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Astragalus algarbiensis is nodulated by the *genistearum* symbiovar of *Bradyrhizobium* spp. in Morocco

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ABSTRACT

Astragalus algarbiensis is a wild herbaceous legume growing in Maamora, the most important cork oak forest in northern Africa. It is a plant of great importance as fodder in silvopastoral systems, and in the restoration of poor and degraded soils. The purpose of this study was to describe the biodiversity of rhizobia nodulating this plant and determine their identity. Out of 80 bacterial isolates, 56 strains isolated from root nodules of *A. algarbiensis* were characterized. ERIC-PCR fingerprinting grouped the strains in two main clusters containing 29 and 27 isolates, respectively, and the amplified ribosomal DNA restriction analysis (ARDRA) generated two different ribotypes. Based on both the ERIC-PCR and ARDRA results, representative strains As21 and As36 were selected for further genetic studies. The nearly complete 16S rRNA gene sequences of As21 and As36 showed that they were closely related to *Bradyrhizobium cytisi* CTAW11^T with similarity values of 99.84% and 99.77%, respectively. Concatenation of *atpD*, *recA*, *gyrB* and *dnaK* housekeeping gene sequences indicated that strains As21 and As36 had a 95.22% similarity but they showed values of 95.80% and 94.97% with *B. cytisi* CTAW11^T, respectively. The sequencing of the symbiotic *nodC* gene of the two strains revealed 97.20% and 97.76% identities, respectively, with that of *B. cytisi* CTAW11^T isolated from *Cytisus villosus* growing in the Moroccan Rif Mountains. Furthermore, the phylogenic analysis showed that the strains isolated from *A. algarbiensis* clustered with *B. cytisi* and *B. rifense* within the bradyrhizobia *genistearum* symbiovar and may constitute two novel genospecies.

Introduction

With a total area of 132,000 hectares (ha), including 66,000 ha of cork oak, the Maamora is the largest cork oak forest in the south Mediterranean region, playing important economic, social and environmental roles [5,17]. This forest, however, is under high edaphoclimatic and anthropic pressures, since it is submitted to different constraints such as overgrazing, climatic changes and mineral element poverty of its soils [18]. These factors provoke erosion and desertification and, therefore, degradation and regression of the forest [37,49].

The vegetation cover of the Maamora area is known for its great diversity, and *Fabaceae* plants occupy second place in importance after the *Poaceae* plant family [1]. The use of legumes to improve soil quality and fertility, as well as protection against desertification, is an ecologically sustainable approach that respects the environment [35]. Legumes are known to be pioneer species and can rapidly restore degraded ecosystems [27] through a symbiosis they elicit with nitrogen (N_2)-fixing bacteria called rhizobia [11,25,42,53,64].

The *Fabaceae* is the third family of the flowering plants with almost 19,000 referenced species and 750 genera [2]. However, only a few leguminous plant species have been studied for nodulation, most of which are those used for animal and human consumption [30]. Wild legumes, such as astragals, are characterized by their excellent adaptation to edaphoclimatic conditions (e.g. drought, salinity, degraded soils) and their multiple uses (e.g. the fight against erosion, animal feed, pastoral, phytoremediation) [22,23,65].

Astragalus is the largest genus in the *Fabaceae* with approximately 2900 species [67], and it is widely distributed in Africa, South America and the Northern Hemisphere [43]. Many species of this genus are used in traditional and modern medicine to cure

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several diseases such as diabetic nephropathy and hemorrhoids, and some astragalosides have an anti-aging effect related to the improvement of brain function and immunomodulatory effects. They also constitute an important source of pharmacologically active molecules with hepatoprotective, immunostimulatory and antiviral effects [55].

In the Mediterranean area, nearly 500 species of *Astragalus* have been reported [45]; however, only three spontaneous species are recorded in the Maamora forest, *A. algarbiensis* Coss. *A. lusitanicus* Lam (R) and *A. solandri* Lowe (N) [1]. *A. algarbiensis* (syn. *Tragacantha algarbiensis*) is an annual wild herb, endemic to Morocco, Algeria and the Iberian Peninsula. According to the European Committee for the Conservation of Nature and Natural Resources [52], *A. algarbiensis* is in the course of extinction in Portugal and Spain, but it is widely distributed in the sandy substrates of the coastal regions of Morocco [5,44,54] where it is a multipurpose plant used as fodder for livestock and to prevent soil erosion and desertification. In addition, *A. algarbiensis* can enrich the soil with nitrogen due to its ability to fix atmospheric N_2 in symbiosis with local rhizobia.

