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Characterization of root-nodulating bacteria on *Retama raetam* in arid Tunisian soils

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Abstract

The aim of this study is to investigate the diversity of *Retama raetam* root-nodule bacteria isolated from arid regions of Tunisia. Twelve isolates, chosen as representative for different 16S rRNA gene patterns, were characterized by 16S rRNA gene sequencing and phenotypic analysis. Isolates were assigned to *Sinorhizobium*, *Rhizobium* and *Agrobacterium*. Symbiotic properties of *Sinorhizobium* and *Rhizobium* isolates showed a large diversity in their capacity to infect their host plant and fix atmospheric nitrogen. Strain RK 22 identified as *Rhizobium* was the most effective isolate.

Keywords: *Retama raetam*; Rhizobia; 16S rRNA gene sequence; Tunisia

1. Introduction

Retama raetam is a spontaneous shrub legume belonging to the Fabaceae; it is one of the most important plants in the east Mediterranean deserts. In Tunisia this shrub legume plays an important ecological role, it is widely used in dune stabilisation and soil fixation [1]. In addition, *R. raetam* is able to produce important quantities of biomass that are used as forage and for treating various human and animal diseases. Due to their capacity to enter into symbiosis with legume nodulating bacteria (LNB) collectively called rhizobia, *R. raetam* could play an important role in the nitrogen cycle. They may be used to restore or increase fertility of degraded and eroded soils.

Studies of nodulating bacteria isolated from indigenous arid legumes species of Tunisia have shown a high diversity; strains belonging to the genera *Sinorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Phyllobacterium* [2–5]. The microsymbionts of *Retama sphaerocarpa* in Spain have been classified as *Bradyrhizobium* species [6]. At present, few studies and little information are available about the diversity and symbiotic properties of rhizobium strains nodulating *R. raetam* in Tunisia. However, Zakhia et al. [5] found that strains isolated from *R. raetam* grown in the infra-arid regions of Tunisia belonged to the branches containing the genera, *Bosea*, *Ochrobactrum*, Starkeya, *Microbacterium* and *Paracraurococcus*, but all these strains failed to nodulate their host of origin. Recently, Mahdhi and Mars [2] studied the genetic diversity of 35 *R. raetam* root-nodulating bacteria growing in the arid areas of Tunisia by some molecular approach including PCR-RFLP of 16S rRNA gene and 16S–23S rRNA gene region. A high genetic diversity was found; bacteria were associated with reference strains belonging to the genera *Sinorhizobium* and *Rhizobium*. However, many other isolates were not identified and they were not related to any reference strains used in the study. Therefore, the objective of this study is first, to confirm the diversity of *R. raetam* root-nodulating bacteria isolated by Mahdhi and Mars [2] and to clarify

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their taxonomic status by 16S rRNA gene sequencing and phenotypic analysis, and also to determine the symbiotic effectiveness of isolates.

2. Materials and methods

2.1. Bacterial strains and cultural conditions

Twelve rhizobial bacteria isolated by a standard method [7] from nodules of *R. raetam* plant growing in the arid regions of Tunisia were used in this study. They were chosen from among those previously characterized by RFLP analysis of 16S rDNA and 16S–23S rDNA region [2]. The isolates used in this study are shown in Table 1.

2.2. 16S rRNA gene sequencing

Twelve isolates chosen as representatives for each 16S rDNA pattern described by Mahdhi and Mars [2] for 35 *R. raetam* microsymbionts were grown and sub-cultured in YMA broth. Genomic DNA was extracted as described by Mhamdi et al. [8]. The 16 rRNA gene region was amplified using universal primers fD1 (FGPS6) and rD1 (FGPS1509) [9]. PCR amplification was carried out in a 25 μl reaction mixture according to Mahdhi and Mars [2]. The PCR amplification product was purified from agarose gel using QIAquick GEL extraction Kit (Qiagen, Courtaboeuf, France). Two forward primers (FGPS6 and 16S-370f) and one reverse primer (FGPS1509) were used to obtain the complete and partial gene sequence. 16S rRNA gene cycle sequencing was performed using the ABI PRISM BigDye Terminator cycle sequencing kit according to the manufacturer's protocol and analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The results of 16 rRNA gene sequences were analysed for homologies to sequences deposited in the GenBank. The determined DNA sequences and various 16S rRNA gene sequences from selected rhizobial species were aligned using the Clustal X and Genedoc software packages. A neighbour-joining tree was reconstructed and bootstrapped with 1000 replications of each sequence using Mega software [10]. The GenBank accession numbers for the 16S rRNA gene sequences reported in this paper are EF437249 (RB1), EF437248 (RB3), EF437250 (RB5), EF437251 (RM1), EF437252 (RM4), EF437257 (RH3), EF437258 (RH18), EF437259 (RH29), EF437253 (RE1), EF437254 (RZ6), EF437255 (RK12) and EF437256 (RK22).

