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Genetic diversity of root nodulating bacteria associated with *Retama sphaerocarpa* in sites with different soil and environmental conditions

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ABSTRACT

The genetic diversity of root nodulating bacteria isolated from *Retama sphaerocarpa* was studied using BOX-A1R PCR and phylogenetic analysis of the 16S rRNA region, as well as the housekeeping genes *atpD*, *glnII* and *recA*. A total of 193 isolates were obtained from eight different sites with different soil and environmental conditions in the Iberian Peninsula. These isolates corresponded to 31 different strains that successfully nodulated *R. sphaerocarpa* seedlings in reinoculation trials. About one-third of the strains clustered with *B. canariense* or *B. cytisi* within *Bradyrhizobium* group I. The remaining strains clustered with *B. elkanii*/*B. pachyrhizi* within *Bradyrhizobium* group II or in separate clades that could represent new lineages. Based on the 16S rRNA and combined *atpD* + *glnII* + *recA* sequences, two to three lineages of root nodulating bacteria were found at each sampling site, except for Collado Garcia where five species were detected. *B. canariense* and *B. elkanii*/*B. pachyrhizi* were the most abundant species, whereas the least abundant were those related to *B. retamae* and a putative new lineage. *B. canariense* was found only in soils with neutral and acid pH, whereas *B. retamae* was the dominant species in alkaline soils.

Introduction

Retama sphaerocarpa (L.) Boiss is a large leguminous shrub, widespread in southwest Europe and northwest Africa, which facilitates the establishment and growth of many other species under its canopy [2,22]. It has an open canopy with photosynthetic stems and a dimorphic root system that has shallow lateral roots and tap roots that can reach down to a depth of 30 m, which have the ability to lift water from deep, wet layers and release it to shallow, dry soils [21]. This hydraulic lift, together with the amelioration of microclimatic conditions, as well as the increase in nutrient content and mineralization rate under the canopy, has positive effects on the establishment and growth of other plants, which is an ecological process known as facilitation [2,18,22,23,26]. Thus, *R. sphaerocarpa* plays an important ecological role in arid and semiarid areas by maintaining plant diversity and ecosystem functioning [4,15], and is a valuable species for revegetation and restoration projects [11,27].

The establishment of effective symbiosis with nitrogen-fixing bacteria allows *R. sphaerocarpa* to grow in poor soils, which contributes to the increase in nitrogen under the canopy [26,35]. Therefore, this symbiosis is relevant for the ecological role of *R. sphaerocarpa* as a benefactor plant species for other plants under its canopy. Nonetheless, root-nodulating bacteria of *R. sphaerocarpa* have been studied only in a few closely located sites from central Spain in southern Europe [9,28,31], and from Algeria and Morocco in northern Africa [3,9]. These studies show that *Bradyrhizobium* species are by far the dominant microsymbionts associated with *R. sphaerocarpa* [3,9,28,31], although *Phyllobacterium myrsinacearum* and *Rhizobium radiobacter* have also been isolated from nodules of *R. sphaerocarpa* plants from central Spain [31,32]. Phylogenetic analyses of 16S and IGS regions cluster most of the bradyrhizobia from nodules of *R. sphaerocarpa* with *B. canariense* and related *B. cytisi* and *B. rifense* type strains [5,6] within *Bradyrhizobium* group I [17]. Recently, the novel *B. retamae* species, which groups with *B. elkanii* and *B. pachyrhizi* and related *B. lablabi* and *B. jicamae* type strains included in *Bradyrhizobium* group II [17], has been isolated from *R. sphaerocarpa* and *R. monosperma* grown in Morocco [9].

R. sphaerocarpa plants can be found in both acidic and alkaline soils, from sea level to an altitude of 2000 m, and in sites with mean annual precipitation of 200–980 mm [2]. Thus, it is possible that the diversity of root-nodulating bacteria associated with *R. sphaerocarpa* could be higher than that reported thus far. Additionally, more information on the rhizobia associated with this leguminous shrub in sites with different abiotic conditions is necessary in order to understand its biology and the specificity of the symbiosis.

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Considering the predicted increased aridity of the Mediterranean region due to climate change [13], this information would be valuable for designing adequate conservation and restoration plans. Therefore, the primary objective of this study was to identify the nodulating bacteria associated with *R. sphaerocarpa* plants growing in different soils and environmental conditions from southeastern to central western Spain. Bacterial isolates were firstly screened using BOX-A1R PCR [28] and the strains considered different were subsequently analyzed using PCR-amplification and sequencing of the 16S rRNA, *atpD*, *glnII* and *recA* genes. The results showed that 22 strains clustered with type strains included in both *Bradyrhizobium* groups I and II. The remaining nine strains could represent at least two new lineages within the genus *Bradyrhizobium* since they did not cluster with any of the previously described species.

