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SYSTEMATIC AND APPLIED MICROBIOLOGY

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Different *Mesorhizobium* species sharing the same symbiotic genes nodulate the shrub legume *Anagyris latifolia* ☆

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Received 14 May 2007

Abstract

The isolation and characterization of six rhizobial strains isolated from *Anagyris latifolia*, a shrub legume endemic to the Canary Islands, is reported in this study. The isolates were characterized by 16S-ARDRA, and sequencing of the ribosomal 16S rRNA gene, the 16S–23S rDNA intergenic spacer region, and the housekeeping gene for glutamine synthetase II (*glnII*). The phylogenies based on the three types of sequences matched, showing that the isolates belonged to three distinct lineages within the genus *Mesorhizobium* that could represent different species. However, the ribosomal and *glnII* phylogenies revealed some discrepancies in the relationships between the isolates and the named species in this genus. Despite their different taxonomic affiliation, all the isolates showed identical *nodC* sequences which were closely related (95% similarity) to that of the *Mesorhizobium tianshanense* type strain, indicating that they must have acquired the nodulation genes by a phenomenon of lateral gene transfer.

Keywords: Nodulation; Legume; *Mesorhizobium*; *Anagyris latifolia*; Phylogeny; Ribosomal genes; *glnII* gene; Nodulation genes; Canary Islands

Introduction

Leguminosae is one of the largest families of plants and it is distributed all around the world, from the Northern to the Southern Hemispheres. They show a great diversity in morphology, from annual herbaceous to tropical trees, as well as habitat and ecology. Legume plants are used worldwide for a wide range of purposes, mainly as grain legumes for human food and as forage, but also to reduce soil erosion, as wind breaks, for medicinal purposes and as ornamentals. Legumes are of particular agricultural, ecological and economic importance in comparison to other plants, due to their mutualistic nitrogen-fixing symbiosis with soil bacteria.

An enormous diversity has been revealed in recent years among the Gram-negative bacteria that can establish nitrogen-fixing symbiosis with legumes. Currently, 57 species of legume nodulating bacteria have been identified

Abbreviations: ARDRA, amplified ribosomal DNA restriction analysis; ITS, internally transcribed spacer

☆Note: Nucleotide sequence data of isolates Ala-1, Ala-3 and Ala-5 are available in the EMBL database under the accession numbers AM491620-AM491622 for 16S rRNA, AM491630-AM491632 for the 16S-23S ITS region, AM491623-AM491625 for *nodC*, and AM491626-AM491628 for *glnII*, respectively; and AM491629 for *glnII* of isolate Ala-6.

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0723-2020/$ - see front matter © 2007 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2007.07.002

[25]. Most of these species (46 species) are *Alphaproteobacteria* belonging to the genera *Rhizobium, Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium*, known by the general name of rhizobia, but some unexpectedly "non-rhizobial" genera of *Alphaproteobacteria* and *Betaproteobacteria* also contain legume-nodulating species [16]. The list of new rhizobial and non-rhizobial species of nodulating bacteria continues to grow, as further isolations from newly studied legumes are carried out.

The Canary Islands is a small territory (7500 km^2) characterized by an enormous richness of endemic flora (27% of endemism). Forty of these endemic species are leguminous, but there are many other wild non-endemics. They are found colonizing a great diversity of environments from the coast to the summits of the islands. Many are of interest from a biological (rare, fertility problems), ecological (colonizing sandy and salty environments) or agricultural (potential as forage) point of view. Only a small number have been studied with regard to their nodulating rhizobia. They include the characterization of several bradyrhizobial isolates from some *Genisteae* legumes (*Chamaecytisus, Teline, Adenocarpus*, *Spartocytisus* and *Lupinus*) [3,4,10,15,23] whose main genotype was described as *Bradyrhizobium canariense* [22]. Therefore, most of the legumes in the Canaries are still unexamined, and a wide diversity of rhizobia is to be expected.