2.3. Phenotypic characterization and plant test assays

Thirty two phenotypic features were used for characterization of isolates. The modified YMA medium [11] was used to analyse the ability of isolates to use carbohydrates (1% glucose, galactose, fructose and sucrose) and amino acids (0.1% L-arginine, L-proline, L-leucine and L-tyrosine) as a sole carbon and nitrogen sources, respectively. The tolerance of isolates to pH was assessed by adjusting pH to 4.0, 6.0, 9.0 and 12 by the addition of sterile acid or alkali to YMA, and the salt tolerance by adding NaCl to final concentrations of 1–4% in YMA. Maximum growth temperature and antibiotic resistance (Ampicillin 60 and 100 μg ml^{-1}, Streptomycin 60 and 100 μg ml^{-1}, Kanamycin 60 and 100 μg ml^{-1} and Nalidixic acid 20, 50 and 100 μg ml^{-1}) were assessed also on YMA as described by Mohamed et al. [12]. Acid and alkali production was determined in YMA medium with bromothymol blue indicator (0.0025%). In all experiments growth was recorded after 48 h in triplicate. Results were coded (1) for positive and (0) for negative. A computer cluster analysis of phenotypic features was carried out using similarity coefficient and a

Table 1
Isolates used in this study

	Host plants	Geographical origin	16S rRNA gene types
Isolates			
Sinorhizobium			
RH 18	*Retama raetam*	Hazwa (34°49′59″N, 7°42′7″E), Tunisia	5
RH 29	*Retama raetam*	Hazwa (34°49′59″N, 7°42′7″E), Tunisia	4
RM 1	*Retama raetam*	Menzel Habib (34°9′20″N, 9°43′59″E), Tunisia	11
Agrobacterium			
RB 1	*Retama raetam*	Parc de Bouhedma (34°42′47″N, 9°28′27″E), Tunisia	1
Rhizobium			
RH 3	*Retama raetam*	Hazwa (34°49′59″N, 7°42′7″E), Tunisia	3
RB 3	*Retama raetam*	Parc de Bouhedma (34°42′47″N, 9°28′27″E), Tunisia	2
RB 5	*Retama raetam*	Parc de Bouhedma (34°42′47″N, 9°28′27″E), Tunisia	6
RM 4	*Retama raetam*	Menzel Habib (34°9′20″N, 9°43′59″E)	12
RE 1	*Retama raetam*	Eljoref (33°40′15″N, 10°41′23″E), Tunisia	9
RZ 6	*Retama raetam*	Zarate (33°41′N, 10° 23′E), Tunisia	10
RK 12	*Retama raetam*	Kettana (33°45′N, 10° 41′E), Tunisia	8
RK 22	*Retama raetam*	Kettana (33°45′N, 10° 41′E), Tunisia	7

Isolates were chosen as representatives for PCR-RFLP 16S rDNA types described by Mahdhi and Mars [2].

dendrogram was constructed by the unweighted pair group method with average (UPGMA) clustering method [13].