Rhizobia are aerobic soil bacteria that form N_2-fixing symbioses with leguminous plants by eliciting nodules on the roots, and sometimes on the stems, of their host plants. To date, rhizobia have been classified into the genera *Rhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Allorhizobium*, *Shinella*, *Pararhizobium*, *Neorhizobium*, *Mesorhizobium*, *Phyllobacterium*, *Aminobacter*, *Bradyrhizobium*, *Blastobacter*, *Photorhizobium*, *Devosia*, *Azorhizobium*, *Methylobacterium*, *Microvirga* and *Ochrobacterium* within the *Alphaproteobacteria*, together with *Paraburkholderia*, *Burkholderia* and *Cupriavidus* (formerly *Ralstonia*) within the *Betaproteobacteria* [56]. Many studies have shown that legumes have an intrinsic capacity to accommodate both symbiotic and non-symbiotic endophytic bacteria within root nodules [15,52,66].

Nitrogen fixing ability has been recorded in approximately 90 species of *Astragalus* [2], which are known to be nodulated by different species of the genera *Rhizobium* [11,12,19,32,41,62,70], *Ensifer* [11,19,41,64,69] and *Mesorhizobium* [11,19,20,21,41,60,61,64,70]. In addition, members of the slow growing genus *Bradyrhizobium* have been shown to establish N_2-fixing symbiosis with *A. henryi*, *A. melilotoides* and *A. sinicus* [10,70].

The analysis of the biodiversity of rhizobial populations associated with *Astragalus* will permit the selection of efficient inocula that could be utilized to improve the growth of this legume in different Moroccan regions. This study aimed to characterize the molecular diversity of 80 isolates from the nodules of *A. algarbiensis* growing wild in the Maamora forest near Rabat (Morocco). 16S rDNA sequence analysis, as well as four housekeeping gene phylogenies, showed that this legume was nodulated by *Bradyrhizobium* species of the symbiovar *genistearum*.

Materials and methods

Isolation of bacteria and culture conditions

Eighty bacteria were isolated from root nodules of *A. algarbiensis* plants. Nodules (4–6/plant) were sampled from 10 young healthy plants growing wild in the area of the Maamora Forest located (34° 07′ N, 6° 3′ W) in the vicinity of Rabat (Morocco). Mean temperature in the forest is 11 °C in winter and 34 °C in summer, and the mean rainfall is 550 mm/year [1]. The soil had a slightly acidic pH (6.4) and a measured electrical conductivity of 143.45 $\mu s\ cm^{-1}$. The nodules were washed under running tap water, then placed in a fine-mesh steel holder and surface-sterilized by immersion in 3% sodium hypochlorite for 3 min, and finally washed thoroughly with sterile distilled water. Nodules were placed independently in Petri dishes and crushed in a drop of sterile water with a sterile glass rod. The resulting suspension was streaked in Petri dishes containing yeast extract-mannitol (YEM) medium [58] supplemented with 0.0025% (w/v) Congo red. After incubation of the plates at 28 °C for 10 d, colony forming units representing all the colony types that could be distinguished by microscopic observation of living cells were chosen. After identification, all the isolates used in the study were routinely grown in tryptone yeast-extract (TY) medium to prevent the overproduction of exopolysaccharide produced on YEM medium [4].

Before further analysis, the symbiotic property of each isolate was checked by the possession of the *nodC* nodulation gene. The *nodC* gene was amplified by PCR, as mentioned subsequently, and isolates that failed amplification were excluded. The final number of *A. algarbiensis* isolates was thus reduced to 56.

Phenotypic tests

Tests were performed in liquid or on solid media inoculated with an exponentially growing liquid culture. The tolerance of the isolates to high temperatures was tested on TY medium at 28, 35, 40, 43 and 45 °C. The ability of isolates to grow on acidic or basic media was determined on solid YEM medium whose pH had been adjusted and buffered to 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 or 9, as described by Zerhari et al. [68]. The salt tolerance of the isolates was tested on TY medium supplemented with 1.7, 85, 171, 342, 513, 684 and 855 mM NaCl.

