



# Nodulation of *Retama* species by members of the genus *Microvirga* in Morocco

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## Abstract

The genetic diversity of bacterial populations' nodulating *Retama sphaerocarpa* grown in the soils of Maamora cork forest (Morocco) was examined. ERIC-PCR fingerprinting of 30 strains distributed them in 2 groups, of which a representative strain from each group was studied by multilocus sequence analysis of the 16S rRNA, *atpD*, and *gyrB* genes. The two representative strains RSM25 and RSM32, grouped with "*Microvirga tunisiensis*". This is the first description of *Retama* nodule bacteria as members of the genus *Microvirga*. A *nodC*-based phylogeny confirmed that the two representative strains RSM25 and RSM32 are affiliated with symbiovar mediterranense. The 2 strains were capable of nodulating not only *R. sphaerocarpa* but also *R. monosperma*, *R. dasycarpa* and *L. luteus*, and unable to nodulate *Phaseolus vulgaris*, *Vachellia gummifera*, *Cicer arietinum*, *Vigna unguiculata* and *Glycine max*. The inoculation of *R. sphaerocarpa* with RSM25 or RSM32 produced a 1.22-, and 1.36-fold increase in the dry weight of the plants compared to those grown in the presence of 0.05% KNO<sub>3</sub>. The 2 strains used monosaccharides and disaccharides as the sole C source, but fructose, glucose, galactose, arabinose and starch were not used. They were unable to grow on glycine as a N source. Phosphate solubilization and siderophore production was not detected, but IAA or IAA-related compounds were produced. The alkaline pH of the sampling site soil in the Maamora forest where *Retama* grows could explain why *Microvirga* was the dominant species in the root nodules of the plants.

**Keywords** Maamora Forest · *Retama* · *Microvirga* · *Bradyrhizobium*, symbiosis · Phylogeny

## 1 Introduction

Plants of the genus *Retama* are wild shrubby legumes distributed throughout the Mediterranean area, more precisely in southwest Europe and northwest Africa (Zohary 1959; León-González et al. 2018). The genus *Retama* belongs to the Genistaeae tribe of the Leguminosae (Fabaceae) family and contains four described species, *R. monosperma*, *R. sphaerocarpa*,

*R. raetam*, and *R. dasycarpa*. The latter is endemic to Morocco, and *R. sphaerocarpa* is widespread in Southern Spain, Morocco, Algeria and Tunisia. The distribution of *R. monosperma* is limited to Morocco and Algeria, and *R. raetam* is found only in the arid areas of Southern Mediterranean, from Morocco to Libya (Hannane et al. 2014).

*R. sphaerocarpa* (yellow retama) has photosynthetic stems and a dimorphic shallow lateral roots that can reach down to a depth of more than 20 m, which enables the plant to lift water from deep, wet layers and release it to shallow, dry soils (Prieto et al. 2010). The hydraulic lift and the open canopy of the plants allow amelioration of the microclimatic conditions and the increase in nutrient content and mineralization rate under the canopy (Rodríguez-Echeverría and Pérez-Fernández 2003). The establishment of effective dinitrogen (N<sub>2</sub>)-fixing symbioses with soil bacteria, collectively referred to as rhizobia, allows *R. sphaerocarpa* to grow in N-deficient, poor soils, which contributes to the increase in nitrogen under the canopy (Rodríguez-Echeverría and Pérez-Fernández 2003). Thus, *R. sphaerocarpa* plays an important ecological role in arid and semiarid areas by maintaining plant diversity

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and ecosystem functioning and is a valuable species for revegetation and restoration programs, dune stabilization and soil fixation (Rodríguez-Echeverría and Pérez-Fernández 2005).