Anagyris is a genus of legumes belonging to the *Thermopsidae*, a small tribe of six genera spread over the temperate areas of the Northern Hemisphere [17]. *Anagyris* contains two species, *Anagyris foetida*, distributed in the Mediterranean area and the Middle East, and *Anagyris latifolia* Brouss. *ex* Willd. endemic to the Canary Islands. *A. latifolia* is a shrub legume (3–5 m high) with large trifoliate leaves (5–6 cm) and spectacular yellow flowers (known in the Islands as "oro de risco," gold of the rocks). At present, only small populations survive (with very few individuals) in different, often inaccessible, habitats on the Islands of Tenerife, La Palma, La Gomera and Gran Canaria. It is considered to be one the Canary endemisms in "critical danger of extinction." Traditionally, it has been used as forage (probably the cause of its decline), traditional medicine (purgative, emetic) and recently, as an ornamental. Nodulation in this species has not previously been reported. In this study, we isolated and characterized six rhizobial strains nodulating *A. latifolia* from three natural populations, two in Tenerife, in the north (Icod) and south (Arico), and one in the northeast of La Palma (Mazo).

Materials and methods

Bacterial strains, culture conditions and rhizobia isolation

Sterilized and germinated seeds of *A. latifolia* were grown on soil samples collected where three natural populations of this legume are growing. After 8 weeks, the bacteria from the root nodules were recovered, with isolates Ala-1 and Ala-5 being from the north Tenerife population (Punta de Juan Centella, Icod), isolates Ala-3, Ala-4 and Ala-7 from the south Tenerife population (Barranco de Tamadaya, Arico) and isolate Ala-6 from the northeast of La Palma (Mazo). Redundant strains were eliminated by their ERIC-PCR profiles (data not shown). The isolates were grown at 28 °C in yeast mannitol agar (YMA) [21]. All isolates were stored at −80 °C on YM plus 20% v/v glycerol.

DNA isolation

Total genomic DNA was obtained from bacterial batch cultures grown until late exponential phase using the AquaPure Genomic DNA isolation kit (Bio-Rad). DNA concentrations were visually estimated from a 1% agarose gel electrophoresis by comparing the DNA samples with lambda-DNA *HindIII* digest.

16S ARDRA

The nearly full-length 16S rRNA gene was amplified as previously described [4]. Aliquots of the PCR products were separately digested with five endonucleases, *DdeI*, *MspI RsaI*, *HaeIII* and *HinfI* (Amersham-Pharmacia Biotech), following the manufacturer's recommendations with an excess of enzyme (4–5 U). The digests were separated by horizontal electrophoresis in 2.5% high resolution agarose gel (Sigma Chemical Co., St. Louis, Mo) in TBE buffer at 60 V for approximately 3 h. The digitalized gel images from the restriction patterns of the five endonucleases were combined and analysed with GelCompar II v. 4.2. The image was normalized with the 100 bp ladder marker (Amersham-Pharmacia Biotech) loaded at the centre and both sides of the gel. A dendrogram was constructed from the similarity matrix using the unweighted pair group method with arithmetic mean (UPGMA) and Dice's similarity coefficient. Reference strains used for ARDRA were *Mesorhizobium amorphae* LMG 18977^T, *M. huakuii* LMG 14107^T, *M. loti* LMG 6125^T, *M. tianshanense* USDA 3592^T, *M. chacoense* PR5^T, *S. fredii* USDA 205^T, *S. meliloti* LMG 6133^T, *R. leguminosarum* bv. *viciae* USDA 2370^T, *R. etli* USDA 9032^T, *R. giardinii* H152^T, *R. hainanense* I66^T, *A. tumefaciens* ATCC 23308, *B. japonicum* USDA 6^T, *B. japonicum* USDA 110 and *B. canariense* BTA-1^T.

Sequencing of the 16S rDNA, 16S–23S rDNA (ITS), *glnII* and *nodC* loci

The 16S rRNA gene was amplified as above. The internal transcribed spacer (ITS) of the 16S–23S rDNA region was amplified by using the primers FGPS 1490 and FGPL 132′

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[3]. A partial sequence from the glutamine synthetase II gene (*glnII*) was obtained with primers *glnII* 12F and *glnII* 689R [24]. A 930 bp *nodC* gene was amplified with nodCF and nodCI as previously described [8]. The PCR-amplified products were run on 1% agarose gel, and the excised band was purified (Qiaex II Gel extraction, Qiagen) and sequenced in an ABI-377 sequencer (Applied Biosystems Inc.) using the BigDye terminator (v. 3.0).