Symbiotic effectiveness was expressed in percent of dry weight of the aerial biomass of the test plant to that of nitrogen control plants, which were maintained with Jensen's medium containing 0.1 M KNO_3. Seeds were surface-sterilised in 96% sulphuric acid for 2 h and germinated in Petri dishes at 20 °C. One seedling was transplanted into sterile plastic pots filled with autoclaved vermiculite. The pots were placed in a growth chamber at 23 °C with a 12–16 h photoperiod. Inoculation was performed for 48 h, with 10^8–10^9 cells of each isolate. The uninoculated plants (TN and T0) were included as control treatments. Plants were harvested 10 weeks after planting and observed for nodulation. Shoots were cut off and dried at 70 °C for 48 h, then weighed. Nitrogen fixing effectiveness of nodules was determined by comparing the dry-shoot weight of inoculated plants with the dry-shoot weight of +N control plants.

3. Results

3.1. 16 rRNA gene sequencing

The nearly full length sequences of 16S rRNA gene (about 1375 bp) of 11 isolates and partial 16S rRNA gene sequence (599 bp) of one isolate (RB5) were obtained. The studied isolates are clearly related to known LNB and are closely distributed to the genera *Rhizobium*, *Sinorhizobium* and *Agrobacterium*. In the reconstructed phylogenetic tree (Fig. 1), three isolates (RH 29, RH 18 and RM 1) representing 16S rRNA gene types 4, 5 and 11 group in *Sinorhizobium* branch. Strain RH 18 is related to *Sinorhizobium kostiense* LMG 15613^T. RH 29 and RM 1 are related to *Sinorhizobium meliloti* LMG 6133^T and *Sinorhizobium* sp. ORS 1444, respectively. Strain RB 1 representing 16S rRNA gene type 1 [2] is identical with *Agrobacterium tumafaciens* 2001025242. The other isolates representing 16 rRNA gene types 2, 3, 8, 7, 9, 10 and 12 group in *Rhizobium* branch. RH 3 and RE 1 are closely related to *Rhizobium* sp. STM 381. RK 12 and RK 22 are similar to *Rhizobium* sp. ORS 1465. RZ 6, RM 4 and RB 3 are, respectively, related to *Rhizobium giardinii* H152^T, *Rhizobium leguminosarum* LMG 8820 and *Rhizobium sullae* IS123^T. In addition, partial 16S rRNA gene sequence of RB5 (599 bp), representing 16S rRNA gene type 6, reveals that this isolate is similar to *Rhizobium* sp. CCBAU 25179 16S.

3.2. Phenotypic analysis

Twelve isolates were examined with 32 phenotypic characteristics. Results of phenotypic characteristics of the isolates and eight reference strains are shown in Table 2. All tested isolates were fast growers and acid producers. They grew at 28, 35 °C, 40 °C (five isolates) but not at 42 °C. All isolates were able to grow at pH 6.0–9.0, except for RB 3 and RZ 6 (tolerate pH 4.0). Regarding sugar and amino acid utilization, the majority of the isolates were able to use a wide range of carbon and nitrogen sources. All isolates were sensitive to Streptomycin (100 μg ml^{-1}) except for RM 1 and RM 4. Most of the isolates were resistant to 50 μg ml^{-1} of Nalidixic acid. The 32 variable features found among the isolates were used in cluster analysis. Isolates were divided at 70% similarity into five phena (Fig. 2). Strain RB 3 was separately positioned. Phenon 1 (100% internal similarity level) subsumes two isolates (RH 18 and RH 29) identified as *Sinorhizobium* by 16S rRNA gene sequencing. These two isolates were able to grow at 40 °C and tolerated NaCl concentrations from 1% to 3%. Phenon 2 contains two isolates sharing 87% phenotypic homology. These *Rhizobium* isolates (RK 12 and RK 22) were sensitive to 60 μg ml^{-1} of Streptomycin and Kanamycin. They were able to grow at 40 °C and tolerated 3% NaCl. Phenon 3 (75% internal similarity level) consisted of two *Rhizobium* isolates and one *Agrobacteium* isolate (RB 1). These isolates were sensitive to 100 μg ml^{-1} of Ampicillin, Streptomycin and Kanamycin. They were not able to grow at 15 °C and 40 °C. Phenon 4 (70% internal similarity level) contained two *Rhizobium* isolates (RZ 6 and RE 1). They were sensitive to 60 μg ml^{-1} of Streptomycin and one of them (RZ 6) was able to grow at pH 4.0. Phenon 5 included two isolates (RM 1 and RM 4). They were sensitive to 60 μg ml^{-1} of kanamycin and were not able to use L-arginine as sole nitrogen source.

