Identification at species and symbiovar levels of strains nodulating *P. vulgaris* in saline soils of Marrakech region (Morocco) and analysis of *otsA* gene putatively involved in osmotolerance

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Abstract

Salinity is an increasing problem in Africa affecting rhizobia-legume symbioses. In Morocco, *Phaseolus vulgaris* is cultivated in saline soils and its symbiosis with rhizobia depends on the presence of osmotolerant strains in these soils. Here we identified 32 osmotolerant rhizobial strains nodulating *P. vulgaris* at species and symbiovar levels by analysing core and symbiotic genes, respectively. The most abundant strains were closely related to *R. etli* and *R. phaseoli* and belong to symbiovar phaseoli. A second group of strains was identified as *R. gallicum* sv gallicum. The remaining strains, identified as *R. tropici*, belong to the CIAT 899^T *nodC* group which has not been described as symbiovar yet. In representative strains, the *otsA* gene involved in the accumulation of trehalose and putatively in osmotolerance was analysed. The results showed that the phylogeny of this gene is not completely congruent with those of other core genes since genus *Ensifer* is closer related to some *Rhizobium* species than several species of *Rhizobium* among them. Although the role of *otsA* gene in osmotolerance is not well established, it can be an useful protein-coding gene for phylogenetic studies in genus *Rhizobium* since at species level the phylogenies of *otsA* and other core genes are coincident.

Key words: Rhizobia, salt stress, *Phaseolus vulgaris*, phylogeny, *otsA*, trehalose

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Genebank Accession numbers of gene sequences: 16S rRNA: JQ085247-JQ085247 for strains RHM14, RHM15, RHM19, RHM47, RHM54 and RHM67 respectively; *atpD*: JQ085253-JQ085258 for strains RHM14, RHM15, RHM19, RHM47, RHM54 and RHM67 respectively; *recA*: JQ085274- JQ085279 for strains RHM14, RHM15, RHM19, RHM47, RHM54 and RHM67 respectively; *nodC*: JQ085259- JQ085264 for strains RHM14, RHM15, RHM19,

52 RHM47, RHM54 and RHM67 respectively; *otsA*: JQ085265- JQ085273 for strains RHM14,
53 RHM15, RHM19, *Rhizobium gallicum* R602sp^T, *R. loessense* CCBAU7190B^T, *R. lusitanum*
54 P1-7^T, *R. mongolense* USDA1844^T, *R. phaseoli* ATCC14482^T, *R. yanglingense* SH22623^T.
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Introduction

Common bean (*Phaseolus vulgaris*) is one of the most important grain food legumes in the world [56, 62] and Morocco is the main producer of green bean in Africa with 187497 tonnes per year in 2009 [14]. Common bean was brought to Africa via Spain, the Canary Islands or Portugal after the discovery of America in the 16th century. American rhizobial strains nodulating this legume were distributed in other continents harbored in bean seeds since at least *R. etli* has been found in bean testa [34]. Other American species, *R. tropici* and *R. leucaenae* (formerly *R. tropici* type A), nodulating *P. vulgaris* could have also been transported by bean seeds to Europe and Africa. In Africa, strains identified as *R. etli*, *R. tropici* and *R. gallicum* have been found in common bean nodules [4, 5, 10, 26, 31, 35], some of them isolated in Morocco from soils affected by salinity. High concentrations of sodium chloride in soils is an increasing problem which significantly limits the crop production in arid and semi-arid areas [61], particularly when nitrogen nutrition is provided by symbiotic nitrogen fixation [11, 18]. In *P. vulgaris* symbiosis, it has been reported that a best symbiotic N₂ fixation under salinity conditions could be achieved if both symbiotic partners, as well as the different steps of their interaction are all tolerant to this stress [8]. Several rhizobial strains have developed active adaptative mechanisms to survive in hyperosmolarity conditions accumulating intracellular compatible solutes such as glutamate, trehalose or related compounds [6, 9, 12, 51]. Therefore the analysis of strains nodulating common bean in saline soils is crucial since the survival or success of the symbiosis can depend on the inoculation with salt-tolerant rhizobial strains showing high symbiotic nitrogen fixation efficiency [7]. This can be especially important in saline soils under arid climate such as Marrakech region soils, since aridity is an additional constraint affecting crop yield. Moreover, up to date no report is available on the genetic characterization of indigenous rhizobial strains of *P. vulgaris* and their phylogenetic relationships in Marrakech region.

