Environmental Sustainability (2023) 6:161–171
https://doi.org/10.1007/s42398-022-00258-1

ORIGINAL ARTICLE

Bioprospection of naturalized soybean-nodulating *Bradyrhizobium* strains in Uruguayan soils: a genetic and symbiotic approach

N. E. Rodriguez Rodriguez[1] · E. Beyhaut[2] · M. Sicardi[3] · A. Rodríguez-Blanco[1]

Received: 31 May 2022 / Revised: 29 September 2022 / Accepted: 6 December 2022 / Published online: 5 January 2023

Abstract

Soybean (*Glycine max* (L.) Merr.), an economically relevant crop, establishes a symbiotic association with rhizobia to obtain nitrogen (N_2) from the air by biological nitrogen fixation with important benefits. In Uruguay inoculants formulated with two strains, *Bradyrhizobium elkanii* U1301 and U1302, are recommended since 1984. Besides that, the study of native-naturalized rhizobia populations is relevant because these strains could compete with applied inoculants and may present a better symbiotic efficiency. The aim of this work was to study, genetically and symbiotically, naturalized soybean nodulating rhizobia isolated from Uruguayan soils. A collection of ten naturalized rhizobia was studied and compared with Uruguay's commercial strains and neighbouring countries (*B. elkanii* U1301 and U1302, *Bradyrhizobium japonicum* E109 and *Bradyrhizobium diazoefficiens* SEMIA5080). Using a multilocus sequence analysis (MLSA) (*16S rRNA, atpD, gyrB* and *rpoB* genes), five naturalized strains were identified as *B. elkanii* and four as *B. japonicum*. The other naturalized strain UYS-CA02 is suggested to belong to *Bradyrhizobium ferriligni*, considering a second MLSA with *16S rRNA, gyrB, rpoB, dnaK* and *recA* genes. Analysis of symbiotic genes (*nodY/K* and *nifH*) indicates that strains U1301 and U1302 may have transferred these genes horizontally to strain UYS-CA02 or its ancestor. Symbiotic efficiency was evaluated in axenic conditions, in which shoot dry weight, total nitrogen in shoots, number of nodules and nodules dry weight, were determined. In that assay, the U1301:U1302 blend outstood in front of other commercial strains. Multivariate analysis of symbiotic efficiency data shows a better performance of *B. elkanii*-like strains than *B. japonicum*-like ones.

Keywords *Glycine max* · Rhizobia · MLSA · Phylogeny · Biological nitrogen fixation · *Bradyrhizobium*

Introduction

Biological nitrogen fixation (BNF) is a process in which some prokaryotes called diazotrophs transform nitrogen (N_2) from the air into NH_3 (Stein and Klotz 2016). As a consequence, diazotrophs provide a large nitrogen input to the soil, reducing the need for applying synthetic fertilizers in crop areas. Soybean (*Glycine max.* L. [Merr.]) obtains between 50 and 60% of nitrogen in average from BNF (Salvagiotti et al. 2008). This is achieved due to the symbiotic relationship between this legume and diazotrophic bacteria known as rhizobia. An infection of the soybean roots by rhizobia is preceded by a species-specific chemical communication and involves the exudation by the roots of a particular type of flavonoids, which generate chemotaxis and the release of lipo-chitooligosaccharids in rhizobia (Abdel-Lateif et al. 2012; Antolín-Llovera et al. 2012). More recently, Compton et al. (2020) challenged the role of flavonoids suggesting that other components released by the seed may be responsible for attraction. Generally, each legume species is only infected by a few species of rhizobia (Wang et al. 2019). In fact, only *Bradyrhizobium* and *Sinorhizobium* are publicly recognized as capable of establishing an effective symbiosis with soybean (Wang et al. 2019). Among *Bradyrhizobium* species, ten are reported as soybean-nodulating: *B. elkanii*, *B. japonicum*, *B. diazoefficiens*, *B. daqingense*, *B. liaoningense*, *B. huanghuaihaiense*, *B. ottawaense*, *B. brasiliense*, *B. shewense* and *B. ferriligni* (Amsalu et al. 2017; Costa et al. 2020; Wang et al. 2019; Yao et al. 2015).

✉ A. Rodríguez-Blanco
andrearb@fagro.edu.uy

[1] Depto. de Biología Vegetal, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay

[2] Bioinsumos, Instituto Nacional de Investigación Agropecuaria, Las Brujas, Uruguay

[3] Instituto de Ecología y Ciencias Ambientales, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay

In Uruguay, soybean accounts for more than half of the crop-cereal sowing area and is one of the most relevant products in export income (DIEA 2020; OPYPA 2020). In virtue of the environmental and economic benefits of inoculating with rhizobia, all soybean crops in our country are inoculated with a mix of *B. elkanii* U1301 (SEMIA587) and U1302 (SEMIA5019), which are recommended since 1984 by the Uruguayan Ministry of Livestock, Agriculture and Fisheries (*Ministerio de Agricultura y Pesca*, MGAP). It has been reported that rhizobia suffered a phenotypic and genotypic variation due to frequent multiplication in the laboratory and in the soil (Brunel et al. 1990; Flores et al. 1988). Provided that rhizobia persist in soils and that there is a high rate of genetic modification of the inoculated strains, new evolved strains should arise, which may be more adaptable to the local environment and likely to alter its symbiotic properties. Indeed, many authors have reported naturalized rhizobia with better symbiotic performance, isolated from the soil and nodules (Arsese et al. 2012; Chibeba et al. 2017; de Almeida Ribeiro et al. 2015). In addition, the naturalized rhizobia population could compete with inoculants in soybean nodulation, thus reducing its efficacy (Hungria et al. 1998; Iturralde et al. 2019).

Considering that Uruguay has been using soybean inoculants for many decades, the presence of naturalized soybean-nodulating rhizobia is highly possible. Thus, the objective of this study was to gain knowledge about the rhizobia population indigenous to Uruguay that had not been previously researched and is a key factor in soybean yield. More specifically, we aimed to characterize, both symbiotically and genetically, naturalized soybean-nodulating rhizobia from Uruguayan fields.

Material and methods

Rhizobial strains

Origin of commercial and naturalized rhizobial strains

Four commercial strains were used as a reference in the genetic characterization and symbiotic efficiency trial: *B. elkanii* strains U1301 (SEMIA587) and U1302 (SEMIA5019), recommended by MGAP for commercial soybean inoculants in Uruguay, *B. japonicum* strain E109 (SEMIA5085), used in commercial soybean inoculants in Argentina, and *B. diazoefficiens* SEMIA5080, used in Brazilian inoculants. These strains were obtained from the official collection of the Soil Microbiology Laboratory (*Laboratorio de Microbiología de Suelo*) of INIA Las Brujas.