Sequence analysis

The sequences obtained were aligned with Clustal W 1.83 [18] and manually edited for obvious errors. The phylogenetic analyses were conducted using MEGA version 3.1 [6]. The phylogenetic trees were inferred by the neighbour-joining method (NJ) [14] using Kimura's-2-parameter model [5]. Confidence in the nodes was assessed using bootstrap proportions (1000 replicates).

Nodulation tests

The isolates were tested for nodulation on *A. latifolia, Macroptilium atropurpureum, Lotus corniculatus, Glycine max, Trifolium* sp., *Medicago sativa, Phaseolus vulgaris* and *Glycyrrhiza uralensis* (5 replicates for each strain). Germination of *Anagyris* and *Glycyrrhiza* seeds required previous scarification for about 45 and 15 min, respectively, with concentrated sulphuric acid. Seed surface sterilization was carried out with 50% diluted commercial hypochlorite for 5–7 min for *L. corniculatus, Trifolium* sp., *M. sativa*, and *G. uralensis*, and 10–15 min for *A. latifolia, M. atropurpureum* and *G. max*. Sterilized seeds were washed six times with sterile water and placed on plates with water-agar (1%) in the dark. A well grown rhizobial culture was used as the inoculum suspension. Seedlings were inoculated by submerging them in the bacterial suspension for an hour and then transferring them to Leonard jars with vermiculite-sand (1:1) and a nitrogen-free nutrient solution [2]. Uninoculated plants served as control. Plants were harvested after 6–8 weeks and the number of nodules was recorded. Nitrogen fixation capacity was deduced from the appearance of plants and nodules.

Results and discussion

16S ARDRA

All isolates produced a single band of the expected length (1500 bp) in the 16S rDNA PCR amplification. The combined restriction patterns, obtained with five endonucleases, of the six isolates and reference strains of *Mesorhizobium*, *Sinorhizobium*, *Rhizobium* and *Bradyrhizobium* were used for cluster analysis. A Dice-UPGMA dendrogram (data not shown) revealed that the six isolates of *A. latifolia* belonged to the genus *Mesorhizobium*, but they clustered into three different branches. The isolate Ala-5 and the type strain of *M. amorphae* presented identical profiles and were grouped in a branch from *M. huakuii* at a 97.7% similarity level (S_D). The isolates Ala-3, Ala-4 and Ala-7 (S_D 100%) grouped into a branch at 93.9% S_D with the reference strain of *M. tianshanense*, and 92.8% S_D from the previous group. The isolates Ala-1 and Ala-6 (S_D 100%) were recovered more distantly in a branch at 87.6% S_D from the other *Mesorhizobium*. Therefore, ARDRA permitted us to distinguish the existence of three genotypes nodulating the legume *A. latifolia*, however, no more conclusions could be made as only five *Mesorhizobium* species were included in the restriction analysis. The strains Ala-1, Ala-3 and Ala-5 were chosen as representatives from each branch for further analysis.

16S rDNA sequencing

The nearly full-length 16S rDNA sequences were obtained for the isolates Ala-1, Ala-3 and Ala-5, which represented the three 16S-ARDRA groups. The three sequences showed similarity values 99.2–99.5%. High similarities were also shared with the type strains of *Mesorhizobium* species. Similarities of 99% or above, for the three isolate sequences, were found with the type strains of *M. amorphae*, *M. huakuii*, *M. mediterraneum*, *M. plurifarium*, *M. temperatum* and *M. tianshanense*, and values of 97.8–98.5% were obtained with the type strains of *M. loti*, *M. ciceri* and *M. chacoense*. *M. thiogangeticum* produced scores under 96% with all *Mesorhizobium*. The phylogenetic tree built from these sequences (Fig. 1) showed that, in agreement with 16S-ARDRA, the three representative sequences of the *Anagyris* isolates clustered into three different branches. Ala-5 grouped in a branch with *M. amorphae* (99.8% sequence similarity) and *M. septentrionale* (99.7% similarity), but with a low bootstrap support value (69%). The strains Ala-1 and Ala-3 represented two different branches within the big cluster of *Mesorhizobium* species with no bootstrap support to any of the type strains. Thus, it was possible to infer from the phylogenetic tree based on the ribosomal RNA sequences that the three isolates of *A. latifolia* represented three distinct and new lineages within the genus *Mesorhizobium*. However, as expected for such conservative sequences, the resolution of the branches was poor, making taxonomic proposals at the species level unreliable.