Rhizobia include genera *Rhizobium*, *Ensifer*, *Allorhizobium*, *Pararhizobium*, *Neorhizobium*, *Shinella*, *Mesorhizobium*, *Aminobacter*, *Phyllobacterium*, *Ochrobactrum*, *Methylobacterium*, *Microvirga*, *Bradyrhizobium*, *Azorhizobium* and *Devosia* from the alpha-Proteobacteria subclass; as well as *Paraburkholderia*, *Cupriavidus* and *Trinickia* from the beta-Proteobacteria subclass (Estrada-de los Santos et al. 2018; de Lajudie et al. 2019). Despite its ecological role, root-nodulating bacteria of *R. sphaerocarpa* have been studied only in a few sites from Algeria, Morocco and Spain. Rhizobia isolated from *R. sphaerocarpa* in central-western Spain affiliate with *Bradyrhizobium* and were later identified as *B. canariense* (Ruiz-Díez et al. 2009). Similarly, the rhizobial strains isolated from wild-grown *R. sphaerocarpa* in different ecological-climatic areas of northeastern Algeria formed a phylogenetic clade within the *Bradyrhizobium* genus (Boulila et al. 2009). *B. retamae*, originally isolated from *R. monosperma* grown in the subhumid area of Saidia in Northern Morocco was also able to infect *R. sphaerocarpa* (Guerrouj et al. 2013). All of the 31 strains infecting *R. sphaerocarpa* growing in eight different sites with different soil and environmental conditions in the Iberian Peninsula clustered with different members of the *B. japonicum* and *B. elkanii* superclades described by Menna et al. (2009) or with the species *B. retamae* (Rodríguez-Echeverría et al., 2014). Although these studies show that *Bradyrhizobium* is the dominant microsymbiont isolated from nodules of *R. sphaerocarpa* (Boulila et al. 2009; Guerrouj et al. 2013), the endophyte *Phyllobacterium myrsinacearum* has also been isolated from nodules of *R. sphaerocarpa* plants from central Spain (Ruiz-Díez et al. 2009, 2012). More recently, Ahnia et al. (2018) isolated *B. algeriense* from N<sub>2</sub>-fixing nodules of *R. sphaerocarpa* in Northern Algeria, and Lamin et al. (2019) reported that some strains related to “*Ensifer aridi*” obtained from the root nodules of *R. monosperma*, were also able to nodulate *R. sphaerocarpa*.

*R. sphaerocarpa* grows both in alkaline and acidic soils ranging from sea level to 2000 m, in sites with mean annual precipitation of 200–980 mm (Armas et al. 2011), reasons why we hypothesize that rhizobia associated with the plant could be more diverse than that reported thus far. The Maamora is the largest cork oak forest in the south Mediterranean region, where it plays economic, social and environmental roles (El Boukhari et al. 2016). Legumes occupy the second place in importance after the Poaceae family in Maamora, and *Retama* is one of the main diverse legume genera (Aafi et al. 2005).

Therefore, the aim of this study was to identify the microsymbionts associated with *R. sphaerocarpa* grown wild in the Maamora forest.

## 2 Materials and methods

### 2.1 Bacterial strains

Bacteria were isolated from root nodules of *R. sphaerocarpa* grown in soils of the Maamora Forest (34° 02' 76 82 N; -6° 71' 22 23 W) located in the vicinity of Rabat (Morocco).

Root segments bearing nodules were collected, washed under running tap water, surface-sterilized by immersion in 5% sodium hypochlorite for 3 min, and finally washed thoroughly with sterile water. Individual nodules were then placed in Petri dishes and crushed with a sterile glass rod. The obtained extracts were then streaked onto Petri dishes containing solid yeast extract mannitol (YEM) medium (Vincent 1970) supplemented with Congo Red dye (0.025 g l<sup>-1</sup>). After incubation for 10 d at 28 °C, the single colony-forming units (CFUs) appeared on the plates were checked for purity by repeated streaking on the same medium. YEM medium was routinely used for bacterial culture.

### 2.2 DNA extraction, PCR amplifications and genes sequencing

Essentially, bacterial genomic DNA was isolated after growth of the cells in liquid YEM medium, collected by centrifugation in a microfuge, treated with proteinase K (20 mg ml<sup>-1</sup>) and extracted as described by Brenner et al. (1982). After recovery, the DNA was kept at -20 °C until use. The quantity of DNA was determined using a Nanodrop spectrophotometer (NanoDrop ND1000). Enterobacterial repetitive intergenic consensus (ERIC)-polymerase chain reactions (ERIC-PCR) were performed using primers ERIC1R and ERIC2 and reactions conditions previously published (Versalovic et al. 1991). PCR amplifications of the 16S rRNA gene fragments were carried out using the two opposing primers fD1 and rD1 as indicated earlier (Weisburg et al. 1991). The primer pairs atpD255F/atpD782R (Vinuesa et al. 2005) and gyrBF/gyrBR (Martens et al. 2007) were used for amplification of the *atpD* and *gyrB* genes, respectively. For the amplification of *nodC* and *nodA* genes, we used the primers nodCFn/nodCI and nodA1F/nodAb1r as previously described (Laguerre et al. 2001; Chantereuil et al., 2001). All the primers and amplification protocols used in this study are reported in Table 2S.

