Nodulation of *Sulla flexuosa* ecotypes by *Rhizobium sullae* symbiovar sullae (sv. Nov) in Northern Morocco

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Research Article

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Abstract

Sulla flexuosa is a protein-rich Mediterranean pastoral and forage legume that is widely used for animal feed. In this work, we analyzed the diversity of the plant as well as the phylogenetic affiliations of its microsymbiotes. To our knowledge, this is the first evaluation of *Sulla* natural ecotypes in Morocco. We found that the plant populations constitute two distinct ecotypes, related to their geographical origin in Northern Morocco. The phylogenetic analysis of the 16S rRNA gene sequences of two representative strains, KS6 and BG1, isolated from the root nodules of each *Sulla flexuosa* L. ecotype, showed that they were closely related to *Rhizobium sullae* IS 123^T with a similarity of 99.78%. This result was confirmed by MLSA using *atpD*, *recA* and *glnII* housekeeping gene sequences. Indeed, the phylogenetic analysis of the tree genes concatenated sequences sowed that the strains have 98.60 to 98.67% of similarity with *R. sullae* IS 123^T. Furthermore, the phylogenic analysis of the *nodC* and *nifH* symbiotic genes showed that the two ecotypes *Sulla flexuosa* are nodulated by the same symbiovar of *Rhizobium*, for which we suggest the name of sullae (sv. nov.). The two representative strains tolerate water stress but are very sensitive to salinity, and can't grow at temperatures higher than 35°C.

1. Introduction

The legume family or Fabaceae is one of the largest families of flowering plants. Economically and ecologically important, legumes are found in almost all environments, where they play a central role in natural and agricultural systems. This family has long been subdivided into three classical subfamilies, Faboideae, Mimosoideae and Cesalpinoideae. However, a new classification based on the matK plastid gene sequences of about 20% of all legume species in the world, has grouped the legumes into six subfamilies.

Sulla flexuosa [L.] Medik [1] also known as *Hedysarum flexuosum* L. or Sulla is a pastoral, herbaceous, annual legume, in the tribe Hedysareae, subfamily Faboideae. Native to the Mediterranean basin, the plant is adapted to different environmental stress conditions [2]. It produces high levels of green matter with up to 500 q/ha [3] and good quality forage [4]. Consequently, the plant has been widely exploited as a forage crop to feed animals in both pastoralism and livestock production [5]. Legumes have high protein contents of up to 30% of their dry matter. This basic ration is often supplemented to adjust the feed supplied to the needs of the animals, whether for energy, protein or more specifically minor compounds in quantity such as vitamins or minerals [6].

Some authors have shown the existence of significant morphological and genotypic diversity within wild populations or ecotypes of *S. flexuosa* in Algeria and Morocco [7, 8]. An ecotype describes a geographic variety or population within a species, adapted to specific environmental conditions and expressing significant qualitative and quantitative variation [9].

In Morocco, *S. flexuosa* L. is represented by a reduced number of populations covering only small areas, depending on the state of degradation of the site. The extension of Sulla, in different potential cultivation areas, has been limited by nodulation problems, due to the high degree of host specificity of its compatible rhizobia [10–13].

Legumes can establish symbiosis with soil nitrogen-fixing bacteria, which are collectively called rhizobia, with nodule formation at the roots or sometimes stems of the plant. Nodulation involves multiple stages of interaction, from the initial attachment of bacteria and establishment of infection to late nodule development, and is characterized by complex molecular signaling between plants and rhizobia. This symbiotic association can be highly specific; each rhizobium species/strain interacts only with a specific group of legumes, or very broad, with one species that can nodulate legumes of several genera, or tribes.

Most rhizobia belong to the alpha-Proteobacteria subclass of the Bacteria domain, including the genera *Rhizobium*, *Ensifer*, *Allorhizobium*, *Pararhizobium*, *Neorhizobium*, *Shinella*, *Mesorhizobium*, *Aminobacter*, *Phyllobacterium*, *Ochrobactrum*, *Methylobacterium*, *Microvirga*, *Bradyrhizobium*, *Azorhizobium* and *Devosia*. Other three symbiotic N_2-fixing genera, *Paraburkholderia*, *Cupriavidus* and *Trinickia* belong to the family *Burkholderiaceae* of the beta-Proteobacteria subclass [14].