Ten naturalized rhizobia strains were selected from a collection obtained by Lagurara (2018). Rhizobia were isolated from soybean nodules collected throughout six field experiments conducted in five Uruguayan departments, using soil either with or without soybean cultivation history and inoculated with reference strains U1301, U1302, SEMIA5080 and E109 (Table 1). Naturalized rhizobia were selected using PCR-BOX fingerprinting (Lagurara 2018). For this study, the isolates whose similarity to the reference strains (U1301, U1302, SEMIA5080 and E109) was less than 80% were considered as naturalized rhizobia. Ten new BOX-PCR profiles were identified and one isolate from each profile was chosen to continue its genetic characterization and symbiotic efficiency study (Table 1).

Growing conditions and storage of the rhizobia

Rhizobia strains were stored at −20 °C in 25% (v/v) gyicerol:TY at the Microbiology Laboratory (*Laboratorio de Microbiología*) of *Facultad de Agronomía* (*Universidad*

Table 1 Origin of naturalized rhizobia strains including site (with geographical coordinates), strain inoculated in the treatment plot or no inoculation, and whether it has previous history with soybean

Strain	Site	Treatment applied in the plot	Soybean history
UYS-CA01	Canelones (34° 40´S, 56° 23´W)	*B. elkanii* U1301	No
UYS-CA02	Canelones (34° 40´S, 56° 23´W)	No inoculation	Yes
UYS-CL01	Cerro Largo (32° 22´S, 54° 10´W)	*B. diazoefficiens* SEMIA5080	Yes
UYS-CL02	Cerro Largo (32° 22´S, 54° 10´W)	*B. elkanii* U1301 + U1302	Yes
UYS-CL03	Cerro Largo (32° 22´S, 54° 10´W)	*B. diazoefficiens* SEMIA5080	Yes
UYS-CO1	Colonia (33° 56´S, 57° 48´W)	*B. elkanii* U1301 + U1302	Yes
UYS-CO2	Colonia (33° 56´S, 57° 48´W)	*B. diazoefficiens* SEMIA5080	Yes
UYS-SJ01	San José (34° 43´S, 56° 42´W)	No inoculation	Yes
UYS-SO01	Soriano (33° 10´S, 57° 43´W)	No inoculation	Yes
UYS-SO02	Soriano (33° 10´S, 57° 43´W)	No inoculation	Yes

de la República, Uruguay). The strains were grown on yeast extract-mannitol agar (YEMA) medium and were incubated at 28 °C between 5 and 10 days until they reached sufficient colony growth levels to observe morphological features and purity. For the DNA extraction and the inoculum preparation, reference and naturalized strains were grown in TY broth (Howieson and Dilworth 2016) for 4 days at 28 °C under stirring at 120 rpm until reaching 10^7 cells mL^{-1}.

Genetic characterization

DNA extraction

DNA from four reference strains and ten naturalized strains was extracted using GeneJET Genomic DNA Purification Kit #K0721 (Thermo Scientific, USA). DNA purity was confirmed by electrophoresis in agarose 1% (w/v) gels and Tris/EDTA buffer (TE). Good View Nucleic Acid Stain (SBS Genentech, USA) was incorporated in gels to visualize fragments under UV light.

Housekeeping (16S rRNA, atpD, gyrB and rpoB) and symbiotic gene (nifH and nodY/K) sequencing

The amplification conditions of housekeeping (*16S rRNA*, *atpD*, *gyrB, rpoB, recA* and *dnaK*) and symbiotic genes *(nifH and nodY/K)* are shown in Supplementary Table S1 (Arsese et al. 2012; Lane 1991; Martens et al. 2008; Rivas et al. 2009; Stepkowski et al 2005). PCR products were purified using GeneJET PCR Purification Kit #K0701 (Thermo Scientific, USA), except for the *nodY/K* gen, which was purified using Monarch DNA Gel Extraction Kit #T1020S (Biolabs, USA). Gene purity was confirmed by electrophoresis in agarose 1% (w/v) gels and Tris/EDTA buffer (TE). Good view Nucleic Acid Stain (SBS Genentech, USA) was incorporated in gels to visualize fragments under UV light.

Genes were sequenced by Macrogen Inc. (Seoul, South Korea) using Capillary Electrophoresis Sequencing. The sequences obtained were deposited in *GenBank* under the following accession numbers: MT859674-MT859687 (*16S rRNA*), MT904311-MT904324 (*gyrB*) MT904325-MT904338 (*rpoB*), ON262778 (*recA*), ON262777 (*dnaK*) and MT904339-MT904352 (*atpD*), MT904353-MT904366 (*nifH*) and MT904367-MT904379 (*nodY/K*).

Phylogenetic analysis

The phylogenetic analysis of our sequences and the sequences available in GenBank (Supplementary Tables S2 and S3) were performed using MEGA-X v10 software. Sequences from several *Bradyrhizobium* species reported as Soybean-nodulating or not, were included, such as *B. elkanii*, *B. japonicum* and *B. yuanmingense*. Soybean-nodulating *Sinorhizobium fredii* LMG 6218^T was also used as an outgroup for housekeeping genes. ClustalW was used for alignments, and phylogenetic trees were built with Maximum-Likelihood. Statistical using the Tamura-Nei model, support for tree nodes was evaluated by bootstrap analyses using 1000 replicates.

Symbiotic performance test

Seed preparation, trial management and experimental design

The ten naturalized rhizobial strains were evaluated for symbiotic efficiency in soybean in Leonard jars (Vincent 1970). The experiment was carried out in a greenhouse with a period of 12 h of light at 28 °C for 30 days in a completely randomized design including fifteen treatments with five replicates each. Soybean (*Glycine max.* L. [Merr.]) seeds of the Nidera 5909 variety were used. Seeds were surface-disinfected in ethanol (70%) for 1 min, then in sodium hypochlorite (2%) for 2 min and finally washed three times with sterile distilled water. Disinfected seeds were pre-germinated in a Petri dish containing 0.6% agar and incubated at 28 °C for 48 h. After germination, two seedlings were transplanted into sterilized Leonard jars, with sterilized sand in the upper part and supplemented with sterilized Broughton and Dilworth´s N-free nutrient solution (Somasegaran and Hoben 1994) in the lower part. Plants were thinned five days after sowing (DAS), leaving one plant per jar.

Five DAS, the inoculated treatments received 1 mL of a bacterial culture containing 10^7 CFU mL^{-1}. The ten naturalized rhizobia were compared with three positive control treatments, inoculated separately with the reference strains *Bradyrhizobium elkanii* (U1301 and U1302 1:1), *B. japonicum* (E109), and *B. diazoefficiens* (SEMIA 5080), and two negative control treatments without inoculation, with or without N fertilization.