DNA extraction and ERIC-PCR fingerprinting

For DNA extraction and PCR amplifications, genomic DNA was isolated from bacterial cells as previously described [26]. The quantity of DNA was determined by using a NanoDrop spectrophotometer (NanoDrop ND2000/2000c, Thermo Fisher Scientific, USA). Enterobacterial repetitive intergenic consensus (ERIC)-PCR was performed as indicated earlier [25], using primers ERIC1R and ERIC2 [57] to reduce the number of strains and avoid any duplicates or clonality. Comparative analyses of the electrophoretic ERIC-PCR patterns were performed with GelCompar II software (version 2.5 Applied Maths, Belgium) by using the unweighted pair group method with arithmetic averages (UPGMA).

DNA ribotyping

For amplified ribosomal DNA restriction analysis (ARDRA), 7 μL of each purified PCR amplified product were separately digested with the restriction endonucleases *Hae*III and *Msp*I, (Promega Corp. Madison WI, USA), following the manufacturer's instructions. Digested DNA was separated by horizontal electrophoresis in a 2% (w/v) agarose gel (Bioline) prepared with TAE buffer. Electrophoresis was carried out at 70 V for 3 h. The gels were stained with ethidium bromide to a final concentration of 0.4% (w/v), visualized under UV radiation and photographed with a digital camera. An *in silico* ARDRA with 20 restriction enzymes was also performed using the Silent Mutator program. The 16S rRNA sequences were introduced for strains As21 and As36, together with 34 bradyrhizobial type strains and *R. gallicum* R602^T as an outgroup. The sequences downloaded from GenBank were restricted with the enzymes *Apo*I, *Bpm*I, *Bsi*HKAI, *Bsm*FI, *Bsp*1286I, *Bst*KTI, *Bts*CI, *Cvi*QI, *Fai*I, *Fok*I, *Hae*III, *Hga*I, *Hha*I, *Hph*I, *Hpy*CH4IV, *Msp*I, *Rsa*I, *Sfa*NI, *Tai*I and *Set*I. The choice of the 20 enzymes was based on the results obtained with the Restriction Comparator program [6]. The Silent Mutator software [7] was used to scan through the sequences and recognize sites for each restriction enzyme. Then a database containing the concatenated sequences was built and, finally, the Statistica software version 7.0 [50] was used to generate a dendrogram of similarities based on UPGMA analysis.

DNA amplifications, sequencing and analysis

PCR amplifications of 16S rRNA gene fragments were carried out using the two universal primers fD1-rD1 previously described [63]. The primer pair nodCF2/nodCI was used to detect nodulation genes following published methods [33]. Amplification products were checked by horizontal electrophoresis in 1% (w/v) agarose (Bioline) gels stained with ethidium bromide to a final concentration of 0.4% (w/v) (2.5 μL laboratory stock solution per 100 mL gel) in Tris-acetic-EDTA (TAE) buffer at 70 V for 1 h, which were finally photographed under UV light. The primer pairs TSatpDf/TSatpDr, TSrecAf/TSrecAr [51], dnaK1466Fd/dnaK1777Rd, gyrB343F/gyrB1043R and rpoB83F/rpoB1061R were used for amplification of the *atpD*, *recA*, *dnak*, *gyrB* and *rpoB* genes, respectively, as described by Martens et al. [38,39].

Amplification products were purified using the PCR product purification system of Qiagen and subjected to cycle sequencing using the same primers as for PCR amplification with ABI Prism Dye Chemistry, and analyzed with a 3130 X l automatic sequencer at the sequencing facilities of the National Centre for Scientific and Technical Research (CNRST) in Rabat (Morocco). The sequences obtained were compared with those from GenBank using the BLASTN program [3] and the EzBiocloud Database (https://www.ezbiocloud.net/). Distances calculated according to Kimura's two-parameter model [29] were used to infer phylogenetic trees with the neighbor-joining analysis [48] and MEGA7 software. The sequences were compared to those of several validly published *Bradyrhizobium* type species using MEGA7 [31].

Accession numbers of the nucleotide sequences of the bacterial strains used in this study are shown in the figure trees.