Species of the genera *Hedysarum* and *Sulla* are highly specific with respect to rhizobia and there is no cross-nodulation between strains of different species [12]. Plants of both genera are predominantly nodulated by *R. sullae* and *Mesorhizobium camelthorni* species [10, 15–20]. However, in Algeria Beghalem et al. [21] reported that members of the genus *Sulla* are nodulated by *Sinorhizobium meliloti* and *Neorhizobium galegae* genera.

In this work, we analyzed the diversity of rhizobia associated with two natural populations of *S. flexuosa* L., in order to assess whether there is any relationship between the plant ecotype and its microsymbionts. We also evaluated the productivity of the plant inoculated

with the two representative strains. A better knowledge of the genetic diversity of Sulla rhizobia is needed to optimize its forage potential.

2. Materials And Methods

2.1. Plant material and symbiotic efficiency

Samples of *S. flexuosa* L. were collected from two eco-geographical regions localized in North of Morocco, at two phenological stages (late vegetative and early flowering stage). Details on geographic and soil characteristics of the different sites are given in Table 1. Symbiotic efficiency was evaluated by scoring relative abundance of root-nodules [22].

Table 1
Physical and chemical soil analysis of sampling sites

	Chemical composition								Physical composition				
	Altitude, above the see level (m)	Water content (%)	pH (water)	N (%)	P_2O_5 (ppm)	K_2O (ppm)	OM (%)	Total $CaCO_3$ (%)	Clay (%)	Fine silt (%)	Coarse silt (%)	Fine sand (%)	Coarse sand (%)
Ksar Sghir	39	4.68	8.1	0.110	8.6	177.6	0.5	7.64	69.52	18.72	0.11	2.67	0.80
Beni Gorfet	94	4.70	8.0	0.112	13.04	108.4	0.2	4.03	58.20	31.75	0.39	0.85	4.55

2.2. Ecotype determination

2.2.1. Seeds sterilization and growth

Seeds of two natural populations of *S. flexuosa* L. were collected from each site. Mature pods were harvested from a minimum of 30 randomly chosen plants, and manually scarified, then surface-sterilized with 70% (v/v) ethanol for 5 min followed by 2min incubation in pure sulfuric acid and finally rinsed thoroughly with distilled water. The seeds were transferred to water-agar (0.9%) plates, and allowed to germinate at 30°C in the dark. Seedlings (2/pot) were planted in plastic pots containing sterile sand and watered daily with dH_2O and weekly with mineral solution supplemented with KNO_3 (0.05%) as nitrogen source [23]. Finally, all pots were maintained under controlled environment in a growth chamber (16/8 h light/dark of photoperiod and 25°C) during 8 weeks.

2.2.2. Protein extracts and isozyme analysis

Protein extracts were prepared by crushing the leaf material in ice-cold TAMET buffer (pH 7) containing (0.5 M Tris Hcl, 0.3M ascorbic acid, 2% β-mercapto-ethanol, 0.01mM EDTA, 2.8% Triton X-100 and 10% (w/w) insoluble PVP). The homogenate was centrifuged at 8000 g for 20 min at 4°C and the supernatant was collected, assayed for total soluble proteins using the Bradford reagent [24], then used as enzyme extract for isozyme analysis. Samples were submitted to non-denaturing polyacrylamide gels electrophoresis (PAGE) at 4°C for 3 h (80V). After electrophoresis, the gels were removed and colored by incubation in a solution composed of 100 mM Tris-HCl (pH 7.5), 40 mM L-aspartate, and 5 mM 2-oxoglutaric acid, for aspartate aminotransferase (AAT) enzyme, and for esterase (EST) enzyme by incubation in a solution containing 0,05M of Tris-HCl (pH 7.2), 0,03% Alpha-naphthyl acetate (dissolved in 50% acetone). After 15 min of incubation, the gels were rinsed with distilled water and staining using the method outlined by Ben Mrid et al. [25].

2.4. Nodule sampling and rhizobial isolation

Root nodule bacteria were isolated, as described by Ezzakkioui et al. [11], from naturally occurring root nodules at the same sites of seeds collection. Pure isolates were stored with 20% glycerol at -80°C.

2.5. Phenotypic characterization

The isolated bacteria were tested for their ability to grow under stress conditions such as drought, salinity, acidity and high temperatures. Thus, the tolerance of the rhizobial isolates to temperatures was tested on YEM-A plats incubated at 30, 37, and 42°C. The salt tolerance of the isolates was carried out on YEM-A in the presence of 0.5%, 1% and 2% NaCl. In addition, the ability of isolates

to grow on acidic or basic media was determined on solid YEM medium adjusted and buffered to 4, 5, 8 and 9 as described by Zerhari et al. [26]. Finally, the response of strains to drought stress were performed in YEM broth set at different osmotic potentials (-0.25, -0.5 and − 1MPa) using PEG 6000 (polyethylene-glycol 6000) as reported by Busse and Bottomley [27].