The amplification products were purified with the Qiaquick PCR purification kit (Qiagen), verified by electrophoresis in agarose gels and subjected to cycle sequencing using the same primers as for PCR amplification with ABI Prism dye chemistry. The products were analyzed with a 3130xl automatic

sequencer at the sequencing facilities of UATRS (*Unités d'Appui Technique à la Recherche Scientifique, CNRST, Rabat, Morocco*) and Estación Experimental del Zaidín, CSIC, Granada, Spain. All the obtained sequences were compared with those from GenBank using the BLASTn program (Altschul et al. 1990) and the EzBioCloud.net (Yoon et al. 2017). Distances calculated according to Kimura's two-parameter model (Kimura 1980) were used to infer phylogenetic trees with the neighbor-joining analysis (Saitou and Nei 1987) with MEGA7 software (Kumar et al. 2016). Identity values were calculated by pairwise analysis and gaps were not considered. The accession numbers of the nucleotide sequences of the bacterial strains used in this study are shown in the phylogenetic trees.

### 2.3 Plant nodulation tests

Selected strains were tested for nodulation of *R. monosperma*, *R. dasycarpa*, *Lupinus luteus*, *Phaseolus vulgaris*, *Cicer arietinum*, *Vachellia gummifera*, *Glycine max* and *Vigna unguiculata*. Seeds of lupine, *V. gummifera*, *R. dasycarpa*, and *R. monosperma* were surface-sterilized by immersion in 95% ethanol for 30 s, scarified using 95% sulfuric acid for 1, 60, and 120 min, respectively, and then washed several times with sterile water. Chickpea, soybean and cowpea seeds were surface-sterilized by adding 95% ethanol for 30 s, H<sub>2</sub>O<sub>2</sub> 15% for 8 min, and washed with sterile water. Finally, the seeds were placed in Petri dishes containing sterile water-agar (0.6%), and allowed to germinate in the dark at 28 °C for 2–4 d. The seedlings were then transferred to 200 × 20 mm Gibson tubes (1 seedling/tube) containing Jensen's N-free mineral solution (Roughley 1984) and inoculated independently with the different bacterial suspensions (approximately 10<sup>8</sup> cells ml<sup>-1</sup>). Uninoculated plants were used as a control. A set of plants was grown in the same mineral solution supplemented with 0.05% KNO<sub>3</sub>. The plants were placed in a growth chamber at 26 °C under a 16.0/8.0 h light/dark photoperiod and checked for nodules appearance 8 weeks after inoculation. The Jensen solution was supplied just once, after 4 weeks culture.

### 2.4 Phenotypic characterization

Tests were performed using YEM medium inoculated with an exponentially growing liquid culture. The ability of the isolates to grow in acidic or basic media was determined on solid YEM whose pH had been adjusted and buffered to 5.5, 6.0, 7.0, 8.0, 9.0, 9.5 or 10.0, as described by Zerhari et al. (2000). The salt tolerance was tested at 0, 170, 340, 510, 680, or 850 mM of NaCl using solid YEM. Utilization of the amino acids lysine, methionine, histidine, glycine and glutamic acid at 1% as sole nitrogen source was investigated on a modified liquid YEM medium in which the yeast extract was replaced

by the different amino acids. Carbohydrate assimilation was carried out on solid YEM containing 0.005% of yeast extract and 0.4% of glucose, fructose, sucrose, maltose, lactose or starch. The carbohydrates were sterilized separately by filtration. The intrinsic antibiotics resistance of the isolates was determined on solid YEM supplemented with the following antibiotics (μg/ml): kanamycin (20), ampicillin (20), bacitracin (20), tetracycline (10), vancomycin (20), streptomycin (20), nalidixic acid (20), and chloramphenicol (30). Hydrolysis of urea and nitrate reduction by the rhizobial isolates was investigated on solid YEM amended with 2% urea, 0.0012% phenol red and 0.1% KNO<sub>3</sub>, respectively, as described by Lindström and Lehtomäki (1988). The gelatinase activity was investigated as reported by Missbah El-Idrissi et al. (1996), and the catalase activity was determined by the method of Graham and Parker (1964).

### 2.5 PGPR characteristics

#### 2.5.1 Phosphate solubilization

The ability of the isolates for phosphate solubilization was tested using PVK medium (Pikovskaya 1948) containing 2.5 g l<sup>-1</sup> of (Ca<sub>3</sub>PO<sub>4</sub>)<sub>2</sub>. Aliquots (5 μl) of the bacterial suspension were plated onto PVK medium in Petri dishes and incubated at 28 °C for 7 d. The ability of the isolates to solubilize phosphate was estimated by measuring the size of the halos that appeared around the colonies and the size of the colonies.