Therefore, the first objective of this work was to identify at species and symbiovar (sv) levels the osmotolerant rhizobial strains nodulating common bean growing under arid climate in saline soils in the Marrakech-Tensift-Al Haouz region in Morocco, by analysing the core genes *rrs*, *atpD*, *recA*, and the symbiotic gene *nodC*. The second objective was to analyse the *otsA* gene encoding a trehalose-6-phosphate synthase involved in the accumulation of trehalose carried by our strains in order to compare it with the *otsA* gene from three osmotolerant strains of rhizobia isolated in Tunisia (Africa) from *P. vulgaris* nodules.

Materials and methods

Isolation of rhizobia

Rhizobial strains used in this study were obtained from *P. vulgaris* plants growing in soils of Marrakech-Tensift-Al Haouz region. The strains were isolated from large pink-colour nodules of field-grown bean plants on YMA plates according to Vincent [58]. The isolated strains of rhizobia were verified for nodulation through re-infectivity tests according to Ramírez-Bahena et al. [37]. Noninoculated nitrogen-free and nitrogen-supplemented plants were used as controls. Five replicates for each treatment were used; plants were grown in a greenhouse and were harvested 6 weeks after planting to determine the number and the size of nodules.

Tolerance to NaCl tests

The tolerance to salinity of free-living rhizobia was determined on YEM agar Petri dishes containing different NaCl concentrations (from 0 to 6.6% NaCl w/v).

RAPD fingerprinting

RAPD patterns were obtained as previously described [41] using the primer M13 (5'-GAGGGTGGCGGTTCT -3') and the GoTaq Flexi DNA polymerase (Promega). PCR conditions were: preheating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 45 °C for 1 min and extension at 75 °C for 2 min, and a final extension at 72 °C for 7 min. 10 µl of each PCR products were electrophoresed on 1.5% agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) at 6 V/cm, stained in a solution containing 0.5 g/ml ethidium bromide, and photographed under UV light. Standard VI (Roche, USA) was used as a size marker. A dendrogram was constructed based on the matrix generated using UPGMA method and the Pearson coefficient with Bionumerics version 4.0 software (Applied Maths, Austin, TX)

3.2. Sequence analyses of *rrs*, *recA*, *atpD*, *nodC* and *otsA* genes

The *rrs* gene was amplified and sequenced as previously described [39], *recA* and *atpD* genes according to Gaunt *et al.* [16], *nodC* gene according to Laguerre *et al.* [22] and *otsA* gene according to Fernández-Aunión *et al.* [12]. PCR amplifications were performed with a REDExtract-N-Amp PCR Kit (Sigma) following the manufacturer's instructions. The band corresponding to the different genes was purified either directly from the gel by room temperature centrifugation using a DNA gel extraction device (Millipore Co., USA) for 10

min at 5000 *g* or by elution of the excised band and filtration through silicagel columns using the Qiaquick DNA Gel Extraction Kit (Qiagen, Germany) in all cases following the manufacturers' instructions. The sequence reaction was performed on an ABI PRISM 3100 sequencer using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., USA) as supplied by the manufacturer. The sequences obtained (1475nt for *rrs*, 520nt for *recA*, 500nt for *atpD*, 906nt for *nodC* and 840nt for *otsA*) were compared to those held in GenBank by using the BLASTN program [2]. They were aligned by using Clustal W software [53]. Distances calculated according to Kimura's two-parameter model [20] were used to infer phylogenetic trees with the neighbor-joining method [46] and maximum likelihood with MEGA5 software [52]. Confidence values for nodes in the trees were generated by bootstrap analysis using 1000 permutations of the data sets.

Results and discussion

Isolation of strains and RAPD fingerprinting

A collection of 70 rhizobial strains was isolated from nodules of common bean from saline soils in the Marrakech region. In agreement with the results of other authors that commonly isolated *Rhizobium radiobacter* in *P. vulgaris* nodules [45], 38 of our strains were classified in this species by *rrs* gene sequencing (data not shown). Since they were unable to reinfect *P. vulgaris* in the nodulation tests, they were not further studied nor included in this study. The remaining 32 strains were able to reinfect *P. vulgaris* forming typical and effective nodules. The genetic diversity of these strains was assessed by RAPD fingerprinting, a strain dependent technique suitable in analysing the intraspecific diversity of rhizobial populations [19, 30, 32, 42, 54]. The numerical analysis of the patterns obtained (Figure 1) showed that they belong to eight groups according to the dendrogram obtained, with a similarity percentage lower than 70% (Figure 1 and Table 1) allowing us the selection of representative strains for gene sequencing.