2.6. Plant inoculation

The ability of purified isolates to renodulate their host plants was tested in glasshouse experiments as described earlier [22]. Seedlings were inoculated at sowing with 1ml (~ 10^8 cells/ml) of each bacterial suspension and irrigate twice a week with N-free mineral solution [23].

2.7. DNA extraction and *NodC* gene amplification

Total genomic DNA was isolated following phenol/chloroform method, as described by Chen and Kuo [28]. The quantity of DNA was determined by using a NanoDrop spectrophotometer (NanoDrop ND2000/2000c, Thermo Fisher Scientific, United States) and set to final concentration of 100ng/µl before amplification. Primer pair *nodCl* and *nodCFn* was used for amplification of *nodC* gene [29]. Amplification products were checked by horizontal electrophoresis in 1% (w/v) agarose (Bioline) gels stained with ethidium bromide to a final concentration of 0.4% (w/v) in 1×TAE (Tris-Acetate-EDTA) buffer at 70 V for 1 h and photographed using the ENDURO™ GDS Gel Documentation System (Labnet International, Inc., US).

2.8. ARDRA fingerprint and rebotype analysis:

PCR amplifications of 16S rRNA gene fragments were carried out using the two opposing primers *rD1* and *fD1* [30]. Amplified DNA were first cheek by 1% (w/v) horizontal gel electrophoresis then subject to ARDRA (amplified ribosomal DNA restriction analysis) using *HaeIII* and *MspI* restriction endonucleases (PromegaCorp. Madison WI, USA), following the manufacturer's instructions. Digested DNA was separated by horizontal electrophoresis in a 2% (w/v) agarose gel (Bioline), and photographed under UV light.

2.9. Multilocus sequence and phylogenetic analysis:

Partial sequences of *atpD*, *glnII*, and *recA* genes were obtained using primers described previously [31–33]. Amplification products were purified using the PCR product purification system of Qiagen and subjected to cycle sequencing using the same primers as for PCR amplification at the sequencing facilities of the National Centre for Scientific and Technical Research (CNRST) in Rabat (Morocco). The sequences obtained were compared with those from GenBank using the BLASTN program, then aligned independently with defined reference species of the genus *Rhizobium* using clustal W program from MEGA7 [34]. A neighbor-joining [35] tree of individual and concatenated genes was constructed with Bootstrap confidence levels calculated for 1000 replicates using the Kimura two-parameter method [36].

2.10. Sequence accession numbers

All sequences used in this study have been deposited in Genbank database in NCBI with accession number shown in the figure trees.

3. Results

3.1. Ecotypes determination

The *in-vivo* analysis of two natural populations of Sulla, originating from two distinct regions, Beni Gorfet and Ksar Sghir, in Northern Morocco, revealed a great variability in many morphological characters (length of the main stem, length of the lateral branches, average number of leaflets per leaf... etc.) according to the geographical origin of the populations. Thus, at low altitude, near the Mediterranean coast, at the site of Ksar Sghir, the populations evaluated are distinguished by an erect state, an intense branching and leaves with a large number of internodes; while those in the site of Beni Gorfet present a prostrate growth with a low number of leaves and inflorescences. These results suggested the presence of two different ecotypes of *S. flexuosa*, adapted to different environmental conditions. Furthermore, isozyme polymorphism analysis of different populations of *S. flexuosa* grown in controlled, greenhouse environments showed significant genetic diversity within the population, as evidenced by the different electrophoretic profiles (Fig. 1).

3.2. Symbiotic efficiency

A total of 25 rhizobial bacteria were isolated from the root nodules of *S. flexuosa* L. collected from Beni Gorfet and Ksar Sghir sites. The characteristics of the sampling sites' are presented in Table 1. The isolates were authenticated by assessing their ability to re-infect

their host plant in axenic conditions. Only 16 isolates were able to form typical and effective pink nodules on the plant roots with dark green leaves color. Then we amplified the *nodC* genes of the 16 symbiotic isolates which produce a single clear band of 930 bp.

3.3. ARDRA ribotyping and 16S rRNA phylogeny

The ARDRA experiments showed that the isolates are grouped in two ribotypes, from which we selected two isolates as representatives for more genomic analysis, based on their efficiency and infective capabilities, namely KS6 and BG1, from Ksar Sghir and Beni Gorfet sites respectively.