16S–23S ITS sequencing

The three representative isolates had ITS regions of different sizes, Ala-3 and Ala-5 produced fragments of

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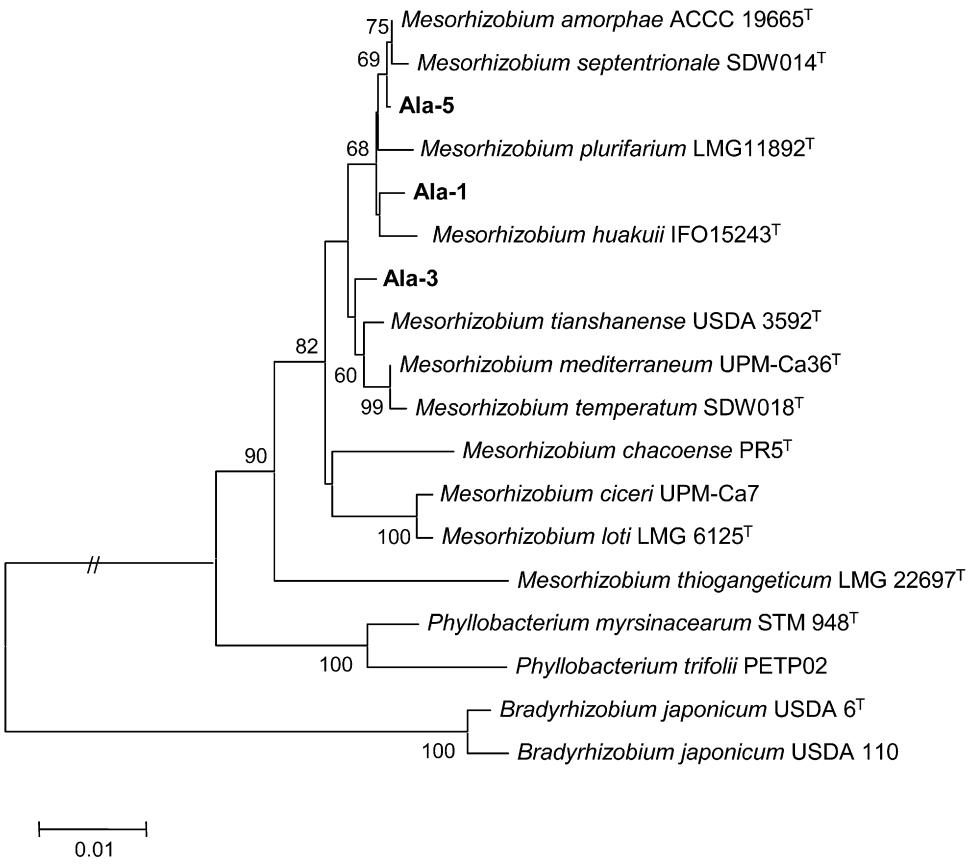

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences (1396 bp) obtained by neighbour joining, showing the relationships of rhizobial isolates from *Anagyris latifolia* and reference strains of *Mesorhizobium*. The tree is rooted on *Bradyrhizobium japonicum*. Isolates are in bold. Numbers at node branches indicate the percentage of bootstrap support of 1000 resampled datasets (only bootstrap values of 60% or above are shown).