Results and discussion

A. algarbiensis is an endangered silvopastoral legume species in Europe, but not in Morocco [44]. Out of 80 bacterial isolates from the root nodules of *A. algarbiensis* growing wild in the Maamora forest near Rabat, 56 contained *nodC* genes that were used for further characterization and identification. All the strains grew on 86 mM NaCl, whereas two failed to grow on 171 mM, only two were able to grow in the presence of 684 mM and none grew at 856 mM NaCl. They all grew on YEM medium with pH values between 6.0 and 7.0 but none of them grew at a pH below 6.0 or higher than 8.5. They all grew very well at 40 °C, and 41% of the strains grew at 45 °C.

ERIC-PCR fingerprinting has been shown to be a powerful tool for assessing the genetic diversity of bacterial strains [26], and this technique was used to group the 56 strains, which showed two different patterns at 46% similarity containing 29 and 27 isolates, respectively (Fig. 1). Out of the 56 strains, 16 isolates showing identical DNA band profiles to other isolates were considered as duplicates and were thus eliminated from subsequent analysis, leaving only 40 strains for further study. The important heterogeneity obtained and the abundance of the repetitive sequences between 200 and 2000 base pairs revealed the high genetic diversity of the strains nodulating *A. algarbiensis* in soils of the Maamora forest. ARDRA assays using the *Hae*III and *Msp*I restriction enzymes showed that the 40 strains were grouped into two ribotypes (Table 1). Taken together, the ERIC-PCR and the ARDRA results allowed selection of two isolates, namely As21 and As36, as the representative strains of each ribotype, respectively, for further analyses.

The nearly complete sequence of the 16S rRNA gene from As21 and As36 showed that the two strains were members of the genus *Bradyrhizobium* within the family *Bradyrhizobiaceae* of the *Alphaproteobacteria*. The phylogenetic tree (Fig. 2) inferred from the 16S rRNA gene sequences revealed that strain As21 showed 99.84% and 99.77% identities with *B. cytisi* CTAW11^T and *B. rifense* CTAW71^T isolated from *Cytisus villosus* growing in the Moroccan Rif mountains [8,9], respectively. Strain As36 had 99.84% identity with *B. ganzhouense* RITF806^T isolated from nodules of *Acacia melanoxylon* [36] and 99.76% identity with *B. guangdongense* CCBAU 51649^T isolated from *Arachis hypogaea* [34] in China.

The 16S rRNA gene is highly conserved among *Bradyrhizobium* species and prevents clear taxonomic affiliation [13,14], which is why the analyses of various core genes (multilocus sequence analysis, MLSA) have been used to date, either individually or in combination, to assess the biogeography and evolutionary genetics of *Bradyrhizobium* species [59]. Rivas et al. [46] suggested that housekeeping genes may suffer horizontal transfer and homologous recombination, therefore, it is recommended to use more than one housekeeping gene for taxonomic inferences. In this study, the *atpD*, *recA*, *dnaK*, *gyrB* and *rpoB* housekeeping genes were used to provide a robust and reliable tool to clarify the taxonomic position of strains As21 and As36.

An *in silico* analysis using 20 restriction enzymes, including *Hae*III and *Msp*I used for the *in vitro* assays, and the 16S rDNA sequences of strains As21 and As36 also showed that the two strains were separated into two different clusters (Supplementary Fig. S1). Strain As21 was close to *B. cytisi* CTAW11^T, whereas strain As36 clustered with *B. rifense* CTAW71^T.

The amplification of the *atpD*, *recA*, *gyrB*, *dnak* and *rpoB* genes gave DNA bands of approximately 0.6, 0.5, 0.7, 0.3 and 1 kilobases (kb), respectively (data not shown). The sequences allowed the construction of phylogenetic trees (supplementary Fig. S2, Fig. S3, Fig. S4 and Fig. S5), which showed that strains As21 and As36 grouped with *B. cytisi* CTAW11^T.

A neighbor-joining tree (Fig. 3) based on the combined sequences of *atpD* + *recA* + *gyrB* + *dnaK* showed that strains As21 and As36 had 95.8% and 94.97% identities with *B. cytisi* CTAW11^T, respectively. They also shared 94.33% and 94.2% identities with *B. ganzhouense* RITF806^T, respectively, and 94.02% and 93.89% with *B. rifense* CTAW71^T.