3.3. Symbiotic properties

Eleven isolates identified as *Rhizobium* and *Sinorhizobium* by 16S rRNA gene sequencing were evaluated for their symbiotic potential. All isolates were capable of nodulation. RB 3 and RM 4 gave the highest nodule numbers per plant. On the other hand, shoot biomass, used as an indicator of relative effectiveness, indicated that *Rhizobium* isolate RK 22 was the most effective with an 80% dry biomass of the TN control (medium containing 0.1 M KNO_3). The least effective isolate was RH 3 with only 61% of the dry biomass of the TN control. The dry biomass of T0 control was 55% of the TN control. Results of the symbiotic performance of all these isolates are shown in Table 3.

4. Discussion

In this study, we characterized 12 nodule isolates, chosen as representative strains for each 16S rRNA gene pattern obtained from 35 *R. raetam* root-nodulating bacteria studied by Mahdhi and Mars [2]. A polyphasic approach [14] including phenotypic analysis and sequencing of the 16S rRNA gene we used showed that the majority of *R. raetam* isolates are grouped in *Sinorhizobium* and *Rhizobium* branches as many indigenous legumes in Tunisia [4,15]. Similar to the previous work [16,17], strain RB1 representing 16S rRNA gene type 1 [2] was identified as *Agrobacteria*, but, failed to nodulate its original host plant. This