Identification at species level

P. vulgaris may be nodulated by different species of genera *Rhizobium* and *Ensifer* and thus the *rrs* gene must be analysed in order to classify bean isolates since this gene is the current basis of rhizobial classification at genus level [16]. Strains from RAPD groups 1, 2 and 8 have identical *rrs* gene sequences and they were represented by strains RHM54 and RHM14 (belonging to the most dissimilar groups) whose sequences were 100% identical to that of *R.*

tropici CIAT 899^T (Figures 2 and S1). Strains RHM15 and RHM47 representative of RAPD group 7 have *rrs* gene sequences identical to that of the type strain of *R. gallicum* R602sp^T and that of strain PhD12, both isolated in Europe from *P. vulgaris*. American strains of *R. gallicum* isolated from a Milpa in Mexico (represented by strain IE4770) were related with strains RHM15 and RHM47 but their *rrs* gene sequences were not identical. Finally, strains from RAPD groups 3, 4, 5 and 6 have identical *rrs* gene sequences and they were represented by strains RHM19 and RHM67 (belonging to the most dissimilar groups) in Figure 2. These strains clustered with several species of genus *Rhizobium*, *R. phaseoli*, *R. etli*, *R. pisi* and *R. fabae*. However they presented differences in the *rrs* gene sequence with respect to all these species being *R. etli* CFN42^T the most closely related with only two nucleotide differences at the positions 170 and 1078 taking as reference the sequence of strain CFN42^T whose genome has been completely sequenced [17]. The *rrs* gene sequence of strain RHM19 was very closely related to those of other strains previously isolated in Morocco, such as RP305 and RP218 [31] and in Ethiopia, such as strain AK-3 [5]. Interestingly these sequences were also related with those of several strains classified in the species *R. etli* (represented by strain IE4804) that were isolated in a milpa of San Miguel in Mexico (Mesoamerica), a domestication center of common bean [48, 49].

The results of concatenated *recA* and *atpD* gene analyses (Figures 3 and S2) are completely congruent with those of *rrs* gene analysis. The strains RHM15 and RHM47 were unambiguously identified as *R. gallicum* since they carry *recA* and *atpD* genes with almost 100% identity with respect to those of the type strain of this species and with respect to those of strain PhD12 isolated from *P. vulgaris* nodules in France [3]. They were also phylogenetically related to several strains classified as *R. gallicum* (IE4868, IE2703, IE992 and IE4770) that were isolated in Mexico (Mesoamerica), a domestication centre of common bean as mentioned above, for which the American origin of this species was hypothesized [48, 49]. However since the American isolates have housekeeping genes different to those of European and African strains, they could possibly have different origin. This would support the second hypothesis of Silva *et al.* [49] that suggests a possible worldwide distribution of *R. gallicum* that would have acquired different symbiotic plasmids along the evolution in different ecosystems. In this case, the Moroccan strains could have the same origin that European strains rather than American ones.

The strains RHM14 and RHM54 were unambiguously identified as *R. tropici* with almost 100% identity with respect to the type strain of this species CIAT 899^T. These genes were also close to those of other American strain Br859 isolated in Brazil. *R. tropici* was isolated in

America from nodules of *Leucaena leucocephala* and it is also able to nodulate *Phaseolus* [25]. Initially this species included two groups, A and B, but recently the strains from group A have been reclassified into a new species named *R. leucaenae* [38]. It is interesting to highlight that curiously the strains isolated in Morocco belong to *R. tropici* [31], whereas those found in Europe belong to *R. leucaenae* [21, 55]. This can be due to a better adaptation of each one of these species to different environmental conditions including the local varieties of common bean in each site.