The rRNA sequences analysis showed that the two strains belong to the genus *Rhizobium*. They share 100% similarity and have percentages of similarity of 99.78% with *R. sullae*, isolated from *S. coronaria* L. syn. *Hedysarum coronarium* L. in Italy [15] and 99.05% with *R. loessense* isolated from *Astragalus mongholicus* in China [37]. Furthermore, the phylogenetic tree (Fig. 2) inferred from the nearly complete sequence of the 16S rRNA gene from KS6 and BG1 showed that the two strains are regrouped with the type strain *R. sullae* IS 123^T.

3.4. MLSA and phylogenetic analysis

To confirm the taxonomic position of the selected strains, we carried out a Multilocus Sequence Analysis (MLSA) using the *recA*, *atpD* and *glnII* housekeeping genes. All the three phylogenetic trees based on the three single genes sequences (Fig. 3, 1S-3S) show that the two strains KS6 and BG1 are closely related, and share similarities between 98.47 and 99.48% with *R. sullae* IS 123^T (Table 3). Furthermore, the three genes concatenated sequences tree confirmed the belonging of the strains to *R. sullae*. The concatenated sequences of strains KS6 and BG1 share 99.93% of similarity and have 98.60 and 98.67% of similarity with *R. sullae* IS 123^T, respectively (Table 3).

Table 3
Percentages of nucleotide identity of the two selected strains from this study and the most related identified rhizobia.

Gene (nucleotides)	Strains	*Rhizobium* sp. KS6	*Rhizobium* sp. BG1
atpD **(392 nt)**	*Rhizobium* sp.BG1	100.00%	100.00%
	Rhizobium sp.KS6	100.00%	100.00%
	Rhizobium sullae IS123^T	99.48%	99.48%
	Rhizobium yanglingense HAMBI 3198^T	95.92%	95.92%
	Rhizobium mongolense USDA1844^T	95.41%	95.41%
recA **(453 nt)**	*Rhizobium* sp.KS6	100.00%	100.00%
	Rhizobium sp.BG1	100.00%	100.00%
	Rhizobium sullae IS123^T	98.87%	98.87%
	Rhizobium mongolense USDA1844^T	90.93%	90.93%
	Rhizobium gallicum R602^T	90.71%	90.71%
	Rhizobium yanglingense HAMBI 3198^T	91.06%	91.06%
glnII **(523 nt)**	*Rhizobium* sp. KS6	100%	99.81%
	Rhizobium sp. BG1	99.81%	100%
	Rhizobium sullae IS123^T	98.47%	98.66%
	Rhizobium yanglingense HAMBI 3198^T	94.46%	94.65%
	Rhizobium gallicum R602^T	94.26%	94.46%
	Rhizobium mongolense USDA1844^T	93.92%	94.12%
atpD **+** glnII **+** recA **Concatenated sequences (1368 nt)**	*Rhizobium* sp.KS6	100%	99.93%
	Rhizobium sp.BG1	99.93%	100%
	Rhizobium sullae IS123^T	98.60%	98.67%
	Rhizobium mongolense USDA1844^T	93.35%	93.43%
	Rhizobium gallicum R602^T	93.20%	93.27%
nodC **(651 nt)**	BG1	100.00%	100.00%
	Rhizobium sp. ICMP 19433	99.53%	99.53%
	R. *sullae* WSM1592	99.50%	99.50%
	R. *sullae* Hc14	99.39%	99.39%
	R. *alkalisoli* CCBAU 01393^T	84.42%	84.42%
	R. *mongolense* USDA 1844^T	81.32%	81.32%
nifH **(683 nt)**	*Rhizobium sullae* Hf_02N (KU173885.1)	100.00%	100.00%
	BG1	100.00%	100.00%

Gene (nucleotides)	Strains	*Rhizobium* sp. KS6	*Rhizobium* sp. BG1
	Rhizobium sullae WSM1592	99.67%	99.67%
	Rhizobium sp. ICMP 19433	99.57%	99.57%
	Rhizobium yanglingense CCBAU 71623^T	91.35%	91.35%
	Rhizobium gallicum R602sp^T	86.67%	86.67%