729 and 757 bp, respectively, while Ala-1 had a larger ITS of 919 bp. As expected, the ITS sequences were much more varied than those of 16S rDNA. The ITS of isolates Ala-5 and Ala-3 were 87.3% similar (all similarity values given for the ITS sequences include gaps), while the ITS sequence of Ala-1 showed around 70% similarity with both Ala-3 and Ala-5. Percentage values of the ITS sequences of the isolates with the included type strains of *Mesorhizobium* ranged from about 68% to 88%. The ITS phylogeny resolved the different genera of rhizobia very well. Thus, the isolates and the reference strains of *Mesorhizobium* were placed on a separate branch from other rhizobia genera. However, the high variability of these sequences, with numerous insertions and deletions, makes careful manual edition necessary to obtain reliable alignments and hinders the comparison of alignments when sequences from distant species or different genera are included. Thus, ITS-trees only including strains within a single genus may be more accurate, which is why we show an unrooted ITS tree for the *Mesorhizobium* genus, as previously presented [7,20]. In the phylogenetic tree built from these sequences (Fig. 2), the isolates and the species of *Mesorhizobium* used here for reference were very well resolved (we used several strains of the same species or related genotypes to delineate the branches better). One branch clustered the isolates Ala-5 and Ala-3, and the reference strains of *M. amorphae*, *M. tianshanense* and *M. huakuii* at a relatively significant bootstrap (77%). Ala-5 and the strains of *M. amorphae* formed a close cluster at a bootstrap value of 99%. Within this branch, Ala-3 was placed on a well-separated subbranch from the other strains. Ala-1 and the other species of *Mesorhizobium* used for reference clustered apart in well-defined branches without significant relationships between them, except for *M. loti* and *M. ciceri* type strains (92.8% similarity). Unfortunately, no ITS sequences are available in the GenBank for *M. chacoense*, *M. septentrionale*, *M. temperatum* and *M. thiogangeticum*, however, no close relationship of these isolates with these species is expected, as deduced from their 16S rDNA sequences.

Contrary to the 16S rRNA sequences, the variation in the ITS region has been shown to be more informative for taxonomic evaluation and species affiliation in the

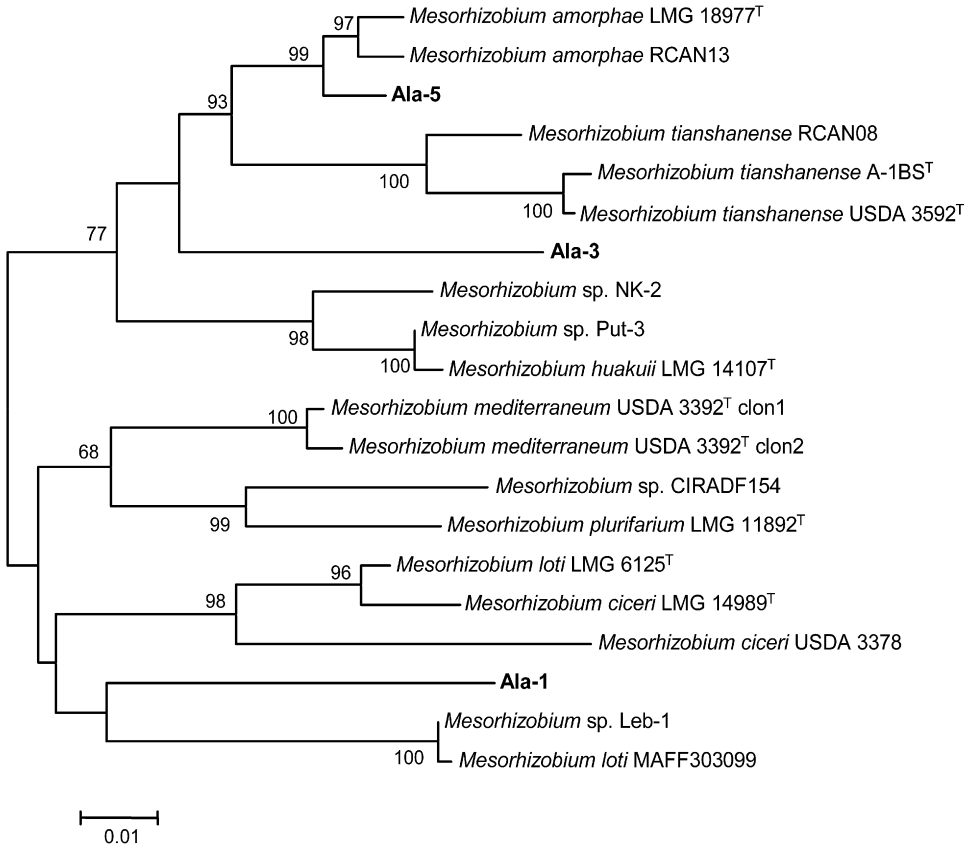

Fig. 2. Phylogenetic tree based on the ITS region sequences (1008 bp, including gaps) showing the relationships of rhizobial isolates from *Anagyris latifolia* with reference strains of *Mesorhizobium*. Further details in the legend to Fig. 1.