Materials and methods

Sampling sites and soil collection

Soil samples were taken from eight *R. sphaerocarpa* shrublands with different soil and environmental conditions. Geographical coordinates, altitude, mean annual precipitation and temperature, and soil physico-chemical properties are shown in Table S1. Sampling was conducted from southeastern to western Spain, thus covering most of the longitudinal distribution of this species in Europe. At each site, 300 g of soil was collected from the upper layer underneath eight adult plants and the distance between sampled plants was at least 5 m in all sites. Soil pH, organic matter (OM), N, P and K were measured following previously described methods [29].

Plant inoculation, isolation of bacteria from nodules and culture conditions

Seeds of *R. sphaerocarpa* were scarified by immersion in concentrated sulfuric acid for 2 h, and then rinsed thoroughly with distilled autoclaved water. Seeds (3/pot) were placed in 1.5 L pots filled with a mixture of autoclaved sand and perlite (2:1, v:v) and inoculated independently with 200 g of each field-collected soil sample. Pots containing uninoculated plants were used as controls. Pots were covered with transparent film to maintain humidity and prevent cross-contamination, and they were placed in a greenhouse with natural light conditions and a day/night temperature of approximately 25/18 °C. After germination, seedlings were thinned out to one per pot and they were watered twice a week with distilled autoclaved water. After growth for six months, 3–8 nodules from all plants in each soil treatment were picked from the roots, pooled together, surface-sterilized and rinsed thoroughly with sterile distilled water, as described previously [26]. Then, at least 25 nodules from plants growing in pots inoculated with the same soil were placed independently on petri dishes and crushed in a drop of sterile water with a sterile glass rod. Bacterial isolates were obtained by streaking the resulting suspension on solid yeast extract mannitol agar (YEM) for 20 days [37], as indicated earlier [28].

Plant nodulation tests

After surface sterilization and scarification, as described above, seedlings of *R. sphaerocarpa* were individually planted in 1.5 L pots containing sterile sand and vermiculite (2:1, v:v), and they were inoculated separately with 1 mL cell suspension (about 10^9 cells/mL) of each one of the 31 strains identified in this study (9 plants/strain). A total of 18 pots were left uninoculated to be used as controls. Plants were grown under greenhouse conditions and watered twice a week with autoclaved distilled water. Plants were checked for nodulation after growth for 4 and 6 months.

DNA extraction and fingerprinting

Genomic DNA was isolated from bacterial cells using the RealPure Genomic DNA Extraction Kit (Durviz, Spain), according to the manufacturer's instructions. DNA concentration was determined using a Nanodrop spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, USA). A preliminary analysis of the bacterial diversity was carried out as previously described [25] using BOX-A1R PCR [36]. After PCR amplification, 9 μL PCR products was subjected to electrophoresis. Digital images of the gels were processed with the software GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). A cluster analysis was performed using the unweighted pair-group method with arithmetic mean algorithm (UPGMA) and the Pearson product–moment correlation coefficient. Isolates with a similarity lower than 80% in the resulting dendrogram were considered different.

DNA sequencing and phylogenetic analysis

PCR amplifications of the 16S rRNA gene were carried out using the primers 41f and 1488r, as previously reported [12]. Partial amplifications of the housekeeping genes *atpD*, *glnII* and *recA* were performed using the primer pairs atpD273F and atpD771R, glnII12F and glnII689R, recA41F and recA640R, respectively [38]. Amplification products were verified by electrophoresis in agarose gel, purified with the Qiagen PCR product purification kit and, finally, quantified as described above.