3.5. Analysis of *nifH* and *nodC* symbiotic genes

The *nodC* and *nifH* genes were used as symbiotic markers to analyze the symbiotic diversity of the two strains and to determine their symbiovars [38]. To our knowledge, this is the first report on the *nodC* nodulation gene phylogeny in strains of *Rhizobium* isolated from members of the genus *Sulla*. The *nodC* sequences analysis showed that the two strains share 100% similarity and have 99.53% similarity with *Rhizobium* sp. ICMP 19433 initially isolated in New Zealand from *S. coronaria* L., a closely related species to the *S. flexuosa* L., and 99.50% similarity with *R. sullae* IS123^T also isolated from *S. coronaria* L. in Italy [15]. Moreover, the phylogenetic tree based on *nodC* sequences (Fig. 4) regrouped the two representative strains isolated from *S. flexuosa* altogether with *R. sullae* strains isolated from other *Sulla* species in a cluster different from the other symbiovars with high bootstrap values. These results suggest that the *R. sullae* strains nodulating members of the genus Sulla constitute a new symbiovar in the genus *Rhizobium*, for which we propose the name of sullae.

The results obtained with *nifH* sequences analysis and phylogeny were similar and grouped the two strains in a separate clade (Fig. 4S). The two strains *nifH* sequences share 100% similarity with *R. sullae* Hf_02N and 99.67% of similarity with *R. sullae* WSM1592, isolated from the root nodule of *S. coronaria* L. respectively in Algeria [10] and in Italy [39].

3.6. Phenotypic characterization

The two representative strains are unable to grow in presence of salinity, even at 0.5% of added NaCl, which is lower than that reported by other authors who found that the tolerance ability of *R. sullae* varied between 2% NaCl and 3% NaCl [12, 13, 37]. Both strains grow at 30°C but not at 37°C.

In other hand, both isolates showed good growth under water stress (up to -1MPa) compared to the isolates obtained by Elboutahiri et al. [40]. Finally, the strain BG1 is capable to grow at different pH values, whereas strain KS6 did not grow at low pH4 and pH5.

4. Discussion

In this work, we assessed the diversity of *S. flexuosa* L., a pastoral Mediterranean legume, known for its high genotypic diversity due to the allogamous breeding regime of the species [8]. In fact, different groups or ecotypes can be distinguished within the species *S. flexuosa* L. [7] as pedoclimatic adaptations. Our results show significant inter-population differences between Sulla populations evaluated for most morphological traits recorded, especially for their growth habits (erect or prostrate). These results is in agreement with previous studies conducted on similar species [41, 42] and suggest a high forage potential, which could be used as grazing, haying or silage material in Mediterranean environments.

The role of rhizobia in increasing plant growth and productivity has been widely reported, but only a few researchers have successfully isolated rhizobial bacteria from Sulla plant nodules and grown them on culture media. Most researchers have reported the isolation and identification of a large number of endophytes instead of bacterial symbiotic partners using the culture-dependent method [17, 43, 44, 45, 46]. These endophytes are preferentially selected by wild legumes because they can help plants cope with stressed environments [18, 47, 48]. On the other hand, the use of direct PCR amplification of the 16S ribosomal RNA gene and nucleotide sequencing of prokaryotic DNA from nodules and other tissues of various Sulla species, showed the presence of bacteria of the genera *Rhizobium* and *Mesorhizobium* inside the nodules, but these bacteria were unable to grow on the common culture medium YEM [17, 45, 46]. In the present study, 16 isolates belonging to *R. sullae* were identified in root nodules of *S. flexuosa* L., in agreement with other studies conducted on spontaneously growing Sulla in Morocco [11], Algeria [10] and Tunisia [49]. Based on phylogenetic analyses of their symbiotic genes such as *nodC* and *nifH*, two strains (KS6 and BG1) were identified as members of a new symbiovar for which we propose the name sullae. Symbiovars (symbiotic variant) are generally used to differentiate between different strains that are distinct

within a species and could be maintained by vertical and lateral transfer of symbiotic genes between rhizobial species [38]. In fact, biovars within *Rhizobium* genera have already been described in different species such as *R. leguminosarum* [50], *R. gallicum* [51], and *R. galegae* [52, 53]. These results show that *S. flexuosa* establishes a specific rhizobial symbiosis with adapted strains in different environments.

5. Conclusion

In this work, we analyzed the diversity of two *S. flexuosa* L. ecotypes and show they are nodulated by a new symbiovar in the genus *Rhizobium* we named sullae. The results obtained indicate that the plant is adapted to the Mediterranean environment and could be useful in other parts of the world, such as Australia and China.