During the experiment, the contents of the jars were periodically completed with a sterilized nutrient solution according to the uptake rate of the plants. A sterilized Broughton and Dilworth´s N-free mineral solution was used to refill the Leonard jars of the inoculated treatments and the control treatment without inoculation, whereas the solution for the N-fertilized treatment included ammonium nitrate (0.35 mM). Soybean plants were collected 30 DAS.

Determination of symbiotic efficiency and statistical analysis

Red and well-developed nodules per plant were counted to determine the number of nodules (NN). Nodules, shoots and roots were dried at 65 °C and then weighed to determine nodules dry weight (NDW), shoots dry weight (SDW) and

roots dry weight (RDW). Dried shoots were sent to the Animal Nutrition Laboratory (*Laboratorio de Nutrición Animal*, *Facultad de Agronomía*) to analyze total nitrogen in shoots (TNS).

Infostat software (Di Rienzo et al. 2018) and a threshold of $p < 0.05$ were used for all statistical analyses. Normality and homoscedasticity were verified with Shapiro–Wilk and Levene tests, respectively. An analysis of variance (ANOVA) followed by a Scott-Knoch test was used for identifying mean differences. A principal component analysis was performed to integrate NN, NDW, SDW and TNS.

Results

Genetic characterization

Phylogenetic trees for housekeeping genes 16S *rRNA* (799 bp), *gyrB* (568 bp), *rpoB* (422 bp) and *atpD* (432 bp) are available in the Supplementary material (Figs. S1–S4). The topology and resolution of 16S *rRNA* and *rpoB* phylogenetic trees were similar: four naturalized strains clustered with *B. japonicum* reference strains and type strains, whereas the other six were in a cluster containing *B. elkanii* reference strains and type strains from various species (*B. elkanii*, *B. brasiliense*, *B. pachyrizi* and *B. ferriligni*). On the other hand, the *gyrB* and *atpD* analysis distinguishes between those species, having one cluster for *B. elkanii* including five naturalized strains, and UYS-CA02 clustered with *Bradyrhizobium* sp. UFLA06-06 (as reported by de Almeida Ribeiro et al. 2015) and a *B. ferriligni* type strain, the latter only in *gyrB* phylogeny, since there are no *atpD* sequences available for *B. ferriligni* strains.

To achieve a more robust result, a multilocus sequence analysis (*MLSA*) was performed by concatenating four housekeeping genes sequences (16S *rRNA*, *gyrB*, *rpoB* and *atpD*) with a total of 2251 bp. The phylogenetic tree obtained is shown in Fig. 1. This phylogenetic tree showed higher bootstrap values than the separate housekeeping genes, although its tree topology was similar to the *gyrB* and *atpD* trees. Naturalized rhizobia were placed in three different clusters: five naturalized strains integrated the same cluster as the *B. elkanii* strains, four naturalized strains were combined with *B. japonicum* strains, and UYS-CA02 was clustered with *Bradyrhizobium sp.* UFLA06-06. The nucleotide identity of *MLSA* for naturalized rhizobia (UYS-CL01, UYS-CL02, UYS-CA01, UYS-CO01 and UYS-SO01) in the first group and *B. elkanii* U1301 and U1302 vary between 99.73 and 99.91%. In the second group, naturalized rhizobia (UYS-CL03, UYS-CO02, UYS-SJ01 and UYS-SO02) showed a greater similarity to *B. japonicum* LMG 6138 ^{T} (nucleotide identity 99.24–99.28%) than *B. japonicum* E109 (98.26–98.97%). On the other hand, UYS-CA02 was 97.57% similar to *Bradyrhizobium sp.* UFLA06-06. Since *B. ferriligni* CCBAU 51502 ^{T} could not be included in this *MLSA* due to the lack of its *atpD* sequences, another *MLSA* was constructed with available gene sequences from 16S *rRNA*, *gyrB*, *rpoB, dnaK* and *recA* (2383 bp) for *B. ferriligni* CCBAU 51502 ^{T}, *Bradyrhizobium sp.* UFLA06-06 and other type sequences of the *Bradyrhizobium* species and the ones obtained for UYS-CA02 (Fig. 2). This analysis showed nucleotide identity of 97.04% between *B. ferriligni* CCBAU 51502 ^{T} and UYS-CA02, 97.43% between *B. ferriligni* CCBAU 51502 ^{T} and *Bradyrhizobium sp.* UFLA06-06, and 98.8% between *Bradyrhizobium sp.* UFLA06-06 and UYS-CA02. Considering that UYS-CA02 is close to *Bradyrhizobium sp.* UFLA06-06, and both are also close to *B. ferriligni* CCBAU 51502 ^{T} in the second *MLSA*, chances are that UYS-CA02 and *Bradyrhizobium sp.* UFLA06-06 may belong to the same species, which could be *Bradyrhizobium ferriligni*.

Symbiotic genes were also studied and trees were obtained for *nifH* (601 bp) and *nodY/K* (315 bp), as shown in Figs. 3 and 4, respectively. Two main groups can be distinguished in both trees: the first cluster (I) contains *B. elkanii* U1301, *B. elkanii* U1302, the five naturalized rhizobia clustered with *B. elkanii* in *MLSA* (UYS-CA01, UYS-CL01, UYS-CO01, UYS-CL02 and UYS-SO01), and UYS-CA02; the other cluster (II) includes *B. japonicum* E109, *B. diazoefficiens* SEMIA5080 and naturalized rhizobia similar to *B. japonicum* strains in *MLSA* (UYS-CO02, UYS-SJ01, UYS-SO02 and UYS-CL03 [*nodY/K* of the latter could not be amplified]). Cluster I had a nucleotide identity of 100% for both genes among their sequences. On the other hand, nucleotide identity between UYS-CA02 and *B. ferriligni* CCBAU 51502 ^{T} was 97.50% for *nifH* (there was no *nodY/K* sequence available for *B. ferriligni* CCBAU 51502 ^{T}). In cluster II, nucleotide identity was 100% between strains for *nifH*. For the *nodY/K* gene, *B. japonicum* E109, *B. diazoefficiens* SEMIA5080 and UYS-SO02 had 100% of nucleotide identity, whereas UYS-SJ01 and UYS-CO02 had 99.67% of nucleotide identity with *B. japonicum* E109.