PCR products were sequenced using the same primers as for PCR amplification with an automatic ABI Prism Dye Chemistry sequencer at the sequencing facilities of the Estación Experimental del Zaidín, CSIC, Granada, Spain. The 16S sequences obtained were compared to those deposited in EZTaxon [14], and *atpD*, *glnII* and *recA* sequences were compared with public databases using BLAST [1]. Similarity matrices for the isolates obtained in this study and already described *Bradyrhizobium* species were obtained for the four sequenced genes using DNADist as implemented in Phylemon 2.0 [33]. The software Geneious 6.0 (Biomatters) was used for sequence assembly and alignment, and to obtain the concatenated sequences of 16S rRNA, *atpD*, *glnII* and *recA*. The best-fit model was found using the Akaike information criterion in jModeltest [20], as implemented in Phylemon 2.0 [33]. Maximum likelihood (ML) analysis was carried out using PhyML [10] with 1000 bootstrap replicates in order to determine the degree of statistical support for branches in the phylogeny. Trees were visualized using FigTree v1.4. The phylogenetic analyses included the corresponding sequences from the current validly published *Bradyrhizobium* species, as well as from strains BC-C1 and BRE-1 as representatives of genospecies alpha and beta, respectively, and from strains LmjB4 and LmjH2p isolated from *Lupinus mariae-josephae* in eastern Spain [34], whose sequences had a high similarity (>96%) with some strains isolated in this study.

Rhizobial richness and diversity

Strain richness at each location was calculated by dividing the number of different strains identified using BOX-A1R PCR by the number of isolates obtained at each location [16]. The genetic diversity (h) at a site was calculated using the formula $h = [1 - \sum p_i^2][N/(N-1)]$, where p_i is the frequency of each strain (n_i/N), n_i is the number of isolates in each strain and N is the total number of isolates per site. $[N/(N-1)]$ is a correction for bias in small samples [3,19].

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Table 1
Number of isolates (*N*), number of strains (*S*), strain richness (*S*/*N*) and strain diversity (*h*), according to BOX-A1R PCR, of bradyrhizobial cells isolated from root nodules of *R. sphaerocarpa* plants grown in soils from eight sampling sites with different soil and environmental conditions.

Sampling site	Isolates (*N*)	Strains (*S*)	Strain richness (*S*/*N*)	Strain diversity (*h*)
Collado García	30	7	0.23	0.82
Tabernas	20	3	0.15	0.42
Rioja	26	3	0.12	0.43
Abrucena	20	5	0.25	0.82
La Calahorra	14	3	0.21	0.47
Castuera	33	3	0.09	0.27
Aliseda	24	3	0.13	0.62
Valencia de Alcántara	26	4	0.15	0.62

Accession numbers

Sequences generated in this study were deposited in the GenBank/EMBL database and their accession numbers are included in the phylogenetic trees.

Results

BOX-A1R PCR and bacterial diversity

A total of 193 isolates were obtained from root nodules of *R. sphaerocarpa* grown at eight locations with different soil and environmental conditions (Table 1). A cluster analysis based on the BOX-A1R PCR data revealed that they corresponded to 31 different strains (data not shown). All 31 strains produced effective symbiosis with their original host plant in the nodulation tests. Indirect effectiveness of the nodules for nitrogen fixation was estimated by visual assay of red leghemoglobin presence in cross-sections and by the dark green intensity of the leaves compared to uninoculated control plants.

The number of isolates obtained from each sampling site ranged from 14 in La Calahorra to 33 in Castuera, which corresponded in most cases to three different strains, although four strains were detected in Valencia de Alcántara, five in Abrucena and seven in Collado García (Table 1). Strain richness ranged between 0.09 and 0.25, with the highest values found in Abrucena (0.25), Collado García (0.23) and La Calahorra (0.21), whereas the lowest value was observed in Castuera (0.09) (Table 1). The diversity (*h*) of strains in each site ranged between 0.27 in Castuera and 0.82 in Collado García and Abrucena (Table 1).

Phylogenetic analyses of 16S rRNA, atpD, glnII and recA genes

The EzTaxon-e analysis inferred from the nearly complete sequence of the 16S rRNA gene indicated that all the obtained strains were members of the genus *Bradyrhizobium* within the family *Rhizobiaceae* of the *Alphaproteobacteria* class, with similarity values above 98% with previously described bradyrhizobial type species (Table S2). An ML phylogenetic tree based on the 16S rRNA gene sequences indicated that 11 out of the 31 strains clustered with *B. canariense* BTA-1, *B. rifense* CTAW71 and *B. cytisi* CTAW11 type strains, as well as strain BC-C1 that was representative of genospecies alpha within *Bradyrhizobium* group I, whereas the remaining 20 isolates were included in *Bradyrhizobium* group II [17].