The phylogenetic trees based on the different single housekeeping gene sequences showed that the strains were closely related to *B. cytisi* CTAW11^T and *B. ganzhouense* RITF806^T. However, the analysis of the *rpoB* gene sequences showed that strain As21 shared 92.75% and 92.39% identities with *B. algeriense* RST89^T and *B. retamae* Ro19^T, respectively. A more divergent position was found for strain As36 whose sequence showed 93.67% identity with *B. huanghuaihaiense* CCBAU 23303^T. In fact, a neighbor-joining tree based on the *rpoB* sequences of strains As21 and As36, and those of other *Bradyrhizobium* type strains, clustered into two clearly separate groups (Supplementary Fig. S6). These results agreed with those reported by other authors showing that the phylogeny of the *rpoB* sequences from species of the genus *Bradyrhizobium* is not congruent with the phylogeny of other housekeeping genes [23,28,46]. Accordingly, the sequences of the *rpoB* gene were not included in the construction of the concatenated phylogenetic tree.

Since 97% identity can be considered as a cutoff level for discrimination between species of the genus *Bradyrhizobium* using concatenated housekeeping genes [16,24], the results of the current study suggested that strains As21 and As36 may represent two novel species. It has also been shown that phylogenetic analyses of 5–6 concatenated housekeeping genes is a good indicator of species affiliation [40,46].

The phylogenetic analysis of the symbiotic *nodC* gene (Fig. 4) grouped strains As21 and As36 in a cluster together with those of the type strains of *B. rifens*, *B. cytisi*, *B. canariense*, and *B. lupini*, which corresponded to symbiovar *genistearum* [59].