#### 2.5.2 Siderophore production

Siderophore production was detected according to Lakshmanan et al. (2015). Isolates synthesizing siderophores were identified by the development of orange halos around the colonies.

#### 2.5.3 Indole acetic acid production

The ability of the isolates to produce indole acetic acid (IAA) and IAA-related compounds was determined on solid YEM medium supplemented with 0.5 g l<sup>-1</sup> tryptophan. Drops (5 μl) of the bacterial suspension were placed on the medium and incubated at 28 °C for 24 to 48 h. The production of IAA was detected using the Salkowski reagent followed by the appearance of a pink halo around the colonies after incubation for 30 to 60 min at 28 °C.

## 3 Results

The sampling site soils are composed of clay (3.15%), fine silt (3.03%), coarse silt (3.30%), fine sand (30.36%), and coarse sand (60.23%). The soil at the sampling site is neutral to

slightly alkaline with a mean pH value of  $7.3 \pm 0.1$ , and an electrical conductivity of  $143.45 \text{ s cm}^{-1}$ ; an average annual precipitation of 500 mm, and  $18 \text{ }^\circ\text{C}$  as mean annual temperature (Benabid and Fennane 1994).

A total of 30 bacterial isolates were obtained from root nodules of *R. sphaerocarpa* grown wild in the Maamora forest, in the vicinity of Rabat (Morocco). The colonies were fast growing and slightly pinkish after 4 days growth on YEM medium supplemented with Congo Red. After ERIC-PCR, the 30 strains distributed into 2 different groups (Fig. S1), of which the strains RSM25 and RSM32 were randomly chosen as the representative strains of each I and II groups, respectively. Members of the group I were all similar; as were the isolates of group II, which shows the high clonality of the isolates. Based on results of the Enterobacterial repetitive intergenic consensus -polymerase chain reactions (ERIC-PCR) the strains RSM25 and RSM32 were used for further genotypic studies.

### 3.1 16S rRNA, *dnak*, and *gyrB* genes phylogenetic analysis

The analysis of the almost complete 16S rRNA gene sequence of the strains RSM25 and RSM32 showed that they were members of the genus *Microvirga* of the family *Methylobacteriaceae* of the Alphaproteobacteria, and the corresponding phylogenetic tree (Fig. 1) showed that the strains clustered with had “*M. tunisiensis*” LmiM8<sup>T</sup> with similarity values of 99.85 and 99.84%, respectively, and 98.33 and 98.20% with *Microvirga makkahensis* SV1470<sup>T</sup>, respectively. PCR amplification of the *atpD* and *gyrB* genes of the strains RSM25 and RSM32 gave DNA bands that were sequenced, and their concatenation confirmed they cluster with *M. tunisiensis* LmiM8<sup>T</sup> (Fig. 2), with which they shared 97.27 and 97.99% similarity, respectively. Phylogenetic trees based on individual *atpD* (Fig. 2S) and *gyrB* (Fig. 3S) gave similar results to those obtained for the concatenated tree, with no further insights into the affiliation of the isolates.

### 3.2 Phylogenic analysis of the *nodA* and *nodC* genes

Utilization of the primer pair *nodCF/nodCI* resulted in amplification of the *nodC* gene from the strains RSM25 and RSM32. The phylogenetic tree (Fig. 3) built with the obtained sequences and those of the *nodC* genes from other *Microvirga* species as well as other *Retama* nodulating rhizobia such as *B. retamae* Ro19<sup>T</sup>, *B. algeriense* RST91<sup>T</sup> and *E. aridi* strains RMT3 and RMT46, revealed that RSM25 and RSM32 grouped with *M. tunisiensis* LmiM8<sup>T</sup>, with similarity values of 90.46 and 92.04%, respectively.

RSM25 and RSM32 have similarity percentages of 82.1 to 82.8% with *Ensifer aridi* strains RMT3 and RMT46; while the similarity percentages with *B. retamae* and *B. algeriense* vary between 83.4 and 83.8 (Table 3S).

Similar results were obtained with *nodA* gene, and the phylogenetic tree (Fig. 4S) revealed also that the two strains RSM25 and RSM32 grouped with *M. tunisiensis* LmiM8<sup>T</sup>, with similarity values of 95.20 and 95.10% respectively.