Bradyrhizobium genus [20,27]. Great variability of the ITS region sequences has also been found in the genus *Mesorhizobium* [7], and this was also the case for our isolates. The ITS sequences permitted us to resolve our isolates and the named species of *Mesorhizobium* into clear branches. Good agreement was found between the 16S rRNA and the ITS phylogenies. As observed in the 16S rRNA phylogeny, the isolates represented three different lineages on the ITS tree, but the branches were better delineated. A close phylogenetic relationship of Ala-5 with *M. amorphae* was more evident in the ITS phylogeny compared to the 16S rRNA. Likewise, the ITS tree showed that strains Ala-1 and Ala-3 represented two independent very well delineated lineages in the *Mesorhizobium* cluser. As observed for *Bradyrhizobium* [28], the strains with percentage similarities of 95% or above in their ITS region sequences always belonged to the same species, although the strains within a species can often present scores under 95%. None of the ITS sequences of the isolates studied here presented similarity values around 95% with any of the reference strains of *Mesorhizobium*, neither did Ala-5 and *M. amorphae*, thus, we cannot assign them to the named species of *Mesorhizobium*.

glnII sequencing

A partial sequence of the glutamine synthetase II (*glnII*) was used to contrast the ribosomal phylogeny. The tree built (Fig. 3) from these sequences agreed with ribosomal analysis in placing Ala-1, Ala-3 and Ala-5 (sequence similarities ranged 86.1–89.8%) on three different branches within the *Mesorhizobium* genus, although none of them included reference strains. On the contrary, in the *glnII* tree topology the three isolates represented three new independent lineages for the genus *Mesorhizobium*. Thus, the phylogeny based on the *glnII* sequences was not congruent with the ribosomal in the relationship found for the isolates Ala-5, since the *glnII*-tree placed Ala-5 distantly from *M. amorphae* (87.4% similarity). Coincident with the ribosomal phylogeny, Ala-1 and Ala-3 were also placed in two distant branches. In this analysis, we included the isolate Ala-6, from the same 16S-RFLP group as Ala-1, and found that they also had highly similar *glnII* sequences (96.6%), and they, consequently, formed a very consistent branch (100% bootstrap). The rhizobial phylogeny based on *glnII* sequences has been showed not to be congruent with the 16S rDNA phylogeny at

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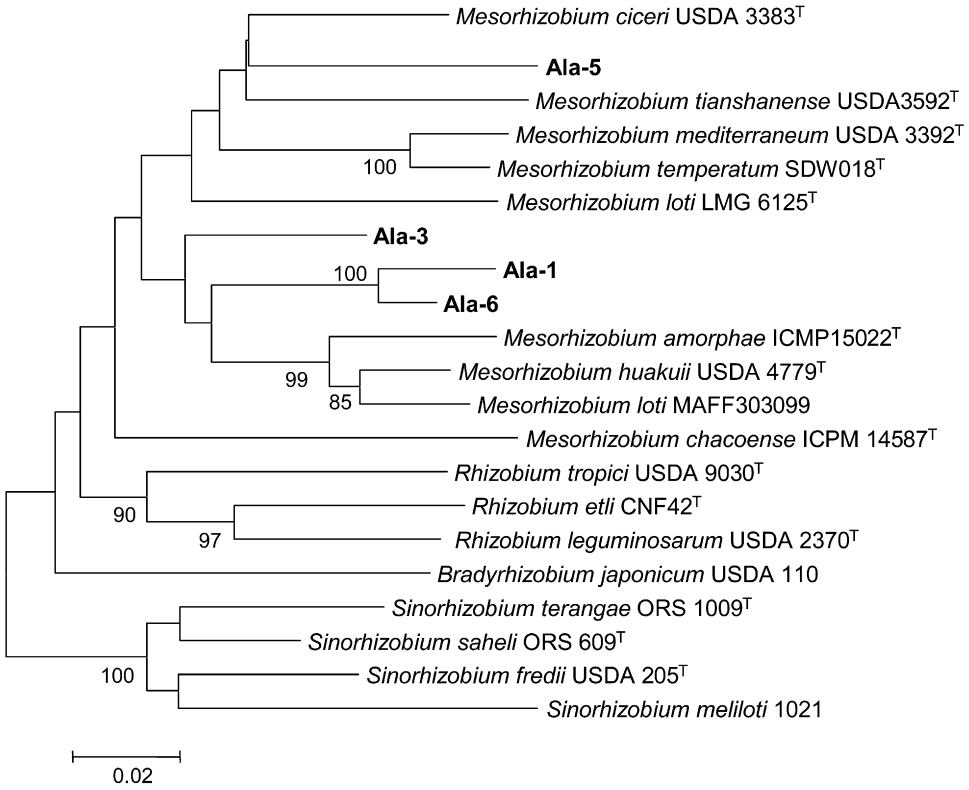