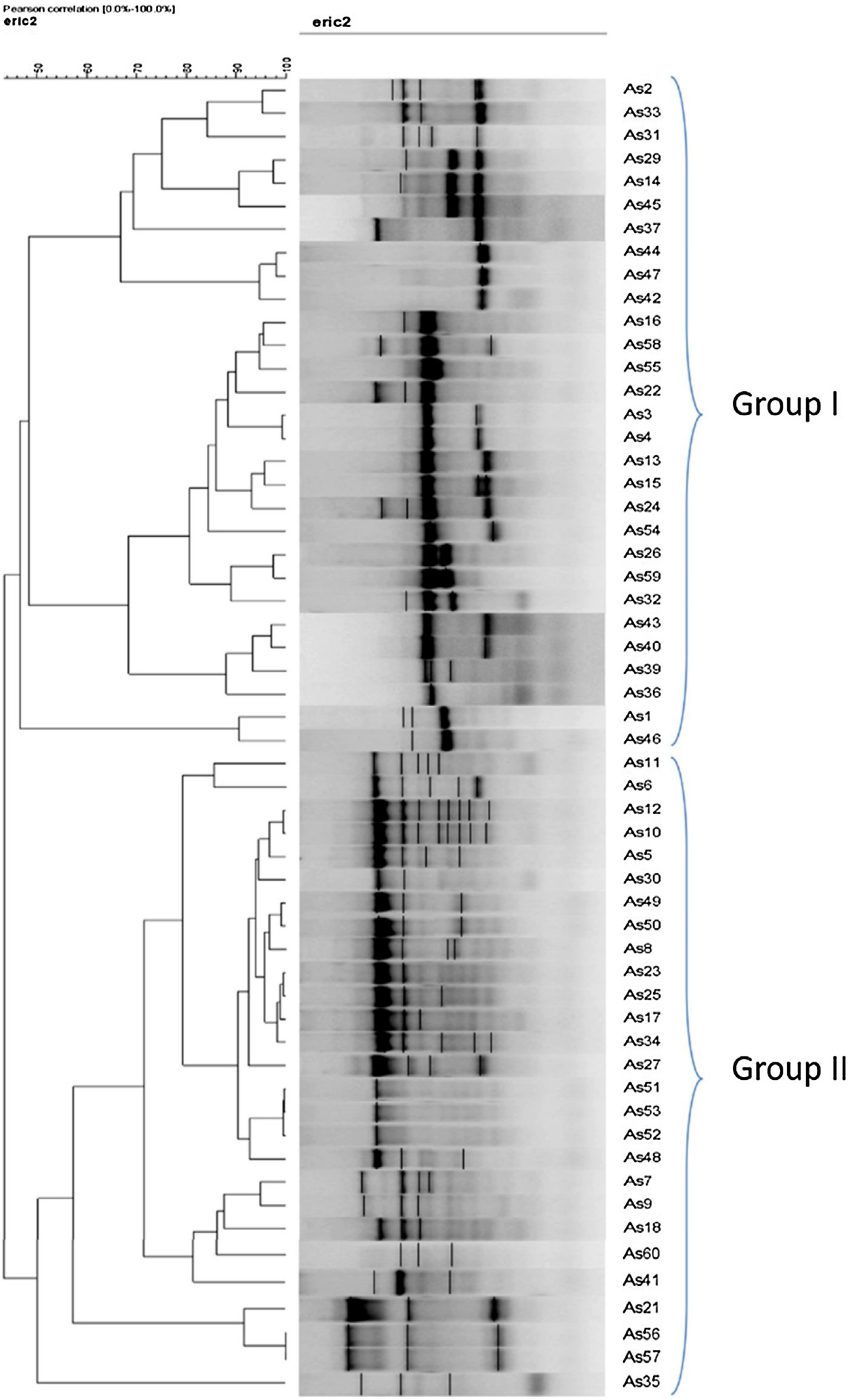

Fig. 1. Dendrogram generated by UPGMA clustering from ERIC-PCR fingerprinting of 56 bacterial strains isolated from *A. algarbiensis* growing wild in the Maamora forest (Rabat, Morocco). Some bands were confirmed and highlighted with the Gelcompar II software.

Table 1
ARDRA ribotypes obtained after digestion with restriction enzymes *Hae*III and *Msp*I.

Restriction enzyme	Ribotype 1	Ribotype 2
*Hae*III	As1, As2, As3, As5, As6, As7, As8, As9, As10, As11, As13, As14, As15, As16, As18, As22, As24, As25, As27, As30, As31, As32, As33, As35, **As36**, As37, As39, As40, As41, As42, As45, As46, As48, As49, As51, As54, As56, As59, As60	**As21**
*Msp*I	As1, As2, As3, As5, As6, As7, As8, As9, As10, As11, As13, As14, As15, As16, As18, As22, As24, As25, As27, As30, As31, As32, As33, As35, **As36**, As37, As39, As40, As41, As42, As45, As46, As48, As49, As51, As54, As56, As59, As60	**As21**