In contrast with the phylogenies obtained for the other groups, the strains RHM19 and RHM67 have *recA* and *atpD* genes divergent to those of *R. etli* in spite of the closeness of *rrs* genes. They were more closely related to *R. phaseoli*, a species distinguishable from the other species of genus *Rhizobium* according to the housekeeping gene analysis [37]. The *recA* and *atpD* genes of RHM19 and RHM67 were nearly identical (more than 99% similarity) with respect to several strains isolated in the Mexican milpa already mentioned [49] and 100% identical to those of a strain isolated in USA (strain KIM5s, see Figures 3 and S2). Although these strains were named *R. etli*, the analysis of *recA* and *atpD* genes suggested that they do not belong to this species [49]. Despite the fact that our strains are closer to *R. phaseoli* than to *R. etli* on the basis of housekeeping gene analysis, the differences found in the *rrs* gene do not allow including these strains in *R. phaseoli*. The genetic interchange among species could explain the phylogenetic position of the strains represented by RHM19 and RHM67, nevertheless before establishing conclusions a taxonomic revision of the complete group *R. phaseoli-R. etli* would be desirable.

Identification at symbiovar level

Whereas the identification at species level is based on core gene analysis, symbiovars identification is based on nodulation genes. Although *nodA* [33, 57] and *nodB* [36, 48, 49] genes have been used with this purpose, currently *nodC* gene is commonly used to define symbiovars [15, 19, 23, 29, 40, 59]. The analysis of symbiovars is in rhizobia as important as that of species, since rhizobial host range, legume promiscuity, coevolution and biogeography studies should be based on symbiovars rather than on species. For example, *P. vulgaris* is not considered a promiscuous legume because it can be nodulated by several chromosomal species of rhizobia, but rather because it nodulates with many different symbiovars (which harbour different *nodC* genes). In this way, common bean is commonly nodulated by symbiovars *phaseoli*, *gallicum*, *giardinii*, *mediterraneense* [44] and by strains from other groups that belong to undefined symbiovars [55]. Among these symbiovars, only sv *phaseoli*

seems to be exclusive for common bean nodulation [3, 55] and therefore the symbiovar phaseoli plasmid should have evolved with native common bean plants in America [1, 24]. Moreover, given that *R. etli* is the major endosymbiont of common bean in the American domestication centres of this legume [1, 49] the combination *R. etli* sv phaseoli is with high probability of American origin. Also, since *R. phaseoli* was considered to be *R. etli* for many years, the combination *R. phaseoli* sv phaseoli is also likely indigenous of American continent. Since the Moroccan strains represented by RHM19 and RHM67 and by the previously isolated strains PR218 and PR305 [31] belong to the symbiovar phaseoli (Figures 4 and S3) and to the chromosomal group *R. etli*-*R. phaseoli* (see Figures 2 and 3), they possibly have an American origin in agreement with Diouf *et al.* [10] that proposed that bean rhizobia had been introduced in Africa from the Americas together with the seeds.

The group of strains identified as *R. gallicum*, represented by RHM15 and RHM47, belong to the symbiovar gallicum and have *nodC* genes identical to that of strain RP421 previously isolated in Morocco from *P. vulgaris* nodules [31] and to that of the type strain of *R. gallicum* R602sp^T isolated in France [3] (Figure 4). However the strain PhD12, whose housekeeping genes are identical to those of our strains, carries a *nodC* typical of symbiovar phaseoli. Since the symbiovar phaseoli was not found in the *R. gallicum* strains isolated in Mesoamerica [49] and considering that this symbiovar evolved with *R. etli*, it is possible that the transfer of nodulation genes typical of this symbiovar to *R. gallicum* strains have occurred in European soils. In any case the presence of symbiovar gallicum in different continents could indicate a worldwide distribution of *R. gallicum* that would have acquired different symbiotic plasmids along the evolution in different ecosystems as suggested Silva *et al.* [49].

As for the group of strains identified as *R. tropici*, RHM14 and RHM54, they were identical to the Moroccan strain RP261 [31] and to several American strains from *R. tropici* and from *R. leucaenae* (Figure 4). They were also identical to *nodC* of *R. lusitanum* type strain, a Portuguese species nodulating common bean that probably acquired its *nodC* gene from *R. leucaenae*, since this last species has been found in common bean nodules in Portugal and carries identical *nodC* gene [55]. Therefore the American origin of the *R. tropici* strains nodulating *P. vulgaris* in Morocco seems to be clear in agreement with the suggestions of Diouf *et al.* [10].

