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Research Article

Different species of *Bradyrhizobium* from symbiovars genistearum and retamae nodulate the endemic *Retama dasycarpa* in the High Atlas Mountains

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Abstract

Retama dasycarpa is an endemic *Retama* species native to the cold semi-arid bioclimates of the High Atlas Mountains in Morocco. In this work, we analyzed the diversity of the microsymbionts nodulating this plant and their different phenotypic and symbiotic characteristics. Phylogenetic analysis of the 16S rRNA gene revealed that the tested isolates clustered in the *Bradyrhizobium* genus. Multilocus sequence analyses of four housekeeping genes (*recA, gyrB, glnII* and *atpD*) for 12 selected strains grouped them into four clusters close to *B. lupini* USDA 3051^T, *B. frederickii* CNPSo 3446^T, *B. valentinum* LmjM3^T and *B. retamae* Ro19^T. The individual phylogenies of these core genes and the symbiotic genes *nodC*, *nodA* and *nifH* were congruent. These isolates showed a broad host range, being able to nodulate different legume hosts, such as *R. sphaerocarpa*, *R. monosperma*, *Lupinus luteus*, *Cytisus grandiflorus* and *Chamaecytisus albidus*, but not *Phaseolus vulgaris* or *Glycine max*. They all had a similar metabolic capacity, using the majority of the carbohydrates and amino acids tested as sole sources of carbon and nitrogen. Furthermore, out of the 12 selected strains, some displayed plant growth-promoting features, with six of them solubilizing phosphate and three of them producing siderophores. The present work provides, for the first time, a detailed description about the microsymbionts associated with the endemic legume *R. dasycarpa*.

Keywords: biodiversity analysis, *Bradyrhizobium*, phylogenetic analysis, plant-growth-promoting activities, *Retama dasycarpa*

Introduction

The Fabaceae (Leguminosae), also known as legumes, is a very important plant family, considered the third largest angiosperm family after the Asteraceae and Orchidaceae. This superfamily is divided into six subfamilies with nearly 770 genera, and 20 000 species distributed worldwide (LPWG 2017). Its members are among the most versatile and important plants, found in a variety of environments with extreme temperature conditions and ubiquitous in different habitats and ecosystems. The legumes family includes a wide variety of plants, from common food crops to nitrogen-fixing trees, to popular ornamentals, with a variety of shapes, sizes and applications. Many species are considered an important source of protein and carbohydrates for human consumption and fodder for livestock.

The Genisteae (subfamily Papilionoideae) is the most represented tribe of legumes in the South Mediterranean, and consists of annuals and shrubs in Morocco, including the genus *Retama*, which contains only four validly described species (*R. monosperma* (L.) Boiss., *R. raetam* (Forssk.) Webb, *R. sphaerocarpa* (L.) Boiss. and *R. dasycarpa* (Cosson.)) (Boulila et al. 2009). Retamas are shrubby perennials, with long branches and few or no leaves, as a sign of their great adaptation to arid environments (León-González et al. 2018). They are widely distributed in different climates and ecosystems, and commonly found in coastal dunes and deserts, in North Africa, Southern Europe, the Canary Islands and East Asia. The three species *Retama sphaerocarpa*, *R. monosperma* and *R. raetam* are widespread in Spain and North African countries (León-González et al. 2018), whereas *R. dasycarpa* is endemic to Morocco (locally known as 'R'tem' or 'Algu'), and its geographic distribution is limited to the High Atlas Mountains (Teixidor-Toneu et al. 2016). These mountains, known for their high altitudes ranging between 1084 and 4167 m, are covered with large amounts of snow during the rainy season. Late snow persists until the summer, but permanent snow covers are infrequent (Boudhar et al. 2016). The climates vary from subhumid to semi-arid following altitudes with harsh long winters and low temperatures, while summers are hot with temperatures exceeding 30°C (Peyron 1980, Chaponnière et al. 2005). The vegetation cover decreases with elevation and only resilient plants can persist at altitudes above 2600 m (Chaponnière et al. 2005).

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Retama spp. plants are known for their various therapeutic properties because of their phenolic content, such as alkaloids, terpenes, steroids and flavonoids (González-Mauraza et al. 2013, Belayachi et al. 2014, Teixidor-Toneu et al. 2016). *Retama* plant species are also used for ecosystem restoration and they form natural plantations without the need for any artificial assistance (Hannane 2016). They are also included in revegetation programs in arid and semi-arid South Mediterranean areas to improve soil fixation and fertilization and to create natural barriers that prevent land desertification (Caravaca et al. 2003). *Retama dasycarpa* is a perennial plant, mainly found in the inner valleys of the High Atlas in cool and cold semi-arid bioclimates. This erect shrub is a pastoral species appreciated by herds and can reach more than 3 m in height, with a trunk of 3.5 to 6 cm in diameter. Its pods are ovoid and densely hairy with yellow seeds (Fennane et al. 2007).

Plants from the genus *Retama* establish N_2-fixing symbioses with soil bacteria commonly known as rhizobia, which allows them to grow in infertile soils unsuitable for other crops, contribute to feeding livestock, increase the fertility and quality of degraded soils, prevent erosion and participate in soil stabilization (Yahara et al. 2013, Ferguson et al. 2019). The process of nitrogen fixation is based on the reduction of N_2 by the bacterium to ammonia (NH_4^+), an assimilable form of nitrogen that is more efficiently metabolized by the host plant. In return, the plant provides a favorable ecological niche for the growth of the bacterium and provides it with carbon substrates originating from photosynthesis (Cheng 2008, Maróti and Kondorosi 2014). Rhizobia belong to 17 genera from both alpha and beta subclasses of proteobacteria (Bouhnik et al. 2021).

Over the last 20 years, several studies have been carried out in the Mediterranean area to analyze the diversity of *Retama* spp. microsymbionts. Species of the genus *Bradyrhizobium* are commonly the most encountered microsymbionts in *Retama* spp. root nodules (Rodríguez-Echeverría et al. 2014; Boulila et al. 2009, Guerrouj et al. 2013, Ahnia et al. 2018, Alami et al. 2021). However, Rejili et al. (2019) found that *R. raetam* is nodulated by *Ensifer meliloti* (former-*Sinorhizobium meliloti*) in arid environments in Tunisia, whereas Lamin et al. (2019) showed that *R. monosperma* microsymbionts in an abandoned lead mine tailings in Eastern Morocco belong to *Ensifer aridi* species. More recently, it has been reported that *Retama* spp. are also nodulated by members of the genus *Microvirga* in the Maamora forest (Lamrabet et al. 2020). However, no study has been conducted on *R. dasycarpa* endosymbionts. Therefore, in this paper, our main objective was to characterize for the first time the diversity of nodulating bacteria associated with this endemic plant in different locations of the High Atlas Mountains.