Declarations

Funding information

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Contribution

A. El Yemlahi and **O. Bouhnik**, conducted the experimentations, acquired and analyzed the Data. **A. El Yemlahi** and **M. Missbah El Idrissi** wrote the Original draft. **M. Bekkali**, **A. Laglaoui**, and **A. Arakrak**, designed the study. **M. Missbah El Idrissi** and **A. Laglaoui** acquired the funding. **A. El Yemlahi** and **M. Missbah El Idrissi** analyzed the data, and contributed to the final version of the paper.

Competing Interests

The authors declare that they have no conflict of interest

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Figures

Figure 1

Aspartate aminotransferase (A) and Esterase (B) zymograms' patterns of tow native ecotypes of *Sulla flexuosa* L. growing in Beni Gorfet (BG) and Ksar Sghir (KS) sites, in Northern Morocco.

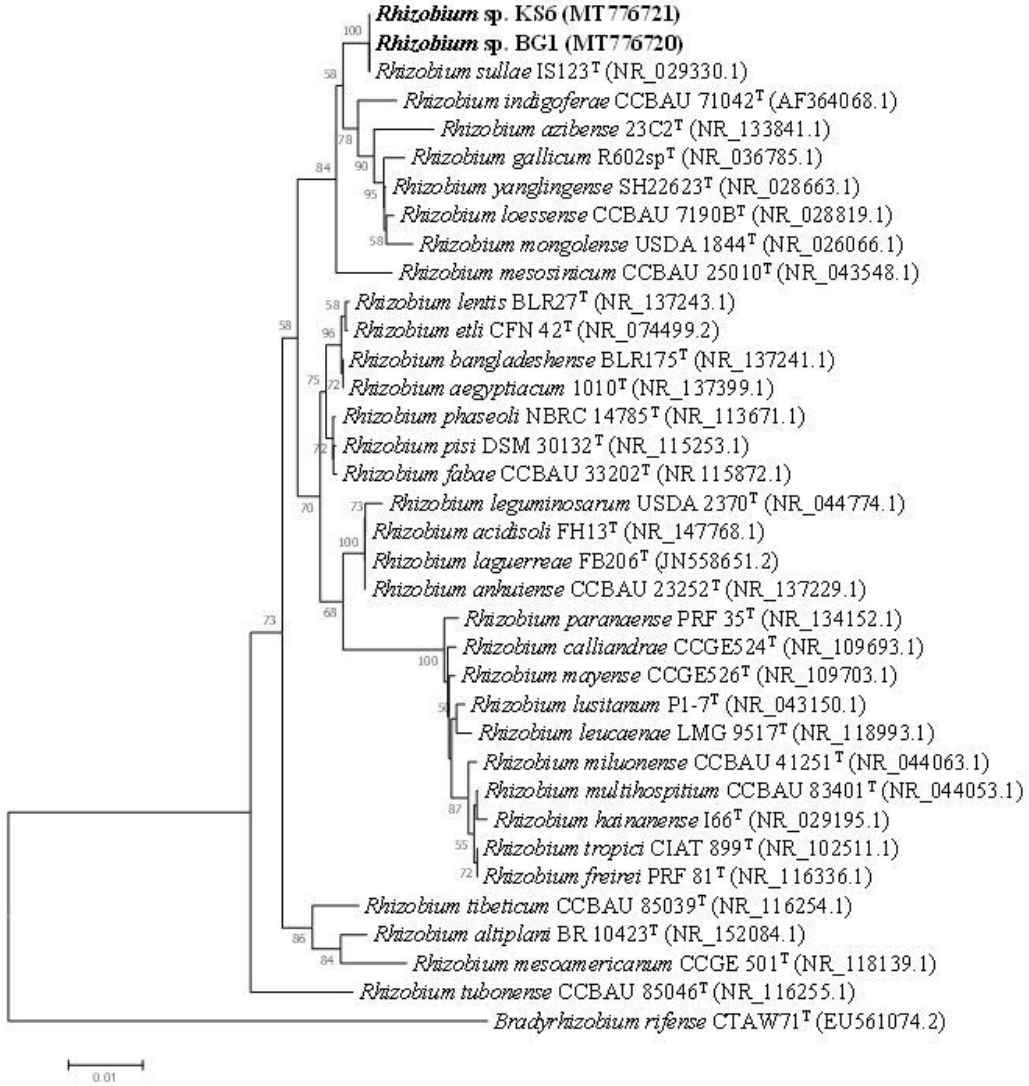

Figure 2

Neighbor-joining phylogenetic tree based on partial **16S rRNA** sequences of strains KS6 and BG1 isolated from nodules of wild-grown *Sulla flexuosa* L. and phylogenetically related species within the genus *Rhizobium*. The isolates are denoted in bold. Bootstrap values are indicated as percentages derived from 1000 replications. Bar, 1 nucleotide substitution per 100 nucleotides. The tree was rooted with *Bradyrhizobium rifense* CTAW71^T.