Osmotolerance and analysis of *otsA* gene

The soils in which we isolated the strains from this study are affected by salinity as well as arid climate, with variable values of electric conductivity (EC) ranging from 2.5-9

mmhos/cm. For this reason we analyzed the strains osmotolerance, which is reported in Table 1. This characteristic was variable, but all the strains were able to growth in presence of NaCl concentrations ranging from 1.5% to 3%. These values were similar to those previously reported for strains isolated in other region of Morocco [8] where the climate is sub-humid instead of arid, and lower than those reported for rhizobial strains isolated from *P. vulgaris* in Egypt, which are tolerant up to 4% NaCl [47]. In the three species detected in this study we found Moroccan strains able to grow in higher NaCl concentration than the corresponding type strain. *R. tropici* CIAT899 was the most osmotolerant type strain (2.5%), whereas *R. gallicum* R602sp grew up to 1% NaCl. The most significant case was *R. etli*, *R. leguminosarum* and *R. phaseoli* whose type strains only grew in presence of 0.5% NaCl, whereas the Moroccan strains phylogenetically related with these species (strains from RAPD groups 3, 4, 5 and 6) were able to grow in higher concentrations, over 2% NaCl in all cases, which demonstrate that this adaptative trait is independent of phylogenies and very variable within a single species. In our case this implies an adaptation of American strains arriving with the bean seeds to the edapho-climatic conditions in saline Moroccan soils characterized by high temperatures, low relative humidity, high rate of evaporation and little rainfall. Hence, it will be very interesting to carry out more studies to find out what has been the adaptive modification that renders bacteria tolerant to salt. The salt stress can produce a failure in the establishment of infection and nodulation in the rhizosphere [60, 61], but osmotolerant rhizobia can stand large modifications in the osmolarity without decrease in the number of viable cells [50]. When bacteria are subjected to severe osmotic stress the accumulation of trehalose was observed [28] and this disaccharide play a major role in the adaptation of rhizobia to osmotic stress conditions [27]. The genes encoding the trehalose-6-phosphate synthase are involved in the accumulation of trehalose process and the *otsA* gene has been sequenced in some osmotolerant strains of rhizobia isolated in Tunisia from *P. vulgaris* nodules [12]. However only six species of genus *Rhizobium* were analysed in this previous work and moreover all of them belong to a close phylogenetic group within this genus. In the present work we have sequenced this gene in the strains isolated in Marrakech (Morocco) as well as in the type strains of six rhizobial species closely related with African isolates (Morocco and Tunisia). Moreover in the phylogenetic analysis of *otsA* gene we also included the strains whose complete genomes are currently available except *Agrobacterium tumefaciens* (currently *R. radiobacter*) C58 in whose genome the *otsA* gene is not present (Figures 5 and S4). In all the remaining strains the *otsA* gene is present, but it has different locations, harboured in the chromosome in *Rhizobium* strains whereas in *Ensifer* it is located

in a symbiotic plasmid. In the case of strain *E. meliloti* AK83 the *otsA* gene is located in chromosome 3, which really correspond to a symbiotic plasmid since it contains *nod* genes. These differences in the location of genes essential for environmental adaptation have been previously found as occurs in the case of *celC* gene which is also located in *Ensifer* plasmids but in *Rhizobium* chromosomes. However in the case of *celC* gene, that encodes a cellulase, its phylogeny is completely congruent with that of other core genes since the *celC* genes of *Ensifer* strains are divergent from those of genus *Rhizobium* sensu stricto [43]. In the case of the *otsA* gene, the phylogeny is not completely congruent with those of other core genes, given that species from genus *Ensifer* are more closely related to the group *R. gallicum*-*R. mongolense* than to other species of *Rhizobium*. Nevertheless, at species level the phylogeny of *otsA* gene is congruent with those of core genes since the strains of concrete species clustered with the corresponding type strains. For example, RHM15 has *otsA* gene identical to that of the type strain *R. gallicum* R602sp^T and to that of the osmotolerant strain 8a3 isolated in Tunisia by Fernández-Aunión *et al.* [12]. Very closely related to this group are located the species *R. mongolense*, *R. yanglingense* and *R. loessense* in agreement with the results of other core genes supporting the hypothesis that they belong to the same species [13, 43, 49]. The *otsA* gene of RHM14 was nearly identical to that of the type strain of *R. tropici* CIAT 899^T in agreement with the results obtained after the analysis of *rrs*, *recA* and *atpD* genes. Finally, RHM19 was closely related to the strain 12a3 isolated in Tunisia by Fernández-Aunión *et al.* [12]. These two strains are more closely related to *R. phaseoli* than to *R. etli* in agreement with the results of the housekeeping gene analysis. It is remarkable that the strain CIAT 652, currently included in *R. etli*, was closer related to *R. phaseoli* ATCC 14482^T than to *R. etli* CFN42^T and as was previously pointed out this strain probably belong to *R. phaseoli* [43]. Both species *R. etli* and *R. phaseoli* were clearly separated between them and from the cluster formed by *R. leguminosarum* that includes non-osmotolerant strains such as the type strain USDA 2370^T and osmotolerant ones such as 31c3 isolated in Tunisia by Fernández-Aunión *et al.* [12].