Symbiotic efficiency

Nodulation, biomass and nitrogen trial results are shown in Table 2. The highest NN and NDW values were obtained from treatments inoculated with a mix of *B. elkanii* U1301:U1302 (1:1), a commercial strain used in Uruguay, and inoculated separately with UYS-CA02, UYS-SO01, UYS-CO01, UYS-CL01, UYS-CA01 and UYS-CL02. In the case of SDW and TNS, the treatment fertilized with nitrogen had the best performance. However, among inoculated treatments plants inoculated with *B. elkanii* U1301:U1302 (1:1) produced similar SDW and TNS than those inoculated with four other naturalized strains (UYS-CO01, UYS-CL01,

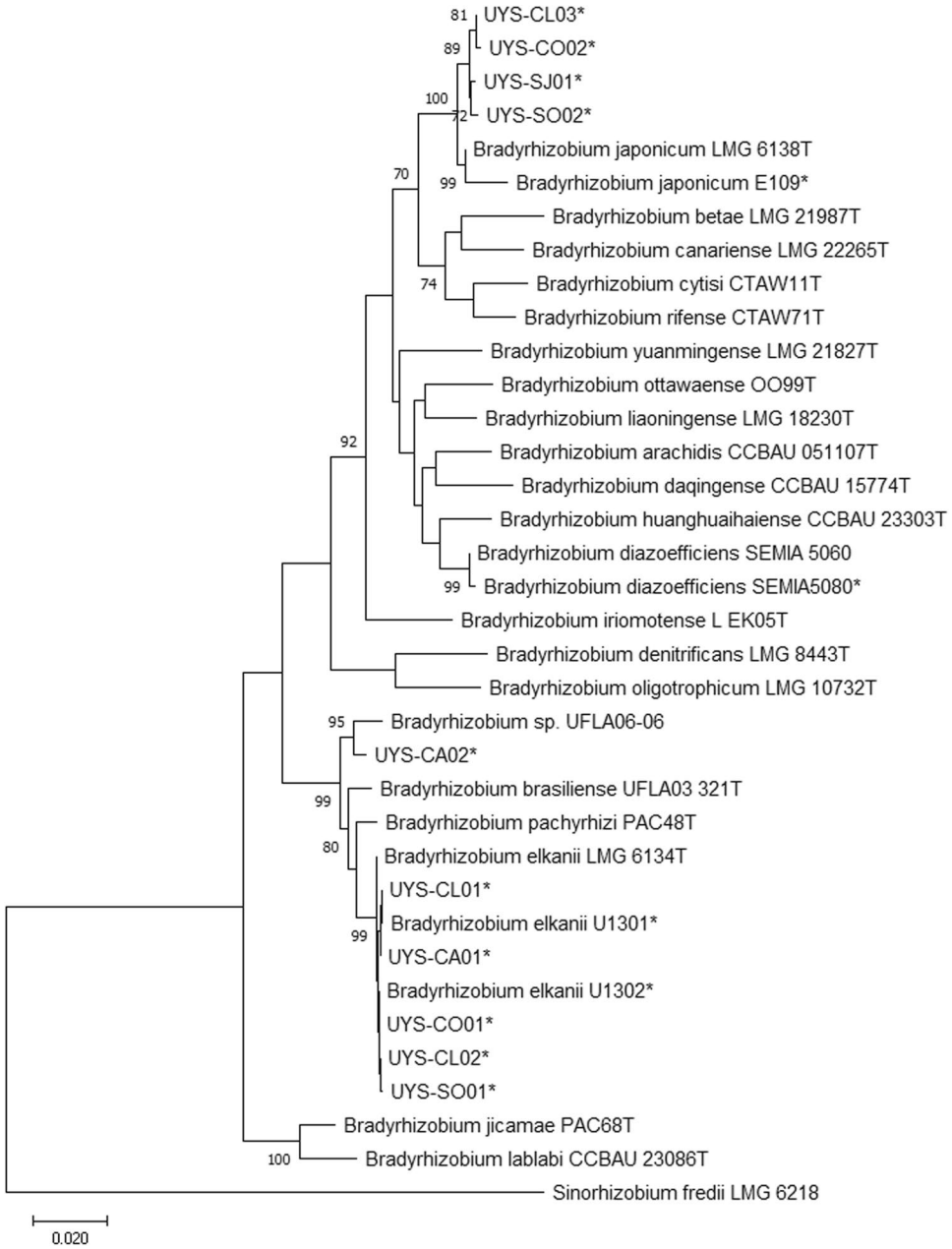

Fig. 1 Maximum likelihood phylogenetic tree based on the concatenated sequence data for housekeeping genes (16S *rRNA*, *gyrB*, *rpoB* and *atpD*) of soybean-nodulating rhizobia. Data from fourteen strains (indicated with an asterisk) were obtained: four commercial strains (U1301, U1302, SEMIA5080 and E109) and ten naturalized strains. Type strains from the *Bradyrhizobium* species were included, as well as *Sinorhizobium fredii* LMG 6218 ^{T} as an outgroup. Bootstrap values higher than 70, based on 1000 trials, are indicated at the nodes. Mega-X was used for this analysis

UYS-CA01 and UYS-CL02), which were significantly higher than treatments inoculated with *B. japonicum* E109, *B. diazoefficiens* SEMIA5080, UYS-SO02, UYS-CL03 and UYS-CO02.

To visualize all the variables in the same plot, a principal component analysis (PCA) was performed (Fig. 5). The PCA was able to represent 98.5% of the variability and show two different groups of treatments. The mix of *B. elkanii* U1301:U1302, UYS-CA01, UYS-CL01, UYS-CO01, UYS-CA02, UYS-SO01 and UYS-CL02 appear on the right side of CP1, producing a better symbiotic performance, and the treatments inoculated with *B. japonicum* E109, *B. diazoefficiens* SEMIA5080, UYS-SO02, UYS-CO02, UYS-CL03 and UYS-SJ01 appear on the left side. In summary, the PCA showed a correlation with clusters formed in the trees of symbiotic genes (*nifH* and *nodY/K*).

Discussion

Genetic characterization

While the 16S *rRNA* gene is widely used as a molecular marker in prokaryotes taxonomy (Howieson and Dilworth 2016), our results confirm that it fails in distinguishing between *Bradyrhizobium* species, in agreement with