PCR amplification of the *atpD*, *glnII* and *recA* genes from each of the 31 strains yielded single bands of approximately 438, 519 and 412 base pairs, respectively (data not shown). Their respective DNA sequences showed similarity values with those of the *Bradyrhizobium* type strains in the public databases ranging from 92.9% to 100% (Table S2). The corresponding single ML phylogenetic tree for each of the *atpD*, *glnII* and *recA* genes (Figs. S1, S2 and S3) confirmed the affiliation of the strains to *Bradyrhizobium* groups I and II, but showed differences regarding the taxonomic position of the isolates. The combined ML phylogenetic tree based on the *atpD* + *glnII* + *recA* sequences (Fig. 1) indicated that 77.4% of the 31 strains obtained in this study clustered with type strains belonging to *Bradyrhizobium* groups I and II [17]. Four strains included in *Bradyrhizobium* group I were closely related to the type strains *B. cytisi* CTAW11 and *B. rifense* CTAW71, and seven strains to *B. canariense* BTA-1. Within *Bradyrhizobium* group II, nine strains clustered in a sister group of *B. elkanii* LMG 6134 and *B. pachyrizi* PAC 48 type strains, and four with the newly described species *B. retamae* Ro 11 isolated from nodules of *R. sphaerocarpa* and *R. monosperma* plants grown in Spain and Morocco [9]. Strains RiojA2, CollC7, AbruD4, AbruD8, CalaE1 and CalaE8 grouped with strain LmjH2p, described by Sánchez-Cañizares et al. [34], in a clade in which no reference strain was included. Finally, strain CollC23 was also placed in *Bradyrhizobium* group II but it was phylogenetically distant from all other species and strains.

Abundance and distribution of Bradyrhizobium species nodulating R. sphaerocarpa

Based on the taxonomic classification inferred from the phylogenetic tree of the combined *atpD* + *glnII* + *recA* genes (Fig. 1) and the abundance of each strain according to the BOX-A1R PCR (Table S2), the most abundant species found in *R. sphaerocarpa* root nodules were those clustering with *B. canariense* (32.6% of the total number of *Bradyrhizobium* isolates) and the *B. elkanii*/*B. pachyrhizi* subgroup (28.0%), followed by *B. cytisi* (15.0%) and *B. retamae* (10.9%). Strains without clear affiliation represented 9.8% of the total number of *Bradyrhizobium* isolates, and the putative new species CollC23 accounted for the remaining 3.6% (Table S2).

The distribution of the species was not uniform across all sites (Fig. 2). The most widespread species were those in the *B. elkanii*/*B. pachyrhizi* subgroup, which occurred in all sites with the exception of La Calahorra, and they were the dominant species in Rioja (73.1% of all isolates in this site) (Fig. 2). The presence of *B. canariense* was observed in five out the eight sampling sites and was the dominant species in Castuera (84.8%), Aliseda (61.5%) and Valencia de Alcántara (53.8%). Strains that grouped with *B. cytisi* were found in three out of the eight sampling sites and were dominant in La Calahorra (92.8%). Strains related to *B. retamae* also occurred in three out of the eight sampling sites, and were dominant in Tabernas (95.0%) (Fig. 2). The putative new species CollC23 occurred only in Collado García, and represented 23.2% of the total number of isolates at that site. It is interesting to note that *B. retamae* was found only in alkaline soils, and that *B. canariense* was the dominant species in soils with neutral and acid pH (Fig. 2).

Discussion

The richness of bradyrhizobial strains associated with *R. sphaerocarpa* varied among the eight sampling sites (Table 1) with values that were lower than those reported in other studies from