Materials and methods

Isolation and culture of bacteria

One hundred and twenty strains were isolated by trapping from root nodules of *R. dasycarpa* plants grown in four different soils in the High Atlas Mountains, where populations of this plant grow wild (Table S1). The climate in these areas is typically semi-arid with cold winters and dry summers (Bouhnik et al. 2022).

Seeds were scarified with 98% sulfuric acid for 1 h 30 min, allowed to germinate then planted in pots and grown in a greenhouse for 90 days. After 4 months of plant growth, the nodules were collected, washed with tap water and surface sterilized by immersion in 0.1% $HgCl_2$ (w/v) for 1 min, 95% (v/v) ethanol for 1 min and finally washed thoroughly with sterile water. Then each nodule was placed in microtubes, containing 0.5 ml of sterile distilled water and crushed with a pellet pestle. The extracts obtained were then streaked on Yeast-Extract-Mannitol-Agar (YEM) medium supplemented with 0.0025% Congo red and then the plates were incubated at 28°C for 10 days. After incubation, the colonies were subcultured several times under sterile conditions until homogeneous colonies were obtained.

DNA extraction and sequencing

Genomic DNA was isolated after cell growth in liquid TY medium (Beringer 1974) using the phenol-chloroform method. The amount of DNA was quantified using a Nanodrop spectrophotometer (NanoDrop ND1000). DNA was stored at -20°C until use. Primers REP1R-I and REP2-I were used for rep-PCR experiments as previously described by De-Bruijn (1992). The amplification of 16S rRNA (*rrs*) gene was achieved using the two primers fD1 and rD1 as reported by Weisburg et al. (1991) . The primer pairs TSrecAf/TSrecAr, TSatpDf/TSatpDr, TSglnIIf/TSglnIIr (Stepkowski et al. 2005) and gyrBF/gyrBR, dnaK1466F/dnaK1777R (Martens et al. 2007) were used to amplify the *recA*, *atpD*, *glnII*, *gyrB* and *dnaK* genes, respectively. The primers nodCFn/nodCI and nodA1F/nodAb1r were used for the amplification of *nodC* and *nodA* genes, as described by Laguerre et al. (2001) and Chaintreuil et al. (2001) . The *nifH* gene amplification was performed using primers nifHf/nifHi as described by Martens et al. (2007) .

The amplification products were sequenced using the same primers used for PCR amplification at the sequencing facilities of Estación Experimental del Zaidín, CSIC, Granada, Spain and Eurofins Genomics Germany. The obtained sequences were compared with those available in the GenBank database using the BLASTn program (Altschul et al. 1990) and EzBiocloud.net (Yoon et al. 2017). Distances were calculated according to Kimura's two-parameter model (Kimura 1980) and used to infer phylogenetic trees by neighbor-joining analysis (Saitou and Nei 1987) with MEGA7 software (Kumar et al. 2016). Identity values were calculated by pairwise analysis. Phylogenetic trees were subjected to 1000bootstrap replications and preferred topologies were plotted.

Shannon–Weiner diversity index

To estimate the diversity of the bradyrhizobial communities, the Shannon–Wiener diversity index (*H'*) was used as a quantitative parameter (Saeki et al. 2008, Ansari et al. 2014):

$$H' = -\Sigma Pi \ \ln(Pi)$$

Pi is the dominance of the isolates expressed as (n_i/N), and n_i and *N* are the number of isolates belonging to a particular REP-PCR fingerprint (at 70% similarity) and the total number of isolates tested, respectively.

Phenotypic characterization

All the phenotypic tests were carried out on YEM or TY media inoculated with fresh bacterial suspensions. The ability of the isolates to grow in acidic or basic media was assessed on solid YEM medium, which had been pH adjusted and buffered to 5.5, 6.0, 7.0, 8.0 or 8.5, as described by Guerrouj et al. (2013) . Salt tolerance was tested at 0, 1, 1.5, 2 and 2.5% of NaCl using solid YEM medium. The ability of strains to grow at 35, 40 and 43°Cwas also tested on solid YEM medium. Utilization of the following amino acids, tyrosine, histidine, tryptophan, lysine, glycine, serine, threonine, alanine, methionine, isoleucine and arginine, as sole sources of nitrogen, was investigated in solid MGS medium (Bouhnik et al. 2021), containing 0.4% (w/v) of each amino acid. Carbohydrate assimilation was assessed on solid MGS medium supplemented

with 0.4% (w/v) maltose, fructose, sucrose, lactose, trehalose, sorbitol, mannose and Myo-inositol. Carbohydrates and amino acids were sterilized by filtration.

PGP activities

The ability of the isolates to solubilize phosphate was tested using PVK medium (Pikovskaya 1948) containing 2.5 g l-1 of $(Ca_3PO_4)_2$. Aliquots (5 μl) of the bacterial suspension were deposited on PVK medium in Petri dishes and incubated for 7 days at 28°C. The ability of the isolates to solubilize phosphate was evaluated by measuring the size of halos that appeared around the colonies and the size of the colonies. The production of siderophore was detected as described by Lakshmanan et al. (2015) . The siderophore synthesizing isolates were identified by the formation of orange halos around the colonies, while the isolates' ability to produce indole acetic acid (IAA) was assessed on solid YEM medium supplemented with 0.5 g l-1 tryptophan. Five microliters of the bacterial culture were plated on the medium and incubated for 24 to 48 h at 28°C. IAA production was determined using Salkowski's reagent, which was followed by the appearance of a pink halo around the colonies after incubation at 28°C for 30 to 60 min. All the tests were run in triplicate.