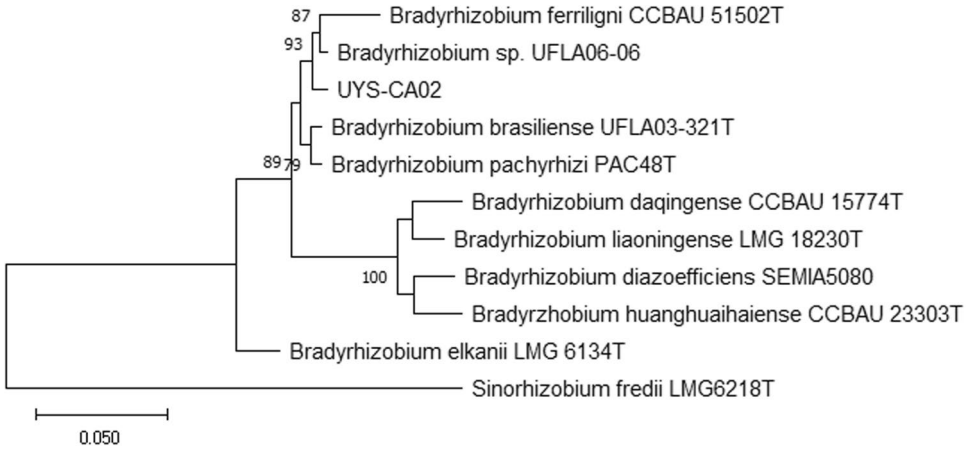

Fig.2 Maximum likelihood phylogenetic tree based on the concatenated sequence data for housekeeping genes (16S *rRNA*, *gyrB*, *rpoB*, *dnaK* and *recA*) of soybean-nodulating rhizobia. Data from naturalized strain UYS-CA02 (indicated with an asterisk) was obtained. Type strains from *Bradyrhizobium* species were included, as well as *Sinorhizobium fredii* LMG 6218 ^{T} as an outgroup. Bootstrap values higher than 70, based on 1000 trials, are indicated at the nodes. Mega-X was used for this analysis

previous reports by Delamuta et al. (2012) and Menna et al. (2009). In contrast, *gyrB* and *atpD* genes were more sensitive than 16S *rRNS* and *rpoB*, in line with results reported by de Almeida Ribeiro et al. (2015). Indeed, the *gyrB* and *atpD* phylogenetic trees succeed in separating *B. elkanii* from other similar species (*B. ferriligni*, *B. brasiliense* and *B. pachyrizi*). *MLSA* had the same topology as the *atpD* gene, but it presented a more robust result, which makes it a useful tool to identify *Bradyrhizobium* species (Arsese et al. 2012; Chibeba et al. 2017; de Almeida Ribero et al. 2015; Delamuta et al. 2017;).

The *MLSA* allowed us to identify five naturalized strains as belonging to *B. elkanii* and four to *B. japonicum*. These species are the ones most reported worldwide as naturalized strains isolated from soybean nodules (Arsese et al. 2012; Chibeba et al. 2017; de Almeida Ribeiro et al. 2015; Helene et al. 2020; Iturralde et al. 2019; Mason et al. 2017), probably because strains of these species are used as soybean inoculants (Hungria et al. 2005). As for the other naturalized strain (UYS-CA02) and another soybean-nodulating strain isolated by de Almeida Ribero et al. (2015) (*Bradyrhizobium sp.* UFLA06-06), we suggest that they may belong to *B. ferriligni*, due to similarities found in the second *MLSA* analysis. This would be the first report of rhizobia belonging to *B. ferriligni* isolated from soybean nodules, although *B. ferriligni* CCBAU 51502 ^{T} isolated from nodules of *Erythriphleum fordii* has been reported as capable of nodulating *Glycine max* (Yao et al. 2015). Considering that soybean is a promiscuous plant that nodulates with a wide variety of species such as *B. elkanii*, *B. japonicum*, *B. diazoefficiens*, *B. daqingense*, *B. liaoningense*, *B. huanghuaihaiense* and *B. ottawaense* (Wang et al. 2019), nodulation by new species is expected to occur. For instance, Costa et al. (2020) reported new strains isolated from soybean nodules belonging to *B. brasiliense* (symbiovar Sojae), although *B. brasiliense* type strains were isolated from nodules of *Vigna unguiculata* (Costa et al. 2017).

In addition, the *nifH* sequence of *Bradyrhizobium sp.* UYS-CA02 was identical to that of the *B. elkanii* type and naturalized strains identified by the *MLSA,* while *B. ferriligni* CCBAU 51502 ^{T} was in the same cluster with only a few differences in its sequence. On the other hand, *B. japonicum*-like and type strains were grouped in a different cluster, including *B. diazoefficiens* strains. A similar phylogeny was obtained for the *nodY/K* tree. Many authors have reported that nodulation and fixation genes present identical taxonomy (Arsese et al. 2012; Delamuta et al. 2017; Mason et al. 2017; Tartaglia et al. 2019). Our results suggest a monophyletic origin of symbiotic genes for *B. elkanii*- and *B. japonicum*-like strains, while UYS-CA02 or its ancestor may have received symbiotic genes from *B. elkanii* strains by horizontal transfer (Arsese et al. 2012; Menna and Hungria 2011). Symbiotic genes are usually located in symbiotic islands that have a great number of insertions and a high horizontal transfer rate (Kaneko et al. 2011).

Symbiotic efficiency

In light of our results, the mix of *B. elkanii* U1301 (SEMIA587) and U1302 (SEMIA5019) strains that are recommended for soybean inoculants in Uruguay by MGAP had a better symbiotic performance in axenic conditions than the other evaluated commercial strains (*B. japonicum* E109 and *B. diazoefficiens* SEMIA5080). Although this result is consistent with de Paiva Barbosa et al. (2017) and Okito et al. (2004), who evaluated soybean plant in axenic