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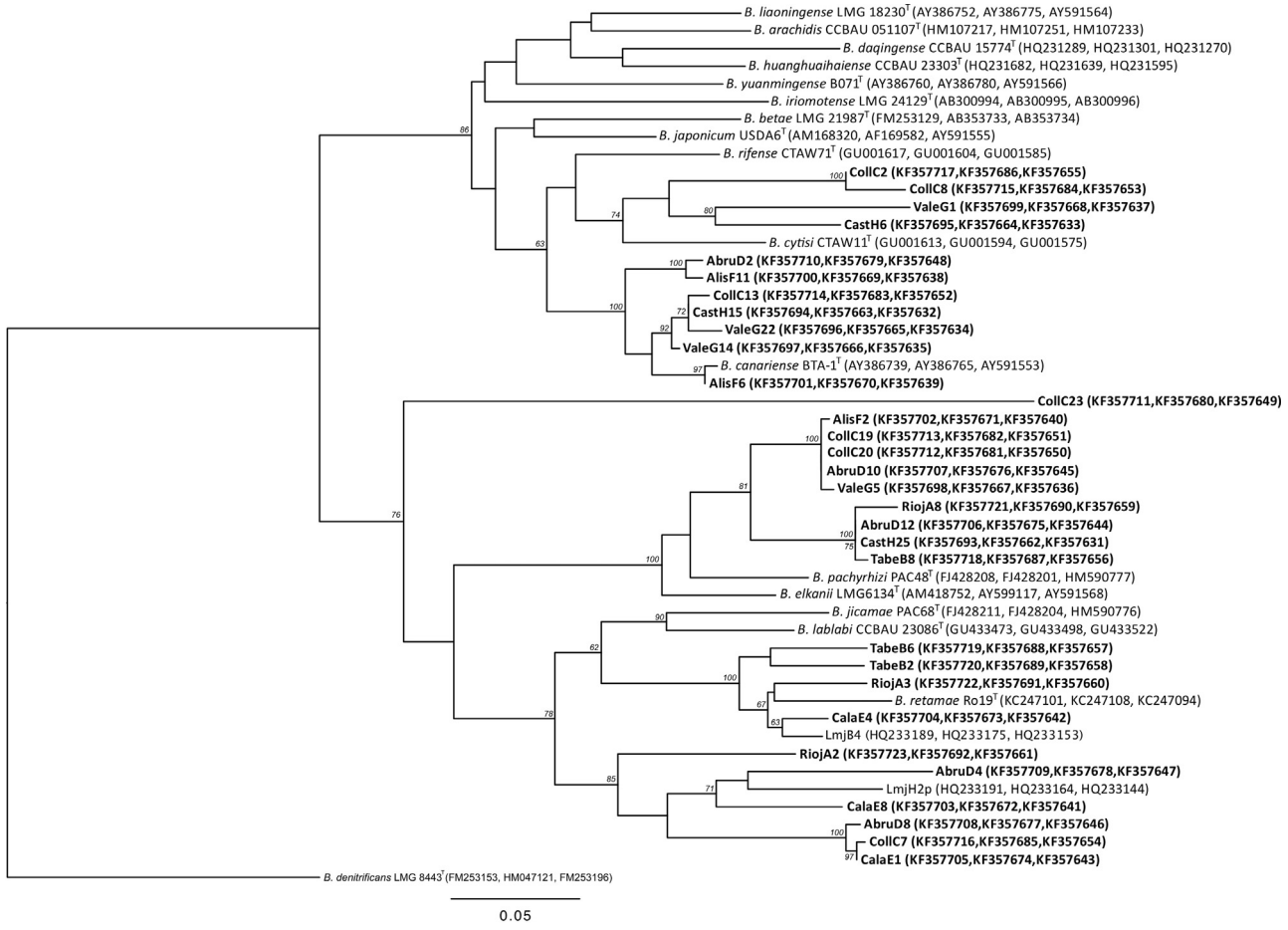

Fig. 1. Maximum likelihood phylogenetic tree based on combined partial *atpD + glnII + recA* sequences of strains from nodules of *R. sphaerocarpa* obtained in this study (in bold) and type strains of *Bradyrhizobium* species. The strains LmjB4 and LmjH2p are also included because their sequences had a high (>96%) similarity with some strains isolated in this study. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 60 are not shown. Scale bar indicates substitutions per site. Accession numbers are shown in brackets. The tree is rooted in *Bradyrhizobium denitrificans* LMG 8443T.

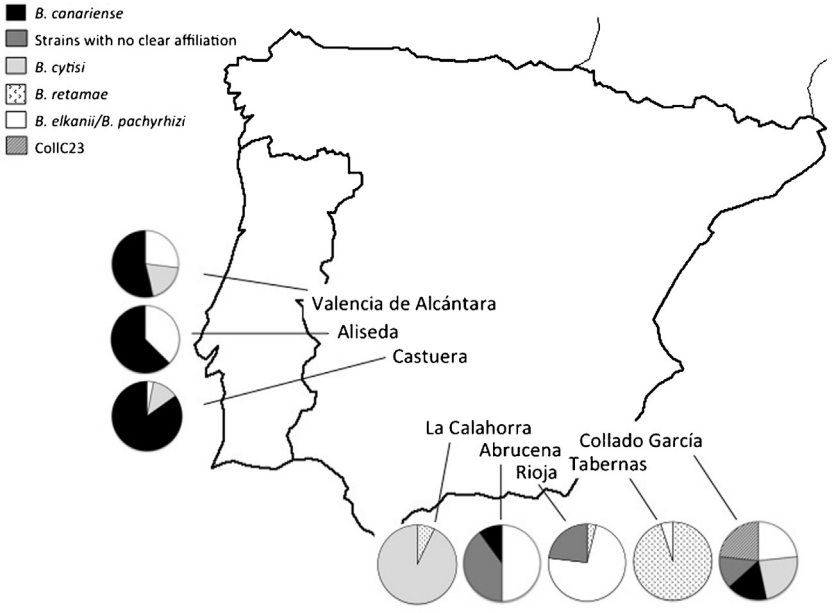

Fig. 2. Geographical situation and abundance of *Bradyrhizobium* species in eight sampling sites with different soil and environmental conditions. Identification is based on phylogenetic analysis based on combined partial *atpD + glnII + recA* sequences and BOX-A1R PCR.