Plant nodulation tests

Four selected strains were tested for nodulation of *R. dasycarpa*, *R. sphaerocarpa*, *R. monosperma*, *Lupinus luteus*, *Cytisus grandiflorus*, *Chamaecytisus albidus*, *Phaseolus vulgaris* and *Glycine max*. Seeds of *R. sphaerocarpa*, *R. dasycarpa*, *R. monosperma* and *C. grandiflorus* were scarified with 95% sulfuric acid, and then washed thoroughly with sterile distilled water. *Phaseolus vulgaris*, Soja and lupine seeds were disinfected for 5 min with 75% sodium hypochlorite and then rinsed with sterile water. Subsequently, the seeds were placed in Petri dishes containing sterile water agar (0.6%) and left to germinate at 28°C in darkness for 2 to 4 days. The seedlings were then transferred to Gibson tubes containing Jensen's N-free mineral solution (Roughley 2022) and independently inoculated with the different bacterial suspensions (approximately 10^8 cells ml^{-1}). Plants were grown at 26°C in a growth chamber under a 16.0/8.0 h light/dark photoperiod and the appearance of nodules was checked 10 to 12 weeks after inoculation. Jensen's solution was supplied only one time after 4 weeks of culture.

Results

Rhizobial isolation and REP–PCR genomic fingerprints

One hundred and twenty strains were isolated by trapping from root nodules of *R. dasycarpa* cultivated in four different soils as previously described, 18 from Tazilda (TZ), 27 from Ijoukkak (RDI), 33 from Ait Benammar (BA) and 42 from Tahanaout (RDT) regions (Fig. S1). All strains are slow growing, with an average time of 7 to 10 days to obtain colonies larger than 1 mm on YEM solid medium. Based on REP–PCR fingerprinting results, the isolates were grouped into nine different clusters at a 70% of similarity level (Fig. S2), from which we randomly selected 40 strains for *rrs* sequences analysis.

rrs sequence analysis

To further analyze the biodiversity of these strains, we conducted a phylogenetic analysis of the *rrs* gene of the 40 selected strains. This analysis revealed that they are all members of the genus *Bradyrhizobium* (Fig. 1), from which we retained 12 strains as representatives for further analysis.

The strains BA2, BA12, BA24 and RDT8 showed high similarities ranging from 99.40% to 100% with *B. lupini* USDA 3051^T, and from 99.1% to 99.7% with *B. canariense* $BTA1^T$, whereas strain TZ32 had percentage identities of 99.40% with *B. lupini* strain USDA 3051 and 99.10% with *B. canariense* strain BTA-1. The strain RDT10 had 99.78% of similarity with both *B. australiense* CNPSo 4014^T and *B. namibiense* 30 3–2^T, while strain RDT25 had similarities of 100 and 99.92 with *B. retamae* strain Ro19 and *B. archetypum* strain CNPSO:4013, respectively. The strain BA46 was close to *B. cytisi* $CTAW11^T$and *B. rifense* $CTAW71^T$ with similarities of 99.71% and 99.64%, respectively. The strain RDI18 was close to *B. retamae* $Ro9^T$ and *B. archetypum* CNPSo 4013^T, with similarity percentages of 99.84% and 99.92% respectively. The strain RDT46 had 99.38% similarity with *B. frederickii* CNPSo 3426^T and 99.3% with *B. guangxiense* CCBAU 53363^T and *B. centrosematis* $A9^T$, while the strain RDT48 shared similarities of 99.06% with *B. ferriligni* CCBAU 51502^T and 98.98% with *B. elkanii* USDA 76^T, *B. australiense* CNPSo 4014^T, *B. namibiense* 510^T, and *B. icense* LMTR 13^T. The strain TZ2 had similarities of 99.92% with strains *B. embrapense* SEMIA 6208, *B. mercantei* SEMIA 6399, *B. viridifuturi* SEMIA 690, and *B. erythrophlei* CCBAU 53325, and 99.77% with four types of strain, *B. namibiense* strain 5–10, *B. australiense* CNPSO:4014, *B. paxllaeri* strain LMTR 21, and *B. icense* strain LMTR 13.

Analysis of the bradyrhizobial communities' diversity

The *H′* diversity index was higher in Tahannaout and Ait Benammar, with values of 1.77 and 1.68, respectively, while the lowest *H′* values 0.26 and 0.21 were found at the Ijoukak and Tazilda sites, respectively (Table S1).

Multi-locus sequence analysis

Six housekeeping genes, *recA*, *gyrB*, *dnaK*, *atpD*, *glnII* and *rpoB*, of the 12 representative strains, BA2, BA12, BA24 and BA46 from the Ben Ammar area, strains RDT8, RDT10, RDT25, RDT46 and RDT48 from the region of Tahannaout, strain RDI18 from Ijoukak, and strains TZ2 and TZ32 from the Tazilda area (Table S1), were analyzed.

The phylogenetic trees of each gene are presented in the supplementary materials (Figs S2–S7). Analysis of the individual genes' sequences (Figs S2, S4 and S5) revealed that the strains BA2, BA12, BA24, BA46, TZ32 and RDT8 were identical to each other, and share similarities ranging from 99.5% to 100% with *B. lupini* USDA 3051^T. The two strains RDT10 and RDT48 are also similar, and their closely related species are *B. valentinum* $LmjM3^T$ with similarities ranging from 94.74% to 97.67%. Strain RDT46 is close to *B. frederickii* CNPSo 3426^T with similarity percentages ranging from 94.33% to 99.08%, whereas strain RDI18, isolated from the Ijoukak region soils, and strain RDT25 from Tahannaout, are closer to *B. retamae* Ro9T, with which they share 95.85% to 98% and 97.5% to 97.7% similarity, respectively.

However, after many trials, using different primer pairs, we were unable to amplify the atpD gene of strain RDT25. Analysis of the concatenated sequences of the *atpD, gyrB* and *glnII* genes of strain TZ2 showed that its closest type of strain is *B. valentinum* LmjM3T with a 95.3% as identity %.