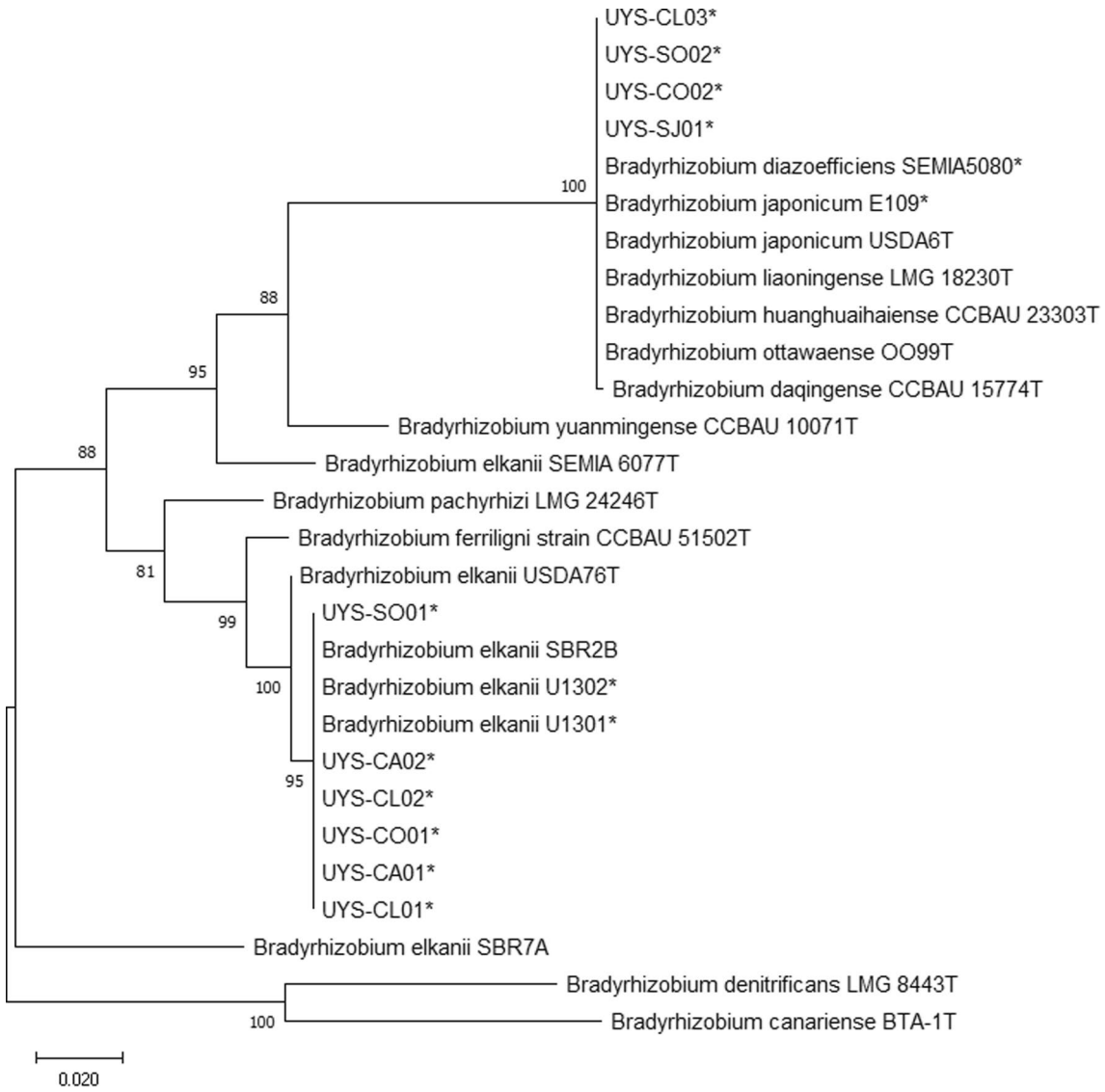

Fig. 3 Maximum likelihood phylogenetic tree based on a *nifH* sequence of soybean-nodulating rhizobia. Data from fourteen strains (indicated with an asterisk) were obtained: four commercial strains (U1301, U1302, SEMIA5080 and E109) and ten naturalized strains. Different strains from *Bradyrhizobium* species were included. Bootstrap values higher than 70, based on 1000 trials, are indicated at the nodes. Mega-X was used for this analysis

conditions inoculated with *B. diazoefficiens* SEMIA5080 and *B. elkanii* U1302 (the former also included *B. elkanii* U1301), it differs from the results of Chibeba et al. (2017), who reported a higher shoot biomass for plants inoculated with *B. diazoefficiens* SEMIA5080 than those inoculated with *B. elkanii* U1301 and U1302. Although all these trials were performed in similar and controlled conditions, the differences between results could be attributed to the different soybean varieties (Chibeba et al. 2017) or to genetic modification during trials (Ribeiro Torres and Kaschuk 2012).

In addition to the evaluated biomass and nodulation parameters, PCA appears as an efficient tool for visualizing and integrating all the parameters more simply (Chibeba et al. 2017). Indeed, this shows a better performance of *B. elkanii*-like naturalized strains, including *Bradyrhizobium sp.* UYS-CA02, beyond *B. japonicum*-like naturalized strains and *B. diazoefficiens* SEMIA5080. This result is consistent with clusters formed in the phylogenetic trees of symbiotic genes. On the other hand, Hungria et al. (1998) evaluated a great number of *B. elkanii* and *B. japonicum* commercial and naturalized strains in axenic conditions. Their results show a better symbiotic performance in soybean plants for *B. japonicum* strains than for those of *B. elkanii*; as noted above, differences in varieties or genetic modifications may account for the contradictions with our results. Furthermore, the high similarity in genetic characterization between

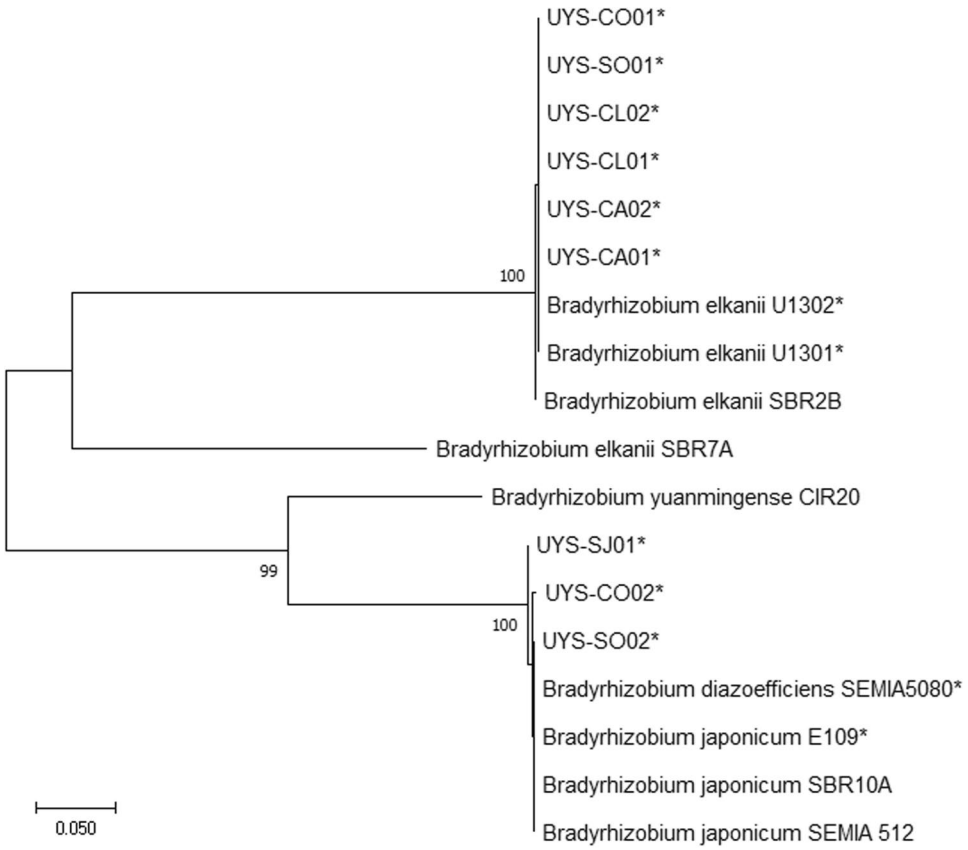

Fig. 4 Maximum likelihood phylogenetic tree based on the intergenic region of a *nodY/K* sequence of soybean-nodulating rhizobia. Data from 13 strains (indicated with an asterisk) were obtained: four commercial strains (U1301, U1302, SEMIA5080 and E109) and nine naturalized strains. Different strains of the *Bradyrhizobium* species were included. Bootstrap values higher than 70, based on 1000 trials, are indicated at the nodes. Mega-X was used for this analysis