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wild legumes [8 and references therein]. However, strain diversity (Table 1), which takes into account both abundance and evenness, was higher than in other studies published earlier [7,8,38]. Nevertheless, it should be noted that Abrucena, La Calahorra and Collado García, the sites with higher values of strain richness, were also located at higher altitude (Table 1). Consequently, whether or not a relationship exists between altitude and richness of *Bradyrhizobium* species nodulating *Retama* plants deserves further study.

Since *Bradyrhizobium* species have highly conserved 16S rRNA gene sequences, several core genes have been used to elucidate the taxonomic relationships between them [17,24,38,40]. In this study, the housekeeping genes *atpD*, *glnII* and *recA* were chosen because they can differentiate species with closely related 16S rRNA genes [5,6,9,38]. 16S rRNA and concatenated *atpD + glnII + recA* gene sequences showed that 11 strains grouped with either *B. canariense* or *B. cytisi* in *Bradyrhizobium* group I [17]. The remaining strains clustered within *Bradyrhizobium* group II, with *B. elkanii*/*B. pachyrhizi* or with the newly described species *B. retamae* [9]. In addition, six strains formed a cluster with no reference species but it contained strain LmjH2p isolated from *L. mariae-josephi* grown in alkaline soils from eastern Spain [34]. In a previous study, strain LmjH2p grouped with RST89 isolated from *R. sphaerocarpa* in Algeria [3], which suggests a close taxonomic position between strains isolated from *R. sphaerocarpa* plants growing in Algeria and southeast Spain. A direct comparison, however, between the isolates obtained in this study and those by Boulila et al. [3] was not possible since the sequenced housekeeping genes were different. Strain CollC23 formed a phylogenetically separated branch from all other strains isolated in this study (Fig. 1). Further identification of these isolates based on genomic and phenotypic information [30] was not pursued in this study.

Most root-nodulating bacteria associated with *R. sphaerocarpa* described to date belong to *Bradyrhizobium* group I [3,28,31] or to the newly described species *B. retamae* in *Bradyrhizobium* group II [9]. However, about two-thirds of the strains isolated in this study clustered within *Bradyrhizobium* group II apart from *B. retamae*. Also, strains related to *B. elkanii*/*B. pachyrhizi* type species were obtained in seven out of the eight sampling sites (Fig. 2). This is surprising since isolation of this species from *R. sphaerocarpa* has not been previously reported. All these results indicated that the rhizobial diversity associated with *R. sphaerocarpa* has been underestimated, most probably as a result of the low number of sites analyzed to date.

Soil pH was an important factor determining the presence of different *Bradyrhizobium* species in *R. sphaerocarpa* nodules. Strains related to *B. canariense* were found only in soils with pH below 7.0, which agrees with the description of this species as well adapted to acid soils [39] and confirms *B. canariense* as a common symbiont of *R. sphaerocarpa* in Spain. In contrast, *B. retamae* occurred only in soils with pH higher than 7.5, which suggests that this species could be better adapted to alkaline soils. Strain LmjB4, isolated from *L. mariae-josephi* in alkaline soils from eastern Spain [34], also clustered in this group. The description of *B. retamae* was performed from isolates obtained in several sites in Morocco and one site in Spain [9], but the pH of these soils was not reported. Thus, more data are needed to confirm the adaptation of *B. retamae* to alkaline soils.

In summary, this study revealed that the diversity of root-nodulating bacteria associated with *R. sphaerocarpa* is higher than previously reported, with about two-thirds of the strains being included in *B. elkanii*/*B. pachyrhizi* and related species within *Bradyrhizobium* group II. Also, putative novel *Bradyrhizobium* species might have been isolated from this legume in southeast Spain. Geographically structured communities of *R. sphaerocarpa*-associated *Bradyrhizobium* could exist due to the capacity of this legume to grow in both acid and alkaline soils.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2013.11.003.

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