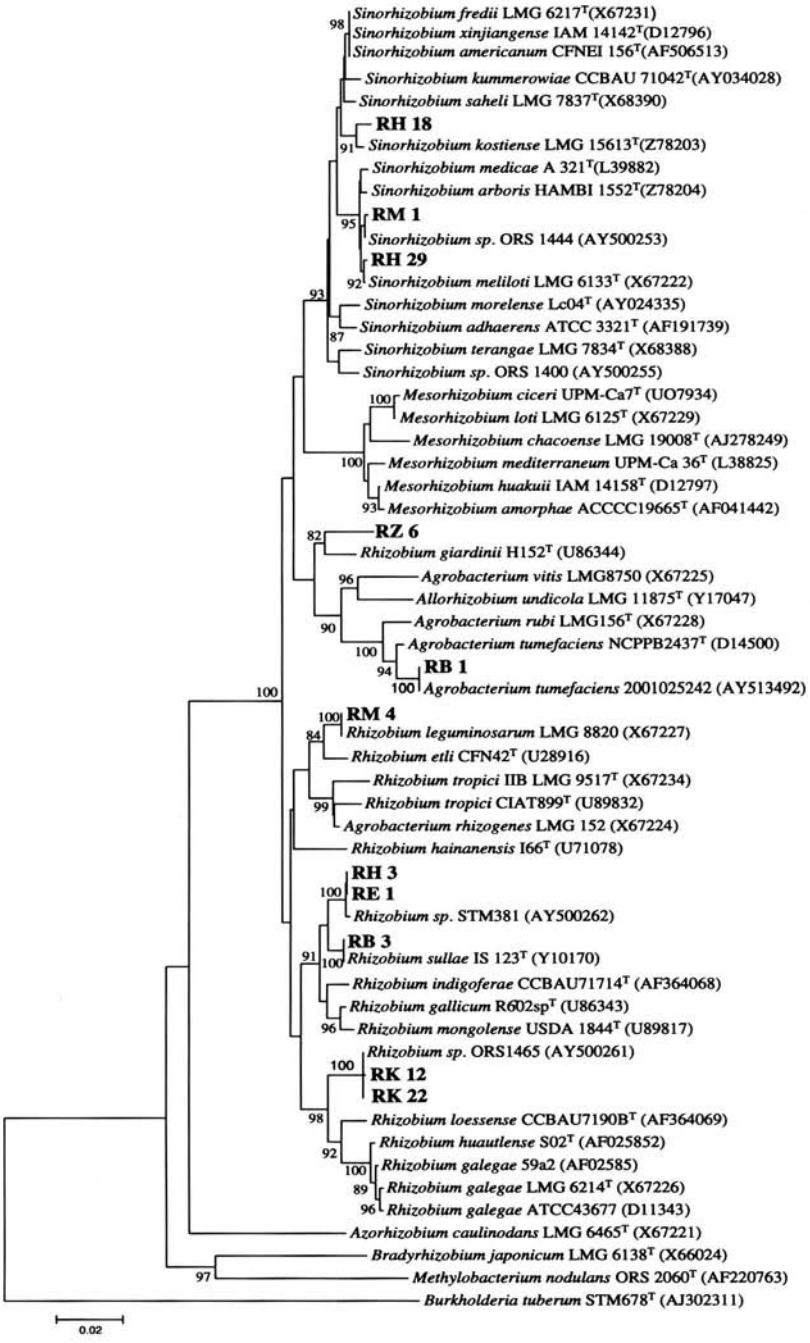

Fig. 1. Phylogenetic tree of 16S rRNA gene constructed by neighbour-joining method, showing the relationships between isolates and rhizobial species. Bootstrap confidence levels greater than 50% are indicated at the internodes. GenBank accession numbers are shown in parentheses.

observation is also reported for other legume in Tunisian soil [8]. The phenotypic analyses also showed an extraordinarily high diversity among the 12 selected isolates and no correlation was found between clusters analysis of phenotypic characteristics and results of 16S rRNA gene sequencing: RM1 and RM4 related to *Rhizobium* and *Sinorhizobium* groups, respectively, were phenotypically similar at 90%. The majority of isolates were resistant to 35 °C and some of them continued to grow at 40 °C. This may be a specific adaptation to high soil temperatures in the arid regions as described by Karanja and Wood [18]. The relatively high tolerance to NaCl reported for LNB from leguminous plants in the arid regions [19,20] was also found with RB 3, RH 3, RH 18, RH 29 and RK12 isolates, as they were able to grow in a medium containing 3% NaCl. All isolates except RB 1 were able to induce nodules when inoculated to *R. raetam* and overall assessments clearly indicated that inoculation of the isolates enhanced the vegetative growth and dry matter. Similar results were reported by Hatimi [21] with isolates nodulating *Retama monosperma* in Morocco and by Rodríguez-Echeverría et al. [6] with bacteria nodulating some shrubby legumes

Table 2
Phenotypic characteristic of isolates and reference strains

Isolates and reference strains	Growth at pH 4	Growth at 15 °C	Growth at 40 °C	NaCl tolerance (3%)	Utilization of carbohydrates				Utilization of amino acids				Antibiotics[a] resistance								Acid production
					Glucose	Galactose	Fructose	Sucrose	L-Leucine	L-Arginine	L-Tyrosine	L-Proline	Am (60 μg ml^{-1})	Am (100 μg ml^{-1})	Str (60 μg ml^{-1})	Str (100 μg ml^{-1})	Ka (60 μg ml^{-1})	Ka (100 μg ml^{-1})	Na (50 μg ml^{-1})	Na (100 μg ml^{-1})	
RH 18	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
RH 29	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
RM 1	−	−	−	−	+	+	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+
RH 3	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
RB 3	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
RB 5	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	−	+	−	−	−	+
RM 4	−	−	−	−	+	+	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+
RE 1	−	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+
RZ 6	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+
RK 12	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+
RK 22	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+
RB 1	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	−	+	−	−	−	+
B. japonicum NZP5549^{T}	−	−	−	−	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	−	−
R. mongolense STM246^{T}	−	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+
R. etli CFN42^{T}	−	+	−	−	+	+	+	+	+	−	+	+	−	−	+	+	−	−	+	+	+
R. galegae HMBI540^{T}	−	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+
R. tropci IIB CIAT899^{T}	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+
M. loti ORS664^{T}	−	+	−	−	+	−	+	+	+	−	+	−	−	−	−	−	−	−	−	−	+
M. mediterraneum ORS2739^{T}	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+
S. meliloti ORS665^{T}	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	+

[a] Am, Ampicillin; Str, Streptomycin; Ka, Kanamycin; Na, Nalidixic acid; (+) positive growth/present; (−) no growth/absent.