Based on the *nodA*, *nodC* sequences and their concatenation, Figs. 3, 4S and 5S also show that the strains isolated from *R. sphaerocarpa* belong to the symbiovar *mediterraneae*. The type strains of *B. retamae* and *B. algeriense* clustered together with *B. valentinum* in the symbiovar *retamae*, while *Ensifer aridi* strains RM3 and RMT46 were very close to *E. aridi* strain JNVU TP6.

### 3.3 Plant nodulation tests

The two strains RSM25 and RSM32 nodulated not only *R. sphaerocarpa*, but also *R. monosperma*, *R. dasycarpa* and *L. luteus*, and were unable to form nodules on *P. vulgaris*, *C. arietinum*, *V. gummifera*, *G. max* and *V. unguiculata*. Under the conditions used in this study, the inoculation of yellow retama with strains RSM25 and RSM32 produced a 1.22-, and 1.36-fold increase in the dry weight of the plants compared to those grown in the presence of 0.05% KNO<sub>3</sub> (Table 2).

### 3.4 Phenotypic characterization

Phenotypic characteristics of the strains RSM25 and RSM32 are presented in Table 1S. The two strains were fast growing with an average generation time of 3 and 5 d to give colonies on solid YEM medium with a diameter higher than 2 mm, respectively. They grew well at 3% NaCl,  $35 \text{ }^\circ\text{C}$ , and within a pH range from 6.5 to 9.0. The two *Microvirga* strains grow in YEM medium with pH values ranging from 6.5 to 9, and at 3% NaCl and produce catalase and urease. Out of the 14 C sources tested in this study, the strains RSM25 and RSM32 were unable to use arabinose, galactose, fructose, starch and glucose. They grew in the presence of arginine, tyrosine, leucine, aspartic acid, tryptophan, histidine, valine, lysine, and phenylalanine as the only N source, and no strain grew on glycine. They are resistant to ampicillin, kanamycin, bacitracin, tetracycline, streptomycin, vancomycin, and nalidixic acid.

### 3.5 PGPR activities

Neither siderophore production nor phosphate solubilization by strains RSM25 and RSM32 was observed; however, they were able to produce IAA (Table 1S).

## 4 Discussion

Although previous work has established that *Bradyrhizobium* is the dominant genus found in nodules of *R. sphaerocarpa*,

