp. nov., isolated from root nodules of *Phaseolus vulgaris* in acid soils in Mexico. Int. J. Syst. Evol. Microbiol. 66, 398–406.
[44] Ruiz-Díez, B., Fajardo, S., Felipe, M.R., Fernández-Pascual, M. (2012) Characterization of rhizobia from legumes of agronomic interest grown in semi-arid areas of Central Spain relates genetic differences to soil properties. J. Basic Microbiol. 52, 66–78.
[45] Ruiz-Díez, B., Fajardo, S., Fernández-Pascual, M. (2012) Selection of rhizobia from agronomic legumes grown in semiarid soils to be employed as bioinoculants. Agron. J. 104, 550–559.
[46] Ruiz-Díez, B., Quiñones, M.A., Fajardo, S., López, M.A., Higueras, P., Fernández-Pascual, M. (2012) Mercury-resistant rhizobial bacteria isolated from nodules of leguminous plants growing in high Hg-contaminated soils. Appl. Microbiol. Biotechnol. 96, 543–554.
[47] Ruiz-Mirazo, J., Robles, A.B. (2012) Impact of targeted sheep grazing on herbage and holm oak saplings in a silvopastoral wildfire prevention system in south-eastern Spain. Agrofor. Syst. 86, 477–491.
[48] Saïdi, S., Ramírez-Bahena, M.H., Santillana, N., Zúñiga, D., Álvarez-Martínez, E., Peix, A., Mhamdi, R., Velázquez, E. (2014) *Rhizobium laguerreae* sp. nov. nodulates *Vicia faba* on several continents. Int. J. Syst. Evol. Microbiol. 64, 242–247.
[49] Saitou, N., Nei, M. (1987) A neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 44, 406–425.
[50] Santillana, N., Ramírez-Bahena, M.H., García-Fraile, P., Velázquez, E., Zúñiga, D. (2008) Phylogenetic diversity based on *rrs*, *atpD*, *recA* genes and 16S-23S intergenic sequence analyses of rhizobial strains isolated from *Vicia faba* and *Pisum sativum* in Peru. Arch. Microbiol. 189, 239–247.
[51] Sbabou, L., Regragui, A., Filali-Maltouf, A., Ater, M., Béna, G. (2016) Local genetic structure and worldwide phylogenetic position of symbiotic *Rhizobium leguminosarum* strains associated with a traditional cultivated crop, *Vicia ervilia*, from Northern Morocco. Syst. Appl. Microbiol. 39, 409–417.
[52] Shamseldin, A., Carro, L., Peix, A., Velázquez, E., Moawad, H., Sadowsky, M.J. (2016) The symbiovar trifolii of *Rhizobium bangladeshense* and *Rhizobium aegyptiacum* sp. nov. nodulate *Trifolium alexandrinum* in Egypt. Syst. Appl. Microbiol. 39, 275–279.
[53] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
[54] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
[55] Tian, C.F., Wang, E.T., Wu, L.J., Han, T.X., Chen, W.F., Gu, C.T., Gu, J.G., Chen, W.X. (2008) *Rhizobium fabae* sp. nov., a bacterium that nodulates *Vicia faba*. Int. J. Syst. Evol. Microbiol. 58, 2871–2875.
[56] Van Cauwenberghe, J., Michiels, J., Honnay, O. (2015) Effects of local environmental variables and geographical location on the genetic diversity and composition of *Rhizobium leguminosarum* nodulating *Vicia cracca* populations. Soil Biol. Biochem. 90, 71–79.
[57] Van Cauwenberghe, J., Verstraete, B., Lemaire, B., Lievens, B., Michiels, J., Honnay, O. (2014) Population structure of root nodulating *Rhizobium leguminosarum* in *Vicia cracca* populations at local to regional geographic scales. Syst. Appl. Microbiol. 37, 613–621.
[58] Ventorino, V., Chiurazzi, M., Aponte, M., Pepe, O., Moschetti, G. (2007) Genetic diversity of a natural population of *Rhizobium leguminosarum* bv. viciae nodulating plants of *Vicia faba* in the Vesuvian area. Curr. Microbiol. 55, 512–517.

[59] Vinuesa, P., Silva, C., Werner, D., Martínez-Romero, E. (2005) Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. Mol. Phylogen. Evol. 34, 29–54.

[60] Wei, G.H., Wang, E.T., Tan, Z.Y., Zhu, M.E., Chen, W.X. (2002) *Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov., respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea*. Int. J. Syst. Evol. Microbiol. 52, 2231–2239.

[61] Xu, K.W., Zou, L., Penttinen, P., Wang, K., Heng, N.N., Zhang, X.P., Chen, Q., Zhao, K., Chen, Y.X. (2015) Symbiotic effectiveness and phylogeny of rhizobia isolated from faba bean (*Vicia faba* L.) in Sichuan hilly areas, China. Syst. Appl. Microbiol. 38, 515–523.

[62] Youseif, S.H., Abd El-Megeed, F.H., Ageez, A., Cocking, E.C., Saleh, S.A. (2014) Phylogenetic multilocus sequence analysis of native rhizobia nodulating faba bean (*Vicia faba* L.) in Egypt. Syst. Appl. Microbiol. 37, 560–569.

[63] Zhang, J.J., Jing, X.Y., de Lajudie, P., Ma, C., He, P.X., Singh, R.P., Chen, W.F., Wang, E.T. (2016) Association of white clover (*Trifolium repens* L.) with rhizobia of sv. trifolii belonging to three genomic species in alkaline soils in North and East China. Plant Soil 407, 417–427.

[64] Zhang, Y.J., Zheng, W.T., Everall, I., Young, J.P., Zhang, X.X., Tian, C.F., Sui, X.H., Wang, E.T., Chen, W.X. (2015) *Rhizobium anhuiense* sp. nov., isolated from effective nodules of *Vicia faba* and *Pisum sativum* grown in Southern China. Int. J. Syst. Evol. Microbiol. 65, 2960–2967.