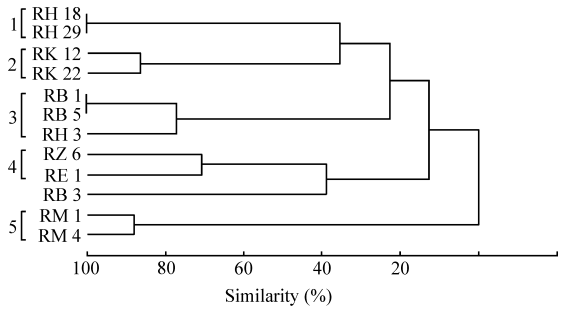

Fig. 2. UPGMA dendrogram showing phenotypic relationships among *Retama raetam* root-nodulating bacteria.

Table 3
Nodule number, shoot dry weight and relative effectiveness of *Retama raetam* plants inoculated with rhizobia native to the soils of the arid regions of Tunisia

	No. Nodules	Shoot dry weight (g/plant)	Relative effectiveness (% of TN control)
Isolates			
RH 18	17 ± 0.857	1.923 ± 0.047	69,083 ± 1.364
RH 29	21 ± 1.163	2.018 ± 0.045	71.886 ± 1.611
RB 5	22 ± 1.335	2.123 ± 0.035	75,627 ± 1.278
RZ 6	15 ± 1.137	1.825 ± 0.058	64.987 ± 2.082
RE 1	21 ± 1.315	2.011 ± 0.031	71.62 ± 1.135
RB 3	26 ± 2.053	1.816 ± 0.032	64.678 ± 1.174
RM 1	19 ± 0.795	1.987 ± 0.042	70.773 ± 1.516
RM 4	27 ± 0.997	1.838 ± 0.043	65,478 ± 1.534
RK 12	16 ± 0.888	1.785 ± 0.048	63,573 ± 1.732
RK 22	24 ± 1.434	2.263 ± 0.027	80.612 ± 0.962
RH 3	17 ± 1.455	1.737 ± 0.032	61.872 ± 1.141
Non-inoculated controls			
Control − N (T0)	0	1.562 ± 0.043	55,638 ± 1.544
Control + N (TN)	0	2.808 ± 0.102	100

Data are means of eight replicates ± SE.

from central western Spain. Strain RK 22 identified as *Rhizobium* was the most effective isolate. Tolerance to high temperature (40 °C) and NaCl concentration (up to 3%) rendered RK 22 as a promising strain for inoculation of *R. raetam*, and provided this strain a high competitiveness for nodule occupancy.

By 16S rRNA gene sequencing, five isolates were related with Tunisian legume nodulating bacteria corresponding to *Rhizobium* sp. ORS 1465, *Rhizobium* sp. STM 381 and *Sinorhizobium* sp. ORS 1444 which were isolated, respectively, from the root nodule of *Anthyllis henoniana*, *Genista microcephala* and *Argyrolobium uniflorum* growing in the same ecological and geographical area of Tunisia [4]. So, it would now be interesting to test the nodulation capacity of *R. raetam* isolates on these legumes and this is under investigation. There are some discrepancies between RFLP of 16S rRNA gene and the sequencing: RH3 and RE1 apparently share the same 16S rRNA gene sequence, however, they belong to different 16S rRNA gene types. These discrepancies can perhaps be explained by a low number of base differences between isolates.

It has been previously reported that *R. sphaerocarpa* in Spain is nodulated by strains belonging to genera *Bradyrhizobium* [6]. Zakhia et al. [5] found that strains isolated from *R. raetam* in Tunisia belonged to several genera not known to include any described LNB species. Surprisingly, all isolates from *R. raetam* investigated in this study are fast growers and group in the known genera of LNB. None of them are phylogenetically related to type strain of *Bradyrhizobium japonicum* LMG 6138.

In conclusion, our results thus confirm those reported by Mahdhi and Mars [2], showing the diversity of *Retama* rhizobia that belong to the *Sinorhizobium–Rhizobium–Agrobacterium* branch. However, none of the isolates identified in this study shows the affinity to the genera described by Zakhia et al. [5]. Some isolates were related to earlier described Tunisian rhizobia. However, rhizobia from other locations that were not covered in this study should be investigated.

Acknowledgements

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