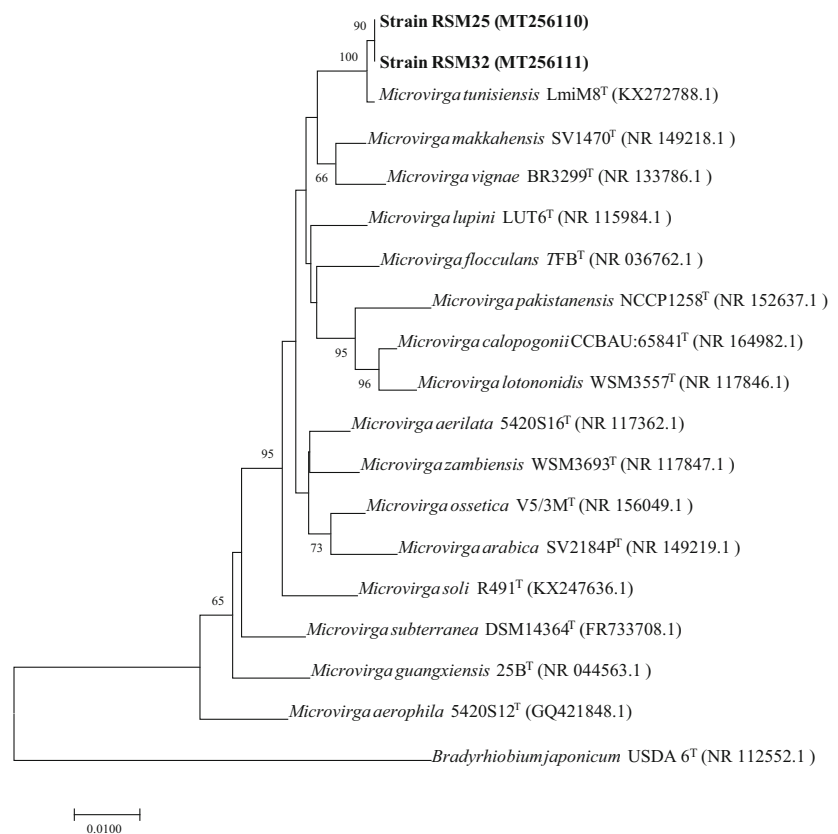


rhizobial strains of *Phyllobacterium* and *Ensifer* have also been isolated (Ruiz-Díez et al. 2009; Guerrouj et al. 2013; Ahnia et al. 2018; Lamin et al. 2019). In this work we extend those findings by showing that *M. tunisiensis* is the most abundant microsymbiont in nodules of *R. sphaerocarpa* growing in soils of the Maamora forest, where, together with legumes of the genera *Teline* and *Cytisus*, and various species of the *Poaceae*, they are overgrazed by an abundant livestock. The sampling sites were located in the cool and temperate sub-humid bio-climate western part of this cork oak ecosystem whose climatic conditions contrast with those of the arid areas of eastern Morocco and the Middle and High-Atlas mountains where the yellow retama thrives spontaneously under hard weather conditions with low and random rainfall as well as very cold winters and hot summers.

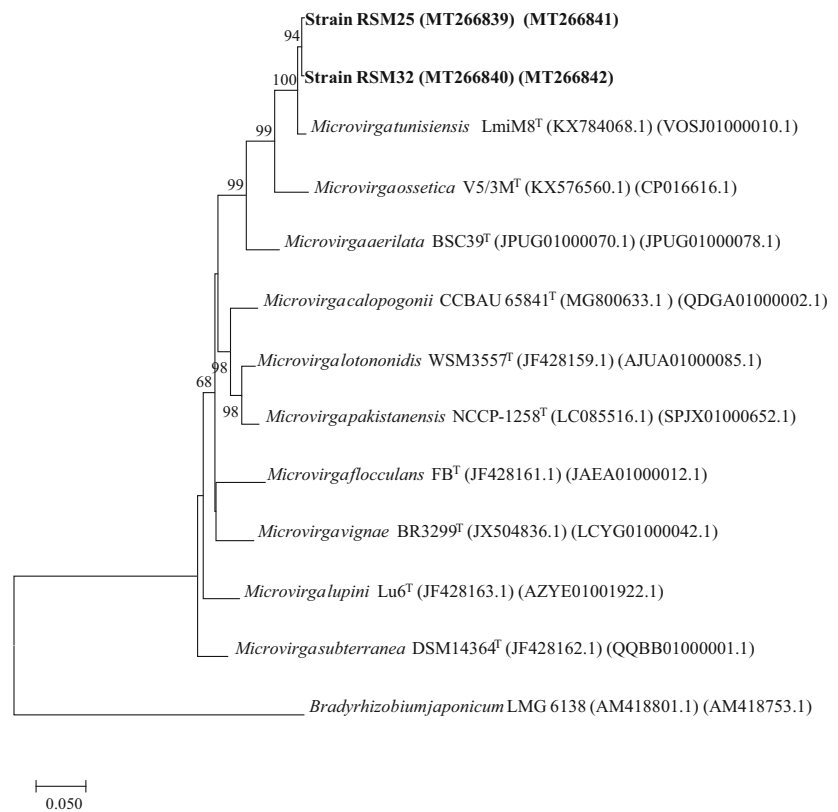
In this study 30 bacterial strains were isolated from nodules of *R. sphaerocarpa* whose overall diversity was determined by rep-PCR using ERIC primers, a technique that has proven useful to avoid clonality as it discriminates among the molecular diversity of the isolates at the intra-species level (Versalovic et al. 1994). ERIC-PCR fingerprinting of the isolates allowed the selection of strains RSM25 and RSM32 that were chosen for further genetic identification.

The phylogenetic analysis of the 16S rDNA sequences and of the combined housekeeping genes *atpD* and *gyrB* revealed that the strains RSM25 and RSM32 are related to *M. tunisiensis* isolated from *L. micranthus* grown in Northern Tunisia (Msaddak et al. 2017b). Members of *Microvirga* were also obtained from nodules of *L. cosentinii* from the Moroccan Maamora forest (Missbah El-Idrissi et al. 2019), and both *R. sphaerocarpa* and *L. cosentinii* can be found in the sub-humid part of the Maamora forest from which *Microvirga* strains have been isolated. Soil structure, pH, salinity, phosphate concentration, moisture and type of soil play a key role in the bacterial communities' biogeography and selection of symbionts by legumes (Han et al. 2009; Rathi et al. 2018; Fan et al. 2018; Wang et al. 2019). The Maamora forest sandy soils are generally acidic under the cork oak trees, with pH values ranging from 4.8 to 6 (El Boukhari et al. 2016). However, in clear areas and under some legumes shrubs such as *Retama* and *Teline*, soils have neutral or slightly alkaline pH (El Boukhari et al. 2016; Belghazi and Mounir 2016; Gaddas 1976; Laouina et al. 1997). The presence of *Microvirga* in root nodules of plants growing on the slightly alkaline soils of the Maamora agrees with previous reports on *Microvirga* strains isolated from *L. micranthus*, *L. luteus* and *L. angustifolius* in alkalized soils of Tunisia (Msaddak et al. 2017a, b; Rejili et al. 2019) and from *L. texensis* and