Fig. 3. Phylogenetic tree obtained by neighbour joining based on a partial fragment (468 bp) of the glutamine synthesase II of isolates and reference strains of *Mesorhizobium, Rhizobium*, *Sinorhizobium* and *Bradyrhizobium*. Further details in the legend to Fig. 1.

the species level [19,26]. There is evidence suggesting horizontal gene transfer for the *glnII* gene among the rhizobia [19]. Interestingly, *M. amorphae* shows high similarity in the *glnII* sequence with *M. huakuii* (94.4%), a rhizobial species whose *glnII* sequence appears to be more related to the *Rhizobium* genus sequences than to *Mesorhizobium* sequences [19]. The distant relationship found in the *glnII* phylogeny between isolate Ala-5 and *M. amorphae* might be explained by the acquisition of *glnII* from different origins. Although the available sequences for *M. plurifarium* and *M. septentrionale* do not overlap with those obtained here for these isolates, no close relationship is expected according to the phylogeny of the 16S rRNA and the ITS.

Therefore, the results presented here show that this single plant species, *A. latifolia*, is nodulated by three different *Mesorhizobium* species. The isolate Ala-5 seems to represent a species which is a very close relative of *M. amorphae*, according to the ribosomal sequence analysis, although the contradictory data from glutamine synthetase makes further studies necessary. However, all the data presented here support the idea that Ala-1 and Ala-3 represent two new species for the genus *Mesorhizobium*. These two representative isolates could represent widely distributed *Mesorhizobium* species in the Canaries, as we have found similar genotypes nodulating some endemic species of *Lotus* in these islands (unpublished). Moreover, they are not restricted to a particular area or island, thus, for instance, the genotype represented by Ala-1 and Ala-6 were isolated from the north of Tenerife and northeast of La Palma, respectively. Data from DNA–DNA hybridization will be necessary to finally determine the taxonomic status.

Phylogenetic analysis of the symbiotic *nodC* gene and infective properties

Despite their taxonomic position based on the ribosomal DNA or *glnII* sequences, all three isolates of *A. latifolia* presented identical *nodC* sequences, and were closely related (95%) to that of the type strain of *M. tianshanense*. In the *nodC* tree (Fig. 4), *M. tianshanense* USDA 3592^T and the isolates clustered in a branch at a 100% bootstrap value.

In the infectivity tests, apart from the original host, the isolates formed nodules on *G. uralensis*, which correlated with the sequencing data of the *nodC* gene (in the same way, *M. tianshanense* USDA 3592^T nodulated *A. latifolia*). The isolates also formed white nodules on *P. vulgaris*, and a few nodules were found on some plants of *M. atropurpureum*, but they did not nodulate on *L. corniculatus*, *M. sativa*, *G. max*, and *Trifolium* sp. Nodulation on *P. vulgaris* and not on *Glycine max*