Table 2 Number of nodules (NN, no. plant^{-1}), nodule dry weight (NDW, g plant^{-1}), shoot dry weight (SDW, g plant^{-1}) and total nitrogen in shoots (mg plant^{-1}) of soybean in a Leonard jar trial

Treatment	NN (plant^{-1})	NDW (g plant^{-1})	SDW (g plant^{-1})	TNS (mg plant^{-1})
B. diazoefficiens SEMIA5080	50,80 A	0,13 A	0.91 A	155 A
B. japonicum E109	52,20 A	0,12 A	0.99 A	204 A
B. japonicum UYS-CL03*	47,20 A	0,14 A	1.,08 A	214 A
B. japonicum UYS-CO02*	38,00 A	0,10 A	1,05 A	159 A
B. japoicum UYS-SO02*	34,20 A	0,09 A	0.90 A	100 A
B. japonicum UYS-SJ01*	54,00 A	0,16 A	1.15 A	273 A
Bradyrhizobium sp. UYS-CA02*	71,50 B	0,21 B	1,28 A	304 B
B. elkanii UYS-SO01*	79,20 B	0,22 B	1.31 A	338 B
B. elkanii UYS-CO01*	66,80 B	0,21 B	1.40 B	345 B
B. elkanii UYS-CL01*	71,40 B	0,21 B	1.57 B	360 B
B. elkanii UYS-CA01*	81,20 B	0,20 B	1.77 B	394 B
B. elkanii UYS-CL02*	82,20 B	0,25 B	1.58 B	402 B
B. elkanii U1301:U1302 (1:1)	93,75 B	0,26 B	1.89 B	449 B
N-fertilized	–	–	4.32 C	1485 C
Not-inoculated	–	–	0.95 A	55 A

Treatments were inoculated with commercial strains (*B. diazoefficiens* SEMIA5080, *B. japonicum* E109 and a blend of *B. elkanii* U1301: U1302 [1:1]) and naturalized rhizobia isolated from Uruguayan soils (*), including N-fertilized and not-inoculated controls. The trial was conducted under greenhouse conditions and plants were harvested 28 days after inoculation

Average of five replicates; same letter indicates no significant difference according to the Scott–Knott test at a significance of $p < 0.05$

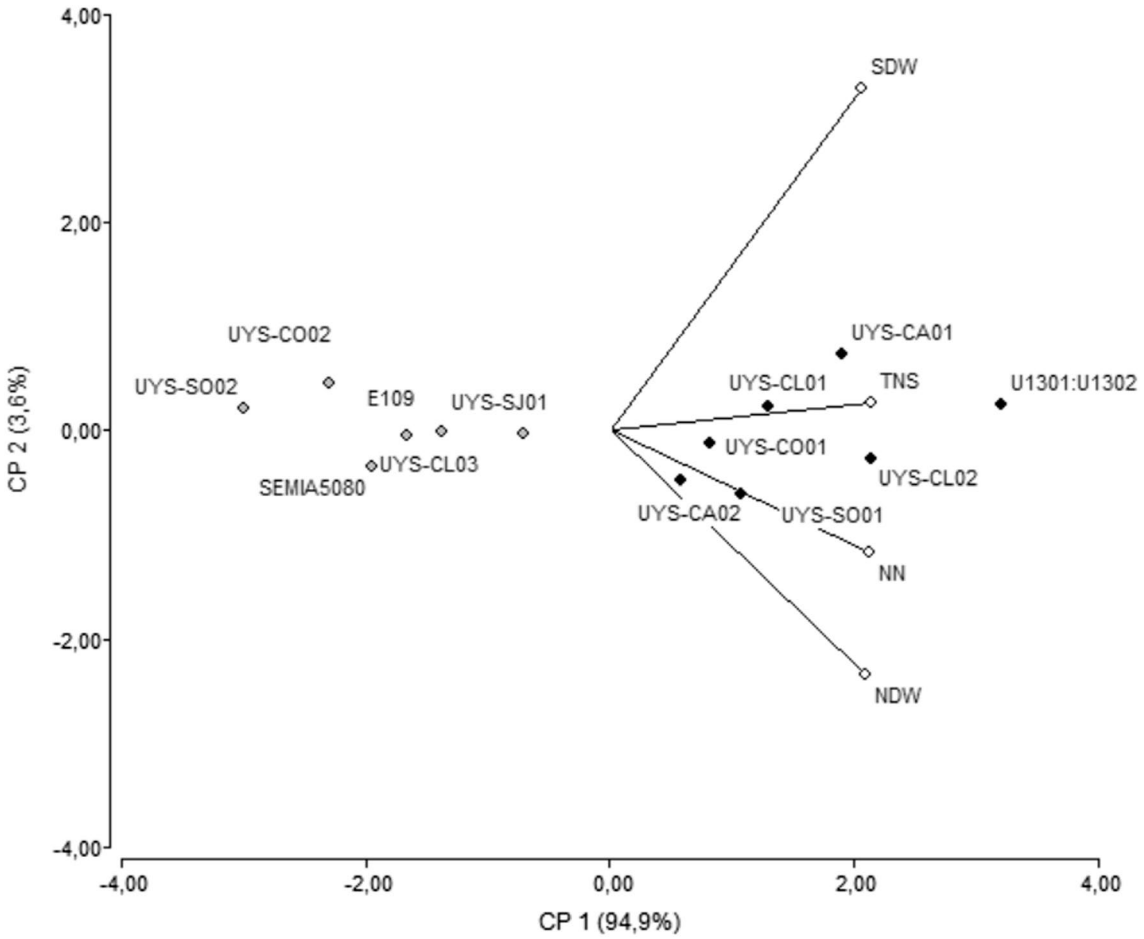

Fig. 5 Principal component analysis of symbiotic parameters including the number of nodules (NN), nodule dry weight (NDW), shoot dry weight (SDW) and total nitrogen in shoots (TNS). Points for treatments inoculated with *B. elkanii* or *Bradyrhizobium sp.* UYS-CA02 are shown in black, and those inoculated with *B. japonicum* or *B. diazzoefficens* in gray

commercial and naturalized strains suggests that the latter may stem from inoculants applied for many years, which is supported by a similar symbiotic performance (Ribeiro Torres and Kaschuck 2012). Finally, we highlight the considerably good symbiotic performance of *B. elkanii*-like naturalized strains that were as efficient as the commercial inoculants used in Uruguay. Other authors have reported naturalized rhizobia with the same or even better results than commercial strains (Chibeba et al. 2017; Iturralde et al. 2019), which encourages research on naturalized strains as an alternative to the inoculants currently in use. Considering that the capacity of rhizobia to promote plant growth is associated to competitiveness and environmental adaptation, symbiotic efficiency should be tested in soil assays, including competence performance, as well as studying the tolerance to abiotic factors such as temperature, salinity and pH with in-vitro assays (Chibeba et al. 2017; Leite et al. 2018). This information may contribute to the characterization of these native strains, and it represents a possible strategy for increasing soybean yields since naturalized rhizobia can be adapted to local conditions.