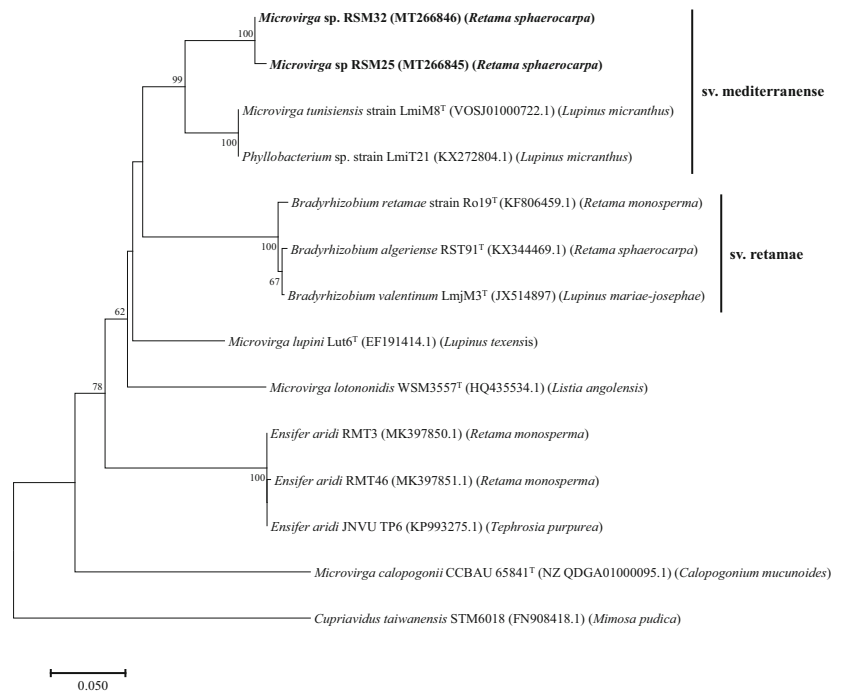
**Fig. 1** Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequences of the strains RSM25 and RSM32 (in bold) from nodules of *R. sphaerocarpa* and phylogenetically related type strains of the genus *Microvirga*. Bootstrap values are indicated as percentages derived from 1000 replications. Scale bar indicates substitutions per site. The tree is rooted with *B. japonicum* USDA6<sup>T</sup>



**Fig. 2** Neighbor-joining phylogenetic tree based on the concatenated partial *atpD* + *gyrB* gene sequences of the strains RSM25 and RSM32 (in bold) from nodules of *R. sphaerocarpa* and phylogenetically related *Microvirga* type species. Bootstrap values are indicated as percentages derived from 1000 replications. Scale bar indicates substitutions per site. The tree is rooted with *B. japonicum* LMG 6138<sup>T</sup>



**Fig. 3** Neighbor-joining phylogenetic tree based on partial *nodC* sequences of the strains RSM25 and RSM32 (in bold) from nodules of *R. sphaerocarpa* and phylogenetically related species of the genus *Microvirga*. Bootstrap values are indicated as percentages derived from 1000 replications. Scale bar indicates substitutions per site. The tree is rooted with *Cupriavidus taiwanensis* STM6018



**Table 1** Nodule number and plant dry weight of *R. sphaerocarpa* inoculated with the strains RSM25 and RSM32. Uninoculated plants were used as a control. A set of plants was supplemented with 0.05% ofKNO<sub>3</sub>. Plants were grown in Gibson's tubes for 2 months. Values represent the mean of 5 replicates followed by the standard error

	Uninoculated plants	Uninoculated, KNO <sub>3</sub> -treated plants	Strain used for inoculation	
			RSM25	RSM32
Nodule number	0.0	0.0	3.33 ± 0.44	2.02 ± 0.21
Plant dry weight (mg/plant)	139.11 ± 18.51	183.31 ± 19.47	224.43 ± 20.9	250.41 ± 2.16
Plant dry weight increase (%)	–	–	22.43	36.60

*L. subcarnosus* grown in soils with pH varying from 7.9 to 8.4 in the United States (Beligala et al. 2017). The soils used in this study are slightly alkaline reddish sandy soils and it is therefore very likely that the pH played a role in the selection of *Microvirga* as microsymbionts of *Retama* instead of members of the genera *Bradyrhizobium* or *Ensifer* as reported previously (Guerrouj et al. 2013; Ahnia et al. 2018; Lamin et al. 2019). All these results support that the soil pH could be a key factor for determination of the biogeography of the *Microvirga* species.