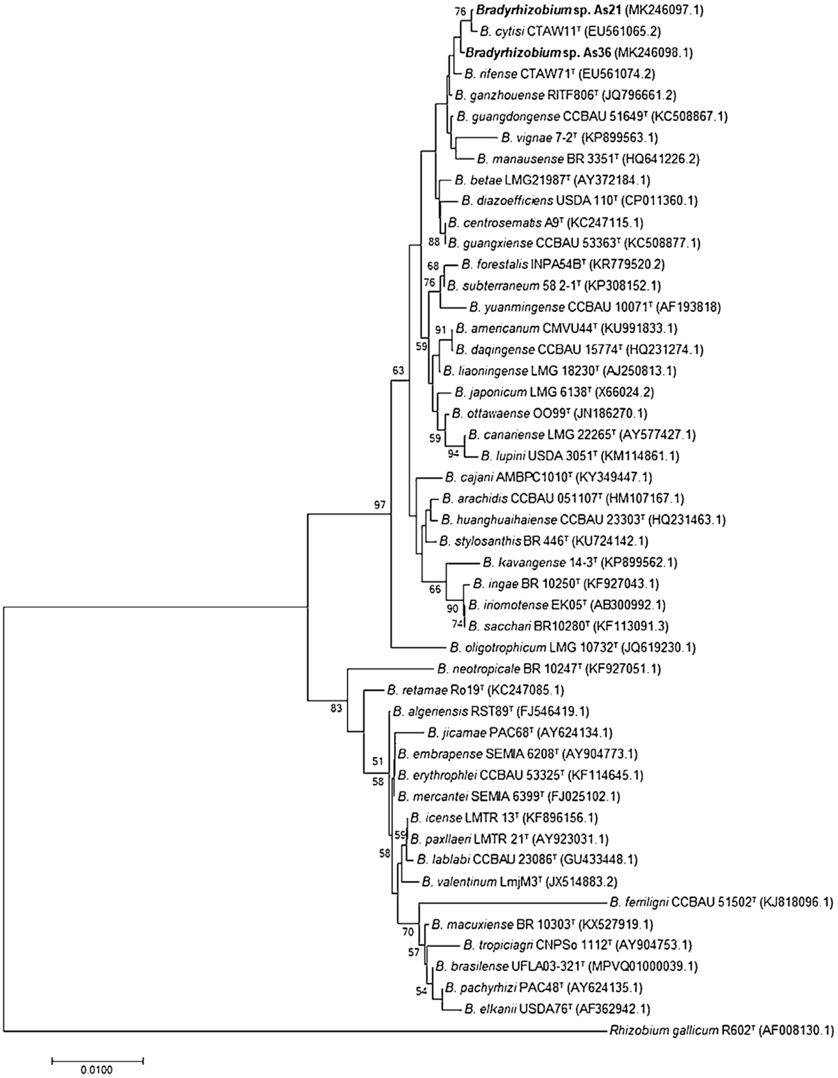

Fig. 2. Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences of strains As21 and As36 isolated from nodules of wild-grown *A. algarbiensis* and phylogenetically related species within the genus *Bradyrhizobium*. The analysis was based on 1328 nucleotides, and isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Bar, 1 nucleotide substitution per 100 nucleotides. The tree was rooted with *Rhizobium gallicum* R-4387^{T}.

The identity between the amino acidic sequences of strains As21 and As36 was 99.53%, whereas strain As21 had 97.2% and 96.74% identities with *B. cytisi* CTAW11^{T} and *B. rifense* CTAW71^{T}, respectively, and strain As36 shared 97.67% and 97.2% identities with *B. cytisi* CTAW11^{T} and *B. rifense* CTAW71^{T}. Previously, Chan et al. [10] had isolated and characterized eleven strains of *Bradyrhizobium* from *A. sinicus* root nodules in China and Japan.

Zhao et al. [70] reported that *Astragalus* species can form nodules with diverse rhizobia and different symbiovars were defined among the nodulating species. Based on the analyses of ribosomal DNA and housekeeping genes, three out of a total of 118 isolates from 13 *Astragalus* species growing in China were identified as *B. japonicum* strains [70]. In the current study, strains As21 and As36 isolated from *A. algarbiensis* clustered with *B. cytisi* and *B. rifense* (Fig. 4), which are included in the symbiovar *genistearum* within the genus *Bradyrhizobium* [8,9]. In contrast, the *nodC* gene of *B. japonicum* strain CCBAU 43170 isolated from *A. henryi* clustered within the symbiovar *glycinearum* (Fig. 4). Symbiovars are generally used to distinguish between different subgroups that are symbiotically distinct within a single rhizobial species [47].

Therefore, the current study identified novel *Bradyrhizobium* species and an additional symbiovar as microsymbionts for *A. algarbiensis*, which demonstrated that different *Astragalus* species might