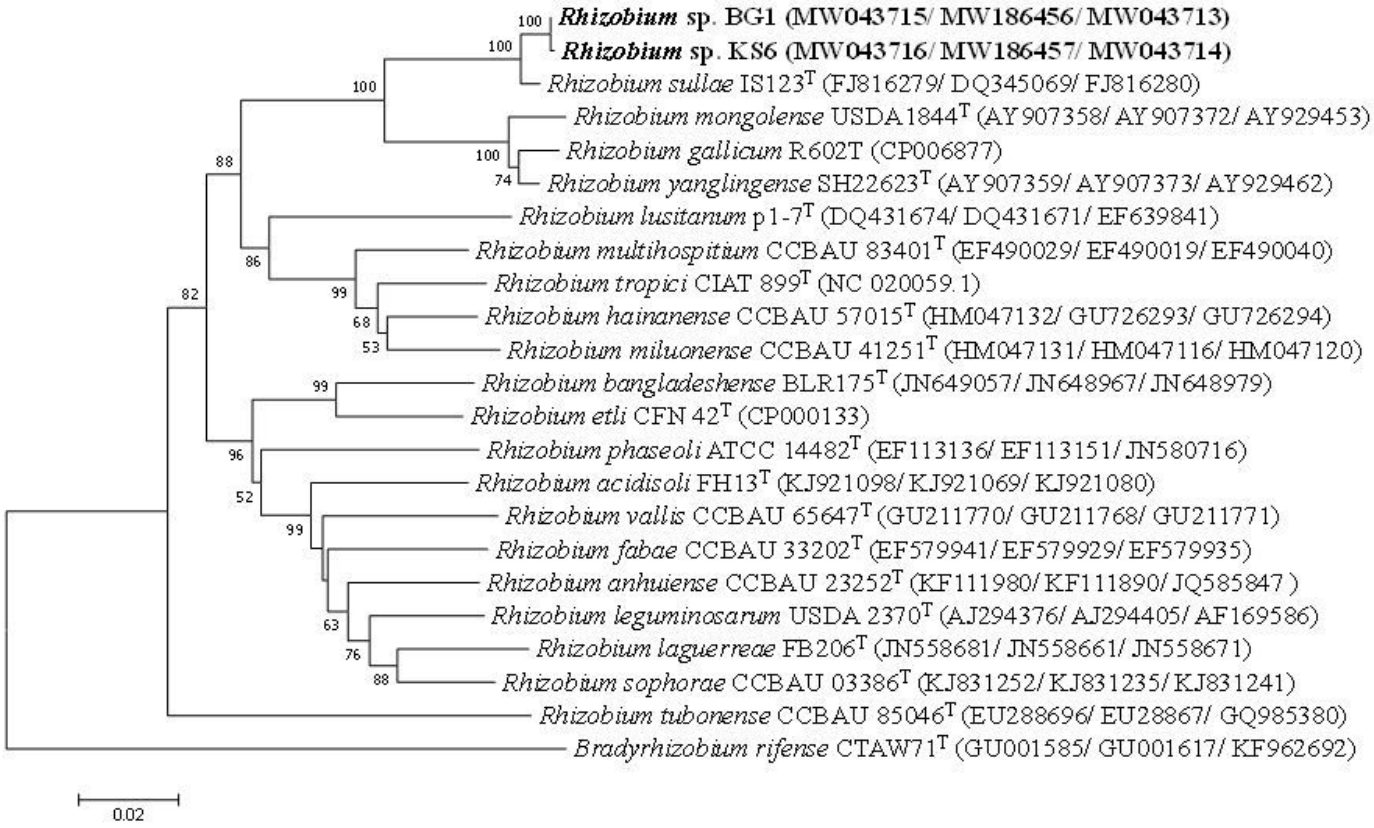

Figure 3

Neighbor-joining phylogenetic tree based on **concatenated** *recA* + *atpD* + *glnII* genes' sequences of strains KS6 and BG1 from nodules of wild-grown *Sulla flexuosa* and phylogenetically related species within the genus *Rhizobium*. The representative isolates are shown in bold and Sequence accession numbers are given in parentheses. Bootstrap values are indicated as percentages derived from 1,000 replications. Superscript 'T' indicates type strains. The tree was rooted with *Bradyrhizobium rifense* strain CTAW 71^{T}.