Hence, for the *recA, dnaK* and *ropB* genes, our strains kept the same repartition as described in the previous genes. However, as is shown in Figs S3, S6 and S7, we were unable to amplify some of these genes, especially for TZ2, TZ32 and RDT25 strains,

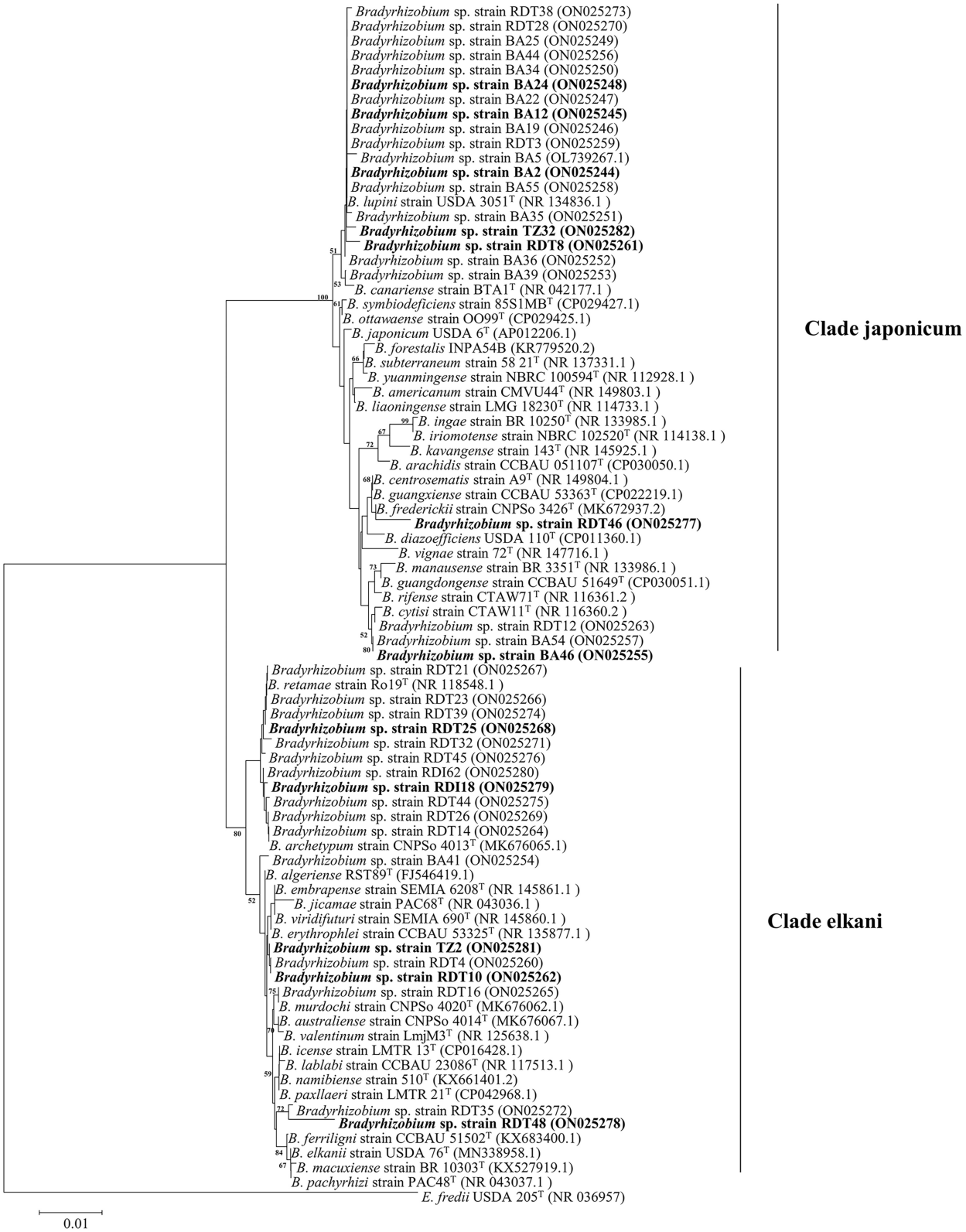

Figure 1. Neighbor-joining phylogeny based on partial *rrs* gene sequences (1370 bp) of strains obtained from *R. dasycarpa* nodules and *Bradyrhizobium* representative species, using the Kimura 2-parameter model in MEGA 7 software. The GenBank accessions are shown in brackets. Bootstrap values calculated for 1000 replications are indicated. Values lower than 50 are not shown. The tree is rooted with *E. fredii* USDA 205^T.

even using a different couple of primers. The sequence analysis of the *recA* gene (Fig. S3) showed that the strains from two different areas labeled BA2, BA12, BA24, BA46 and RDT8 were related to *B. lupini* USDA 3051^T with 100% similarity, while both the strains RDT10 and RDT48 shared similarities of 94.7% and 93.5% with their closet parents *B. valentinum* LmjM3^T and *B. algeriense* RST89^T, respectively. The strain RDT46 was 94.3% related to *B. frederickii* CNPSo 3426^T, whereas the strains RDT25 and RDI18 shared similarities ranging from 97.5% to 97.8% and 96.3% to 96.8% with *B. retamae* Ro9^T and *B. murdochi* CNPSo 4020^T, respectively.

The *dnaK* gene sequences phylogeny (Fig. S6) showed that strains BA2, BA12, BA24, BA46, RDT8 and TZ32 were grouped in the same group; furthermore, they share similarities of 97.2% with the closet relative *B. canariense* LMG 22265^T. The type of strain *B. lupini* USDA 3051^T was not included because its *dnaK* or *rpoB* sequences are not published in any international database. The strains RDT10 and RDT48 were related to *B. algeriense* RST89^T with which they share 99.5% similarity, whereas strain RDT46 was 99.8% close to *B. frederickii* CNPSo 3426^T. The strain RDI18 was closer to *B. murdochi* CNPSo 4020^T, with which it shares a similarity of 97.7%. The analysis of *rpoB* gene sequences (Fig. S7) revealed that the strains BA2, BA12, BA24, BA46 and RDT8 were 99.1% related to *B. canariense* LMG 22265^T, while the strains RDT10, RDT46 and RDT48 were closer to *B. valentinum* LmjM3^T and *B. algeriense* RST89^T and share similarities of 95%, 92.4%, 95.3% and 94.7%, 92.2%, 95.3%, respectively. The strain RDI18 was 98.8% similar to *B. retamae* Ro9^T, and strain TZ2's closest type of strain was *B. paxllaeri* LMTR 21^T with a similarity of 92.8%.