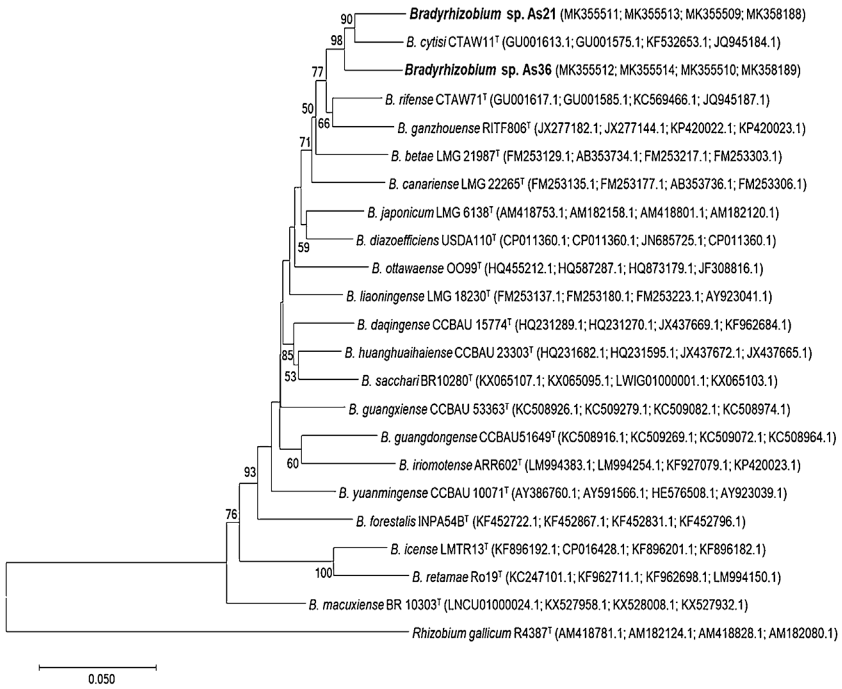

Fig. 3. Neighbor-joining phylogenetic tree based on concatenated partial *atpD* + *recA* + *gyrB* + *dnaK* gene sequences of strains As21 and As36 from nodules of wild-grown *A. algarbiensis* and phylogenetically related species within the genus *Bradyrhizobium*. The analysis was based on 1,570 nucleotides, and isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1,000 replications. Bar, 5 nucleotide substitution per 100 nucleotides. The tree was rooted with *Rhizobium gallicum* R-4387^{T}.

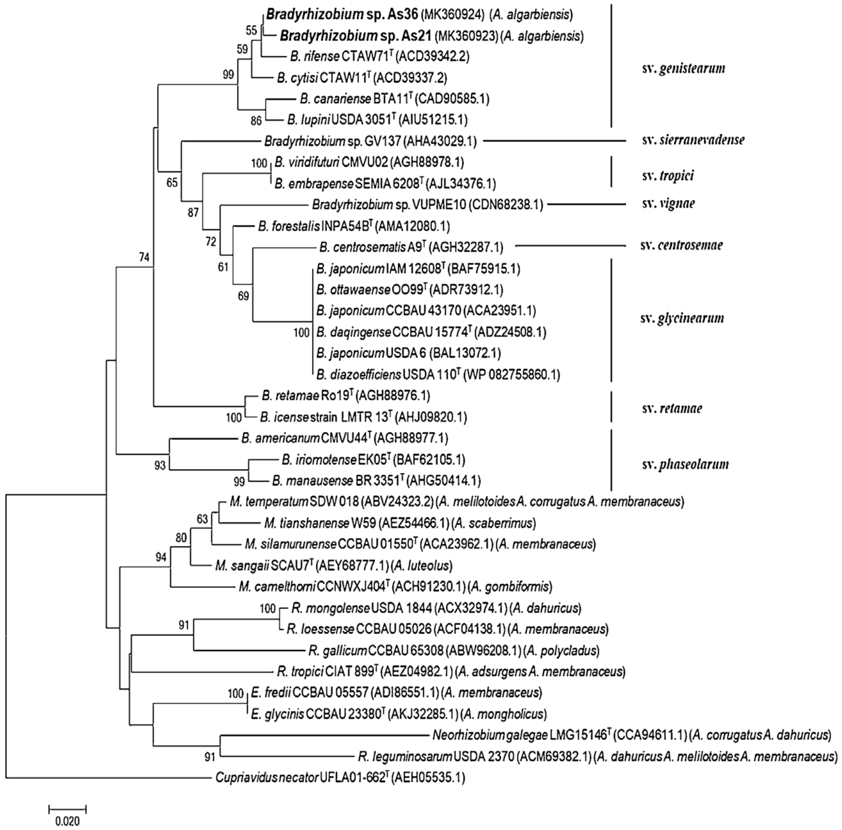

Fig. 4. Neighbor-joining phylogenetic tree based on the amino acid sequences of the nodC gene for strains As21 and As36 from nodules of A. algarbiensis and phylogenetically related species within the genus Bradyrhizobium. The analysis was based on 215 amino acids. Bootstrap values are indicated as percentages derived from 1000 replications. Bar, 2 nucleotide substitutions per 100 nucleotides. The tree was rooted with Cupriavidus necator UFLA01-662^{T}.

nodulate with rhizobia belonging to distinct species/genera harboring divergent symbiotic genes.

Similar to the rhizobia associated with other hosts [69], astragal symbiotic ability would be maintained by vertical and lateral transfer of symbiotic genes among rhizobial microsymbionts, and this might offer the *Astragalus* species more choice to form nodules with the most adapted rhizobia in different environments. However, it is more likely that multiple horizontal transfers could be responsible for members of the *B. cytisi*/*B. rifense* group nodulating legumes of the *Galegeae* tribe.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2019.03.004.

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