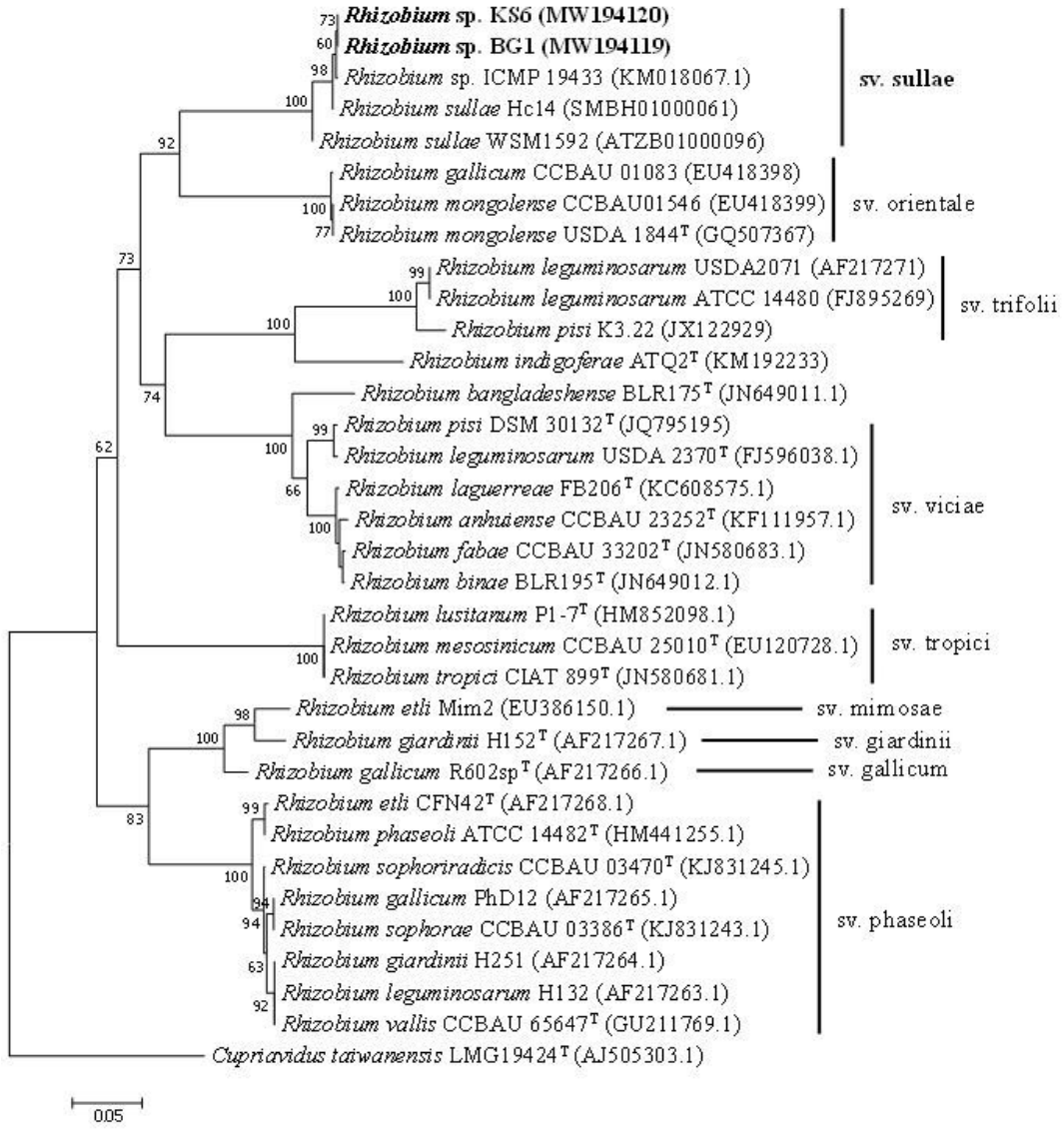

Figure 4

Neighbor-joining phylogenetic tree based on sequences of the ***nodC*** gene for strains KS6 and BG1 from nodules of *Sulla flexuosa* and phylogenetically related species within the genus *Rhizobium*. Bootstrap values are indicated as percentages derived from 1000 replications. Bar, 2 nucleotide substitutions per 100 nucleotides. The tree was rooted with *Cupriavidus taiwanesis* LMG19424^T

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