Because of the unavailability of *B. lupini* USDA3051^T *dnaK* and *rpoB* sequences in the databases, and the inability to amplify some genes (as we mentioned previously) from strains RDT25, TZ2 and TZ32, we retained only nine strains from the 12 for the concatenation of the four genes, *recA*, *gyrB*, *glnII*, and *atpD*. The phylogenetic tree (Fig. 2) revealed that the strains BA2, BA12, BA24, BA46 and RDT8 clustered with *B. lupini* USDA 3051^T, with which they share 99.79% of similarity. However, the closest type strains to RDT10 and RDT48 were *B. valentinum* LmjM3^T and *B. algeriense* RST89^T with, respectively, 95.03% and 94.5% similarity. The strain RDI18 clustered with *B. retamae* Ro9^T and *B. murdochi* CNPSo 4020^T with similarities of 97.7% and 97.68%, respectively, and *B. frederickii* CNPSo 3426^T was the closest type of strain to RDT46, with 95.24% similarity. Furthermore, a concatenation of the only three *gyrB*, *glnII* and *atpD* available genes (Fig. S8), which includes strains from the Tazilda region, confirmed also that the strain TZ32 is affiliated with *B. lupini* USDA 3051^T, with which it shares 99.7% similarity, while the strain TZ2 was related to *B. valentinum* LmjM3^T and *B. algeriense* RST89^T, with similarities of 92.7% and 93.1%, respectively.

Phylogenetic analysis of symbiotic genes

The phylogenetic analysis of the gene *nodC* (Fig. 3) revealed that our strains cluster into genistearum and retamae symbiovars. The strains BA2, BA12, BA24 and BA46 were affiliated with *B. rifense* CTAW71^T and *B. cytisi* CTAW11^T, with which they share 97.45% and 94.6% similarities, respectively, while strain RDT8 showed 97.93% and 94.43% similarities with the same type strain, respectively. The *nodC* sequence of strain RDI18 was 99.84% similar to *B. retamae* Ro9^T and 99.04% similar to *B. canariense* BTA1^T. *Bradyrhizobium retamae* Ro9^T and *B. canariense* BTA1^T were related to strains RDT10, RDT46 and RDT48 with a similarity percentage varying from 97.55% to 99.36%.

We could not amplify the *nodA* and *nifH* genes of some strains either, despite all our attempts with different pairs of specific primers. Therefore, analysis of the *nodA* sequences (Fig. S9) of strains RDT8, RDT10, RDT25, RDT48, RDI18 and TZ2 confirmed that they belonged to both symbiovars (Fig. S8), with strain RDT8 being close to *B. rifense* CTAW71^T and *B. cytisi* CTAW11^T of the symbiovar genistearum, with which it shares 93.3% and 93.4% similarity, respectively. Meanwhile, strains RDT10, RDT25, RDT48, RDI18 and TZ2 are members of the symbiovar retamae, and closer to *B. retamae* Ro9^T and *B. valentinum* LmjM3^T, with which they share similarities ranging from 82.1% to 99.8%.

The phylogenetic tree based on *nifH* sequences (Fig. S10) confirmed also that *B. retamae* Ro9^T is the closest species to strains RDI18, RDT25 and TZ2, sharing 99.8%, 99.6% and 91.5% similarity, respectively. On the other hand, the two strains RDT10 and RDT48 were grouped with *B. algeriense* RST89^T, with which they share similarities of 99.8% and 94.7%, respectively, whereas strain RDT8 was 98.9% similar to *B. cytisi* CTAW11^T, and strain RDT46 was 91.5% similar to *B. lablabi* CCBAU 23086^T.

Phenotypic characterization and PGP activities

The phenotypic properties of the 12 selected strains are reported in Table S3. They all grow well in the presence of 1% (w/v) NaCl, whereas six strains continue their growth at 35°C. All the strains grow at pH 7, but some strains grew at pH 6, whereas none grew at pH 8, and they all produced urease. Out of 12 amino acids tested, no strain was able to utilize glycine or alanine. In addition to mannitol, all strains grew in the presence of maltose, fructose, sucrose, lactose, trehalose, sorbitol and mannose as sources of C; however, no strain was able to utilize myoinositol. On the other hand, only strains RDT25, RDT46 and RDI18 produced siderophores, whereas strains BA2, BA12, BA24, RDT8, RDT46 and TZ32 were able to solubilize phosphate; however, no strain produced IAA. Numeric analysis of all the phenotypic data using the Statistica 7 program, and the UPGMA method, allowed the construction of a phenogram showing the phenotypic relationships between the 12 strains, and their origins (Fig. S11).

Legume host range

The selected representative strains nodulated all the Genisteae species tested, such as *R. sphaerocarpa*, *R. monosperma*, *Lupinus albus*, *Cytisus grandiflorus* and *Chamaecytisus albidus*. However, they were unable to nodulate *Phaseolus vulgaris* or *Glycine max*, members of the Phaseoleae tribe (Table S4).

Discussion

Retama species have a great agronomic and ecological impact, and they constitute an alternative nutritional source for animals in semi-arid and arid areas of the southern Mediterranean. They also play an important role in soil retention and desertification control. In addition, they contribute to limiting the expansion of coastal dunes toward the mainland (Caravaca et al. 2003). Over the last two decades, numerous studies have been conducted on these nitrogen-fixing plants and their associated rhizobia (Rodríguez-Echeverría et al. 2014, Alami et al. 2021, Hannane et al. 2016, Lamin et al. 2021). However, to date, no reports or information are available regarding the diversity of microsymbionts of *R. dasycarpa*. The species is native to the High Atlas Mountains, and thrives at altitudes between 1000 and 3000 m, with very cold winters and hot summers. The climate is mostly semi-arid (Hajhouji et al. 2018) and snowfall is frequent between early fall and late