Symbiobars are generally used to distinguish between different subgroups that are symbiotically distinct within a single legume-nodulating species (Rogel et al. 2011), and they are defined mainly on the basis of *nod* genes involved in legume nodulation.

Since *Retama* is nodulated by members of three different genera, this would suggest that a horizontal transfer of nodulation genes would have occurred between these bacteria. However, analyzes of the three genera *nodC* nodulation genes sequences and their phylogeny showed that strains RSM25 and RSM32 cluster together with *Microvirga tunisiensis* LmiM8T (Msaddak et al. 2019) and *P. sophorae* LmiT21 (Msaddak et al. 2018) in the mediterranense symbiovar, while *B. retamae* and *B. algeriense* clustered together with *B. valentinum* in the symbiovar *retamae*. The two strains of *Ensifer aridi* isolated from *Retama monosperma* in Morocco were closer to *E. aridi* strain JNVU TP6 isolated from *Tephrosia purpurea* in India.

These results show the aptitude of *Retama* to interact a wide range of rhizobia with different types of Nod factors. This ability contributes to the great resilience of the plant, which can develop in different agroecosystems and fix

nitrogen under different conditions. It can thus establish a symbiosis with *Ensifer aridi* under metallic stress (Lamin et al. 2019), or with *B. retamae* (Guerrouj et al. 2013) or *B. algeriense* (Ahnia et al. 2018) under arid conditions, and it could associate with *Microvirga* under milder conditions of climate and soil pH.

To our knowledge, no strain of *Microvirga* has been previously reported to be a *R. sphaerocarpa* endosymbiont. Coincident with other *Microvirga* strains isolated from *Lupinus* (Ardley et al. 2012; Msaddak et al. 2017a, b; Rejili et al. 2019), the strains RSM25 and RSM32 also formed effective nodules on *L. luteus*.

Each strain RSM25 and RSM32 nodulated *R. sphaerocarpa*, *R. monosperma*, and *R. dasycarpa*. Moreover, the two strains established effective symbiosis with their original host, and the inoculation with any of them produced clear increases in the dry weight of the plants after growth for two months under axenic conditions (Table 1).

The strains RSM25, and RSM32 used a variety of carbohydrates as the sole C source and no strain used glycine for growth, a characteristic reported by Guerrouj et al. (2013) for all of the strains isolated from *R. monosperma* and *R. sphaerocarpa*.

Plant growth-promoting rhizobacteria (PGPR) have a favorable effect on plant growth and tolerance against biotic and abiotic stresses. In addition to supplying N to compatible host plants, legume-nodulating bacteria may also act as a crop enhancer and biofertilizer for increased non-legume production (Zaim et al. 2017; Patil et al. 2017; Vargas et al. 2017). However, the strains RSM25 and RSM32 were unable to produce siderophores or solubilize phosphate, but they produced

**Table 2** Nodule number and plant dry weight of *R. sphaerocarpa* inoculated with the strains RSM25 and RSM32. Uninoculated plants were used as a control. A set of plants supplemented with 0.05% of KNO<sub>3</sub> was used as positive controls. Values represent the mean of 3 replicates

	Untreated plants	Nitrate-treated plants	Strains	
			RSM25	RSM32
Nodule number	0.0	0.0	3.33 ± 0.44	2.02
Plant dry weight (mg/plant)	139.11 ± 18.51	183.31 ± 6.47	224.43 ± 2.9	250.41 ± 2.16
Plant dry weight increase (%)	–	–	122.43	136.61

IAA or IAA-related compounds derived from the metabolism of tryptophan.

The results in this study enlarge the scientific information about the diversity of bacteria associated with species of the genus *Retama* in Morocco by adding *Microvirga* to the previously reported species capable of nodulating yellow retama such as *Bradyrhizobium*, *Ensifer*, and *Phyllobacterium*. This, in turn, supports the idea that host plant promiscuity could be an adaptation strategy to grow in diverse ecosystems, with different types of soils and bioclimatic conditions and could aid the future development of bioinocula for use in sustainable agrosystems.

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### Compliance with ethical standards

**Conflict of interest** The authors declare there is no conflict of interest.

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