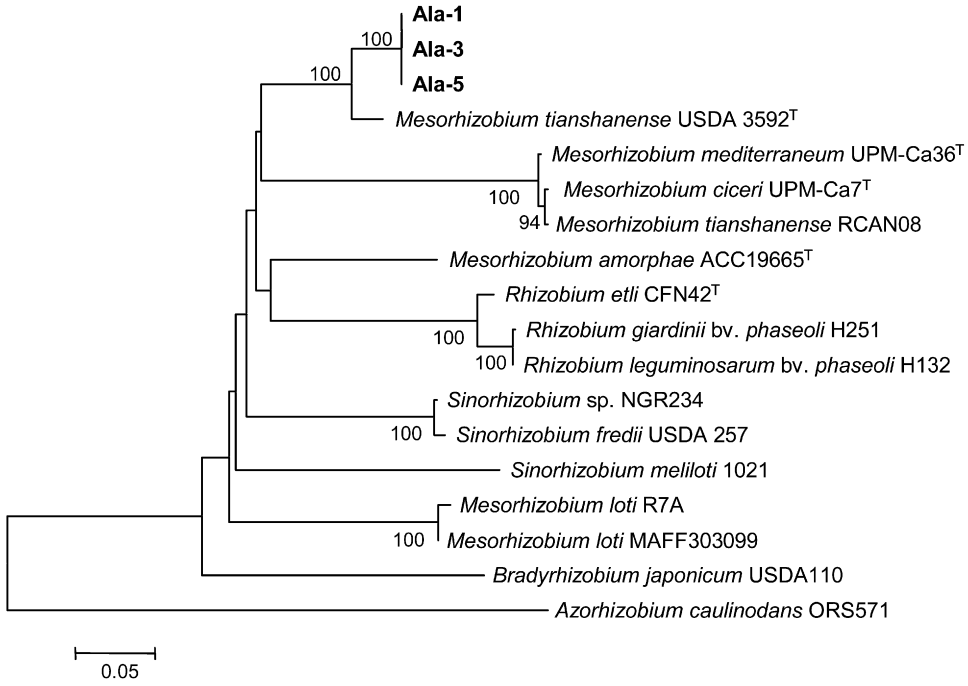

Fig. 4. Neighbour-joining tree showing the phylogenetic relationships based on sequences (717 bp) of the *nodC* gene of isolates from *Anagyris latifolia* and reference strains of *Mesorhizobium, Rhizobium*, *Sinorhizobium* and *Bradyrhizobium*. Further details in the legend to Fig. 1.

contrasts with that of *M. tianshanense* [1]. The capacity to form nodules on *P. vulgaris* is negligible as this plant has proved to be a non-selective legume [11].

The high similarity of the *nodC* sequences of the *A. latifolia* isolates and the type strain of *M. tianshanense*, indicate that they probably acquired these symbiotic genes by a phenomenon of lateral gene transfer. *M. tianshanense* was originally described in China from wild legumes (*Glycyrrhiza*, *Sophora*, *Caragana*, *Halimodendrom*) and *G. max* [1]. Other strains of *M. tianshanense* have been detected in Portuguese soils but the nodulation genes were unrelated to the Chinese strains [9]. Interestingly, no 16S rRNA genotypes similar to *M. thianshanense* were detected among these Canary isolates of *Anagyris*, but it is possible that strains of *M. tianshanense* nodulate other Canary Island legumes, and the *Anagyris* isolates acquired the symbiotic genes from them.

Some species of *Mesorhizobium* were originally described as nodulating a narrow range of legumes, and, in some cases, as for *M. ciceri* and *M. mediterraneum*, they only include the original host [12,13]. However, horizontal gene transfer of the nodulation genes from one species to another is not making it possible to relate a single species of rhizobia with a specific host legume. A few examples of this are the striking presence on the Iberian Peninsula of chickpeas nodulated by *M. tianshanense* strains carrying symbiotic genes like those of *M. ciceri* and *M. mediterraneum* [9], or the diversity of chromosomal genotypes with similar nodulation genes in *Astragalus* isolates [29]. Another case is represented by the results of the present work where novel *Mesorhizobium* genotypes, none belonging to the *M. tianshanense* species, harbour nodulation genes like those originally found in the Chinese strains of *M. tianshanense* [1].

Acknowledgements

This work was supported by Grant BOS2003-05858 from the Ministerio de Educación y Ciencia of the Spanish Government, and a predoctoral fellowship from the Canary Islands Government to J. Donate-Correa. We are grateful to J. García Casanova for his invaluable help in locating the *Anagyris latifolia* populations, and J. Leal, D. Rodríguez-Navarro and A. Buendia for providing seeds.

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Please cite this article as: J. Donate-Correa, et al., Different *Mesorhizobium* species sharing the same symbiotic genes nodulate the shrub legume *Anagyris...*, Syst. Appl. Microbiol. (2007), doi:10.1016/j.syapm.2007.07.002

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Please cite this article as: J. Donate-Correa, et al., Different *Mesorhizobium* species sharing the same symbiotic genes nodulate the shrub legume *Anagyris*..., Syst. Appl. Microbiol. (2007), doi:10.1016/j.syapm.2007.07.002