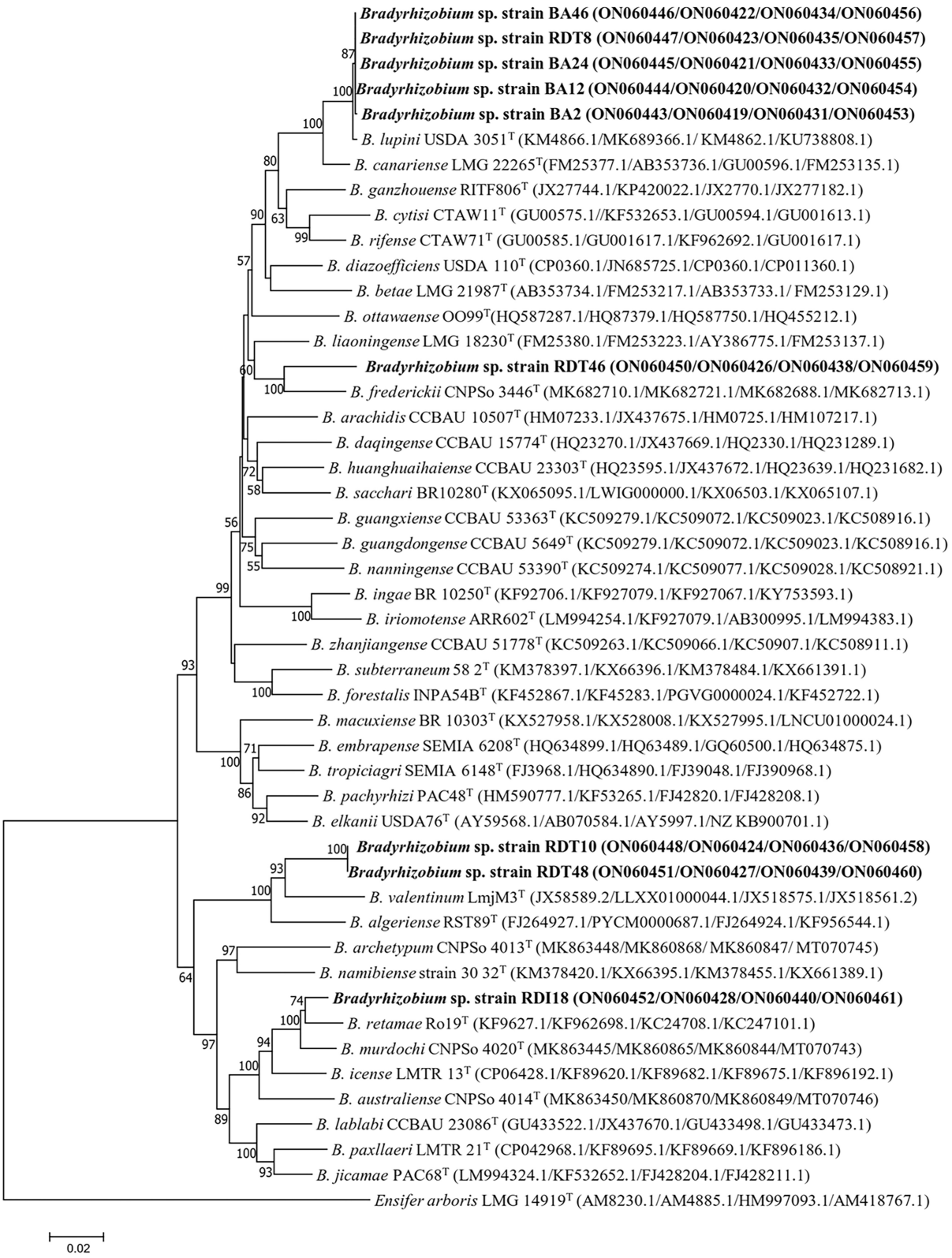

Figure 2. Neighbor-joining phylogeny based on a concatenated alignment of *recA*, *gyrB*, *glnII* and *atpD* (2156 bp) of strains from *R. dasycarpa* nodules and *Bradyrhizobium* representative species, using the Kimura 2-parameter model in MEGA 7 software. The GenBank accessions are shown in brackets. Bootstrap values calculated for 1000 replications are indicated. Values lower than 50 are not shown. The tree is rooted with *Ensifer arboris* LMG14919T.

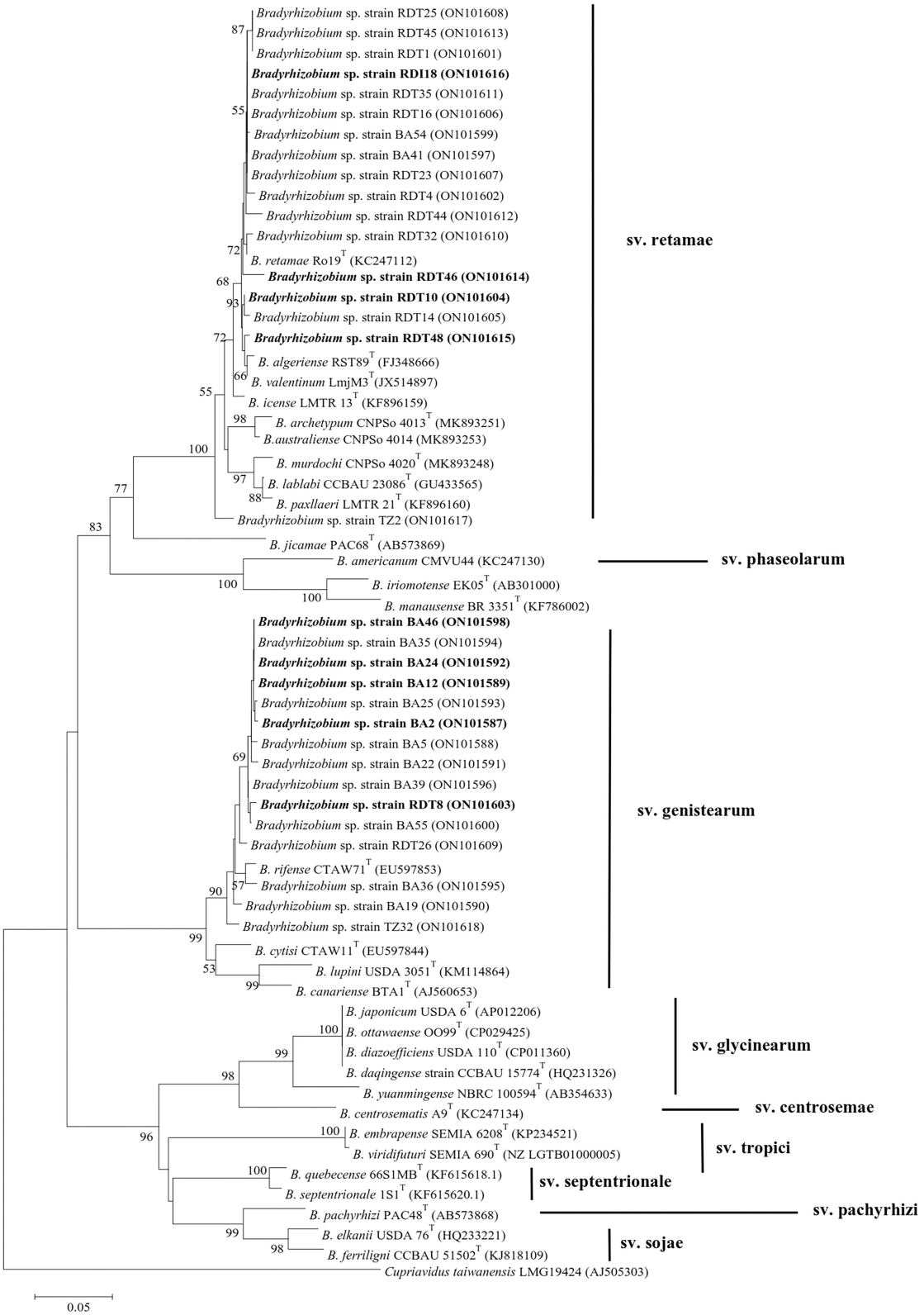

Figure 3. Neighbor-joining phylogenetic tree based on *nodC* (750 bp) sequences of strains from nodules of *R. dasycarpa* and phylogenetically related species within the genus *Bradyrhizobium*. Isolates are denoted in bold. The GenBank accessions are shown in brackets. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 50 are not shown. Bar, 5 nucleotide substitution per 100 nucleotides. The tree is rooted with *Cupriavidus taiwanensis* LMG19426^T.

spring. The snow persists until summer but is rarely permanent. Rainfall is irregular and scarce, with a range from 240 mm.yr^{-1} in the plains to 445 mm.yr^{-1} in the highlands (Boudhar et al. 2016).

In this work, 120 slow-growing bacteria were isolated from *R. dasycarpa* nodules growing in four different sites in the High Atlas Mountains. The genetic diversity of the isolates was first established by the REP-PCR fingerprinting method, a technique that has been successfully used for typing of bacterial isolates (Versalovic et al. 1998). The dendrogram obtained using the Dice similarity coefficient and the UPGMA classification algorithm classified the isolates into nine different groups at a similarity threshold of 70% (Fig. S1), from which 40 strains were then randomly selected for *rrs* gene sequencing. The *rrs* sequences phylogenic analysis revealed that all strains belonged to the genus *Bradyrhizobium* (Fig. 1), and that most of them were close to *B. lupini* USDA 3051^T and *B. retamae* Ro9^T, members of the two clades, japonicum and elkani, of *Bradyrhizobium*.

Although *rrs* gene analysis still provides useful indications for phylogenetic identification at the genus level, it is no longer used to distinguish between species of *Bradyrhizobium* (Ferraz Helene et al. 2020). Therefore, additional multi-locus sequence analysis (MLSA) is usually used to improve the taxonomic relatedness of strains (DeLajudie et al. 2019).

Based on their *rrs* phylogeny, 12 strains were retained as representatives from the 40 selected isolates. Hence, six housekeeping genes, *recA*, *gyrB*, *dnaK*, *atpD*, *glnII* and *rpoB*, were analyzed to refine the phylogenetic analysis of the strains, as these genes are considered conserved among *Bradyrhizobium* species (Menna et al. 2009, De Lajudie et al. 2019). MLSA divided the representative strains into four distinct groups within the *Bradyrhizobium* genus (Fig. 3). Group I, represented by five strains, BA2, BA12, BA24, BA46 and RDT8, affiliated with *B. lupini* USDA 3051^T, with which it shares a 99.7% similarity percentage. Strain RDI18 is closer to *B. retamae* Ro9^T and *B. murdochi* CNPSo 4020^T, with similarities of 97.7 and 97.68%, respectively.

The two strains RDT10 and RDT48 belong to group III and their closest type strain is *B. valentinum* LmjM3^T with 95.03% similarity, while strain RDT46 is closer to *B. frederickii* CNPSo 3426^T, with 95.24% similarity. The degrees of similarity of the concatenated sequences of these latter strains are below the 96% cut-off (Yan et al. 2014, Li et al. 2016), suggesting that they belong to potentially new genomic species. The concatenation of the four housekeeping genes, *recA, gyrB, glnII* and *atpD*, confirmed the results obtained by each gene individually.

Previous studies have shown that *Retama* spp. can be promiscuously nodulated by a wide range of rhizobia under different conditions, suggesting that edaphoclimatic properties may influence the selection between symbiotic partners. Many reports presented *Bradyrhizobium* as the main genus associated with *Retama* spp. (Guerrouj et al. 2013, Hannane et al. 2016, Rodríguez-Echeverría et al. 2014, Ahnia et al. 2018, Alami et al. 2021), although other authors isolated some fast-growing bacteria belonging to *Ensifer* and *Microvirga* genera (Lamin et al. 2019, Lamrabet et al. 2020).

The symbiotic capacity of the strains was confirmed with a first screening by PCR to ascertain the presence of a *nodC* gene copy, using specific primers, and then by the ability of the strains to re-nodulate their original host under axenic conditions. Either located in symbiotic genomic islands or in symbiotic plasmids depending on the rhizobial species, the nodC gene encodes an N-acetylglucosaminyl transferase protein (Rogel et al. 2011), whereas the nodA gene encodes for an acyl transferase. nifH is one of the structural genes (nifHDK) that encode for nitrogenase enzymeresponsible for the reduction of atmospheric nitrogen to ammonium inside the nodule. The nodC is one of the essential genes for the nodulation process, and its phylogeny is important for symbiovars (sv.) determination. Currently, the genus *Bradyrhizobium* includes 11 identified symbiovars (Ferraz Helene et al. 2020), with the sv. retamae encompassing the majority of strains isolated from root nodules of *Retama* species (Guerrouj et al. 2013, Ahnia et al. 2018). The phylogenies of the symbiotic gene sequences were consistent with the core genes, confirming that the strains related to *Bradyrhizobium* species of the elkani group are members of the genistearum symbiovar, while strains of the japonicum group are members of the genistearum symbiovar (Figs 2, S9 and S10).

Analysis of phenotypic characteristics showed that the majority of strains grew at pH 6–7, with optimal growth at pH 7, while no growth was observed at pH 5.6. This result is concordant with the results of Guerrouj et al. (2013) obtained in *B. retamae* strains, isolated from *R. sphaerocarpa* and *R. monosperma* in Eastern Morocco and Spain. Furthermore, all our strains showed optimal growth at 28°C and some grew at 35°C, whereas no growth was detected at 40°C. These strains were isolated from areas with a cold and subhumid climate in winter, with persistent snow between 7 and 10 months minimum per year, and a hotter and drier climate in summer, with high temperatures exceeding 30°C. These strains seem to be adapted to the conditions of their native soil. During the cool seasons, legumes are exposed to low temperatures that limit the development and functioning of symbioses with rhizobia. It is generally agreed that low temperatures limit the growth of bacteria, including rhizobia (Menge et al. 2017)), but rhizobia from arctic areas have been reported to grow at low temperatures (Prévost et al. 1987; Bordeleau and Prevost 1994). Strains from cold areas have higher growth rates at low temperatures than strains from temperate areas, and they can synthesize cold shock proteins under extremely cold conditions, and heat shock proteins at hot temperatures (Cloutier et al. 1992, Bordeleau and Prevost 1994). It has been reported that *B. canariense, B. algeriense* and *B. retamae* also cannot grow at 37°C (Vinuesa et al. 2005 , Guerrouj et al. 2013, Ahnia et al. 2018). Our strains were able to grow in the presence of 1% NaCl, while no growth was observed in 2.5%. The strains used the majority of carbohydrates tested as carbon sources and were able to use a wide range of amino acids as sole nitrogen sources; however, no strain used either glycine or alanine. Similar results were reported by Guerrouj et al. (2013) and Alami et al. (2021) .

Furthermore, there were discrepancies in the phenotypic properties of the strains in correlation with their geographical origin. Hence, the three strains BA2, BA12 and BA24, from Ait Benammar, located at 1422 m above sea level, and strain RDT8 from Tahannaout situated at 964 m, share similar characteristics such as phosphate solubilization ability and growth on sucrose and mannose. None of them grew at pH 6, or in the presence of 1.5% NaCl (w/v). The four other strains, RDT10, RDT25, RDT46 and RDT48, isolated from the Tahannaout site, also share similar phenotypic features. Hence, they grow at pH 6, and 1.5% NaCl, and they grow at 35°C. Strain RDI18 from Ijoukak (1130 m) and the two strains TZ2 and TZ32 from Tazilda (1277 m) have different phenotypic properties, although strain TZ32 was more related to the Ait Benammar strains, but was able to grow in the presence of 2% NaCl. Remarkably, these results confirm the genetic diversity among those strains. The phenogram in Fig. S11 distributed the strains in the two main groups, with the first group including the strains BA2, BA12, BA24, BA46 and RDT8, to which is reattached strain TZ32. Group 2 involves the other RDT strains as well as RDI18 from Ijoukak and strain TZ2 from Tazilda. Remarkably, these results are

in accord with those obtained by the MLSA involving three housekeeping genes (Fig. S8).

Bacterial plant growth-promoting activities such as phosphates solubilization, siderophores and phytohormones production positively affect plant productivity (Ahemad and Kibret 2014). Many rhizobia behave as PGPRs, as they colonize the root nodules, stimulate the growth of the plant and provide protection against abiotic and biotic stresses (Mohammad et al. 2022). Among the selected strains, only three were able to produce siderophores, and six were able to solubilize phosphates, while no strain produced IAA-like compounds. This is very common, and several authors revealed that few strains of *Bradyrhizobium* solubilize phosphates or produce IAA (Boulila et al. 2009, Alami et al. 2021, Lamin et al. 2021).

The mean Shannon–Wiener diversity (H') estimate of *Retama dasycarpa* rhizobia populations in the High Atlas Mountains based on the analysis of REP-PCR fingerprinting confirmed their genetic diversity, with the population of rhizobia originating from the Tahannaout site having the highest genetic diversity estimate ($H' = 1.77$). The lowest diversity was recorded in the Tazilda site ($H' = 0.21$) (Table S1). The Tahannaout site is located at 964 m altitude, with 475 mm rainfall, whereas Tazilda is situated at 1277 m, with an annual rainfall of 290 mm. The strains isolated from Tahannaout are related to three different species of *Bradyrhizobium*, whereas in the Ijoukak site (situated at an altitude of 1130 m, with 327 mm rainfall), all the isolates are related to one species of *Bradyrhizobium*. In Tazilda, the 18 isolates are members of two *Bradyrhizobium* species, whereas in Ait Benammar, although genetically very diversified ($H' = 1.68$), all the isolates belong to one species, *B. lupini*. The richness and diversity of the Tahannaout site in *Retama* rhizobia are certainly due to its lower altitude and milder climate than the other stations. Indeed, it has been reported that variation in rhizobial diversity could be correlated to the differences in sites' agroclimatic and soil conditions (Koskey et al. 2018).

Conclusion

This work reports the first isolation and characterization of microsymbionts from the endemic *Retama dasycarpa* native to the High Atlas Mountains. Phylogenetic analysis of the *rrs* gene and concatenation of four housekeeping genes revealed that they are members of *Bradyrhizobium*, and related to different species, including *B. lupini*, *B. retamae* and two putative novel species. Moreover, the sequences of the three symbiotic genes *nodA*, *nodC* and *nifH* were congruent with the core genes and showed that the strains are members of the genistearum and retamae symbiovars. The genetic and phenotypic diversity of the selected strains, as well as their PGP activities, suggest that microsymbionts are of great importance in the ability of *R. dasycarpa* to survive and adapt to the severe conditions in the High Atlas Mountains. To our knowledge, this is the first report on the diversity of nodulating bacteria associated with *R. dasycrapa*.

Author contributions

Mouad Lamrabet (Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review and editing), Zohra Chaddad (Data curation, Formal analysis, Investigation), Omar Bouhnik (Data curation, Formal analysis, Investigation), Soufiane Alami (Data curation, Formal analysis, Investigation), Kaoutar Kaddouri (Data curation, Formal analysis, Investigation), Meryeme Bennis (Data curation, Investigation), Hanane Lamin (Data curation, Formal analysis, Investigation), Mnasri Bacem (Data curation, Investigation, Validation, Writing – original draft), Sylvain Bourgerie (Data curation, Investigation), Domenico Morabito (Data curation, Investigation), Hanaa Abdelmoumen (Conceptualization, Validation), Eulogio J. Bedmar (Data curation, Writing – original draft), and Mustapha Missbah El Idrissi (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review and editing)

Supplementary data

Supplementary data are available at *FEMSEC* online.

Conflict of interest. None declared

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Data availability

All the sequences described in this work are deposited in the Genbank depository of the National Center for Biotechnology Information (NCBI).

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