Therefore the phylogenetic analysis of *otsA* gene showed that it is closely related in strains from the same species with independence of their osmotolerance. For example the type strains of *R. phaseoli* and *R. etli* are not osmotolerant and have *otsA* genes nearly identical to those of osmotolerant strains from the same species. Therefore the role of *otsA* gene in osmotolerance is not clear and it cannot be used as a marker to identify osmotolerant strains although it can be a good marker to be included as a protein-coding gene useful for phylogenetic studies in genus *Rhizobium*.

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Figure legends

Figure 1. Dendrogram obtained for the strains isolated in this study using Pearson's coefficient and UPGMA analysis of the RAPD patterns.

Figure 2. Neighbour-joining phylogenetic rooted tree based on *rrs* gene sequences (1475 nt) showing the taxonomic location of representative strains from different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

Figure 3. Neighbour-joining phylogenetic tree based on concatenated *recA* and *atpD* genes sequences (520 and 500 nt) showing the position of representative strains from different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

Figure 4. Neighbour-joining phylogenetic tree based on *nodC* gene sequences (906 nt) showing the position of representative strains from different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

Figure 5. Neighbour-joining phylogenetic tree based on *otsA* gene sequences (840 nt) showing the position of representative strains from different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

Table 1

Table 1. Summary of the results obtained in the analysis of strains from this study

Strains	RAPD group	Salinity tolerance (%)	Species	Symbiovar
RHM46, RHM50	1	1.5	<i>R. tropici</i>	unnamed*
RHM54	1	2	<i>R. tropici</i>	unnamed*
RHM60	2	2	<i>R. tropici</i>	unnamed*
RHM66	2	3	<i>R. tropici</i>	unnamed*
RHM1, RHM19, RHM21, RHM25	3	2.5	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
R19, R24	4	2	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
R23	4	2.5	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
R26	4	3	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
RHM69, RHM72	4	2.5	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
RHM70, RHM74	4	2	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
RHM36, RHM37, RHM39, RHM40	5	2	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
RHM41	5	2.5	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
RHM67	6	2.5	<i>R. phaseoli</i> / <i>R. etli</i>	<i>phaseoli</i>
R15, RHM15	7	3	<i>R. gallicum</i>	<i>gallicum</i>
RHM47, RHM56	7	1.5	<i>R. gallicum</i>	<i>gallicum</i>
RHM62, RHM64	7	2.5	<i>R. gallicum</i>	<i>gallicum</i>
RHM3, RHM5, RHM14	8	2	<i>R. tropici</i>	unnamed*
Type strains:				
CFN42 ^T		0.5	<i>R. etli</i>	<i>phaseoli</i>
ATCC 14482 ^T		0.5	<i>R. phaseoli</i>	<i>phaseoli</i>
R602sp ^T		1	<i>R. gallicum</i>	<i>gallicum</i>
CIAT 899 ^T		2.5	<i>R. tropici</i>	unnamed*
CFN 299 ^T		1	<i>R. leucaenae</i>	unnamed*
USDA 2370 ^T		0.5	<i>R. leguminosarum</i>	<i>viciae</i>

* These strains belong to the *nodC* cluster formed by *R. tropici* CIAT 899^T.

Figure 1
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Figure 2 language corrected

0.005

Figure 3 language corrected

Figure 4 language corrected

Figure 5 language corrected