Conclusions

Using *MLSA*, we were able to identify five likely naturalized strains belonging to the *B. elkanii* species and four to the *B. japonicum* species. The other naturalized strain is phylogenetically related to *B. ferriligni* and may belong to that species, while having received symbiotic genes from *B. elkanii* strains by horizontal transfer. In addition, symbiotic genes show two main clusters that were correlated with nodulation and nitrogen fixation in soybean. It is also worth noting that *B. elkanii* likely naturalized strains may have derived from commercial inoculant used in Uruguay due to their high identity in housekeeping and symbiotic genes, and also a similar symbiotic performance in axenic conditions. Differences among strains can be associated with the adaptation

to edaphic environments, some of them are being addressed by our group in new studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42398-022-00258-1.

Acknowledgements We thank ANII (Agencia Nacional de Investigación e Innovación) for financial support through a postgraduate grant (POS_NAC_2017_1_141269) and a project financed by "Fondo María Viñas" (FMV_1_2014_1_104723). We are also grateful to the staff of the Soil Laboratory of INIA Las Brujas for their collaboration during the Leonard jars assay.

Data availability statement Sequences generated from this study are available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/. Other raw data are available upon request through the corresponding author.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

Abdel-Lateif K, Bogusz D, Hocher V (2012) The role of flavonoids in the establishment of plant roots endosymbioses with arbuscular mycorrhiza fungi, rhizobia and *Frankia* bacteria. Plant Signaling Behav 7:636–641. https://doi.org/10.4161/psb.20039

Amsalu AA, Woyke T, Kyrpides NC, Whitman WB, Lindström K (2017) Draft genome sequences of *Bradyrhizobium shewense sp.* nov. ERR11^{T} and *Bradyrhizobium yuanmingense* CCBAU 10071^{T}. Stand Genomic Sci 5:12–74. https://doi.org/10.1186/s40793-017-0283-x

Antolín-Llovera M, Ried MK, Binder A, Parniske M (2012) Receptor kinase signaling pathways in plant-microbe interactions. Annu Rev Phytopathol 50:451–473. https://doi.org/10.1146/annurev-phyto-081211-173002

Arsese AA, Räsänen LA, Aseffa F, Hailemariam A, Lindström K (2012) Phylogenetically diverse groups of *Bradyrhizobium* isolated from nodules of *Crotalaria spp*., *Indigosfera Spp.*, *Erythrina brucei* and *Glycine max* growing in Ethiopia. Mol Phylogenetics Evol 65:595–609. https://doi.org/10.1016/j.ympev.2012.07.008

Brunel B, Boeufgras JM, Bernillon D, Bardin R (1990) Phenotypic drift in *Bradyrhizbium japonicum* populations after introduction into soils as established by numerical analysis. Microb Ecol 19:163–170. http://www.jstor.org/stable/4251111.

Chibeba AM, Kyei-Boahen S, Guimarães MF, Nogueira MA, Hungria M (2017) Isolation, characterization and selection of indigenous *Bradyrhizobium* strains with outstanding symbiotic performance to increase soybean yields in Mozambique. Agric Ecosyst Environ 246:291–305. https://doi.org/10.1016/j.agee.2017.06.017

Compton KK, Hildreth SB, Helm RF, Scharf BE (2020) An Updated Perspective on *Sinorhizobium meliloti* Chemotaxis to Alfalfa Flavonoids. Front Microbiol 11:581482. https://doi.org/10.3389/fmicb.2020.581482

Costa ME, Azarias Guimarães A, Pereira Vicentin R, de Almeida Ribeiro PR, Ribas Leão AC, Balsanelli E, Lebbe L, Aerts M, Willems A, de Souza Moreira FM (2017) *Bradyrhizobium brasiliense sp*. nov., a symbiotic nitrogen-fixing bacterium isolated from Brazilian tropical soils. Arch Microbiol 199:1211–1221. https://doi.org/10.1007/s00203-017-1390-1

Costa ME, de Almeida Ribeiro PR, Soares de Carvalho T, Pereira Vicentin R, Balsanelli E, Maltempi de Souza E, Lebbe L, Willems A, de Souza Moreira FM (2020) Efficient nitrogen-fixing bacteria isolated from soybean nodules in the semi-arid region of Northeast Brazil are classified as *Bradyrhizobium brasilense* (Symbiovar Sojae). Curr Microbiol 77:1746–1755. https://doi.org/10.1007/s00284-020-01993-6

De Almeida Ribeiro PR, dos Santos JV, Costa E, Lebbe L, Silva Assis E, Oliveira Louzada M, Azarias Guimarães A, Willems A, de Souza Moreira FM (2015) Symbiotic efficiency and genetic diversity of soybean bradyrhizobia in Brazilian soils. Agric Ecosyst Environ 212:85–93. https://doi.org/10.1016/j.agee.2015.06.017

De Paiva Barbosa L, Freitas Costa P, de Almeida Ribeiro PR, Rufini M, Guimarães AA, de Souza Moreira FM (2017) Symbiotic Efficiency and Genotypic Characterization of Variants of *Bradyrhizobium spp.* In Commercial Inoculants for Soybeans. Rev Bras Ciênc Solo 41:e0160572. https://doi.org/10.1590/18069657rbcs20160572.

Delamuta JRM, Ribeiro RA, Menna P, Villamil Bangel E, Hungria M (2012) Multilocus sequence analysis (*MLSA*) of *Bradyrhizobium* strains: Revealing high diversity of tropical diazotrophic symbiotic bacteria. Braz J Microbiol 43:698–710. https://doi.org/10.1590/S1517-83822012000200035

Delamuta JRM, Menna P, Ribeiro RA, Hungria MA (2017) Phylogenies of symbiotic genes of *Bradyrhizobium* symbionts of legumes of economic and environmental importance in Brazil support the definition of the new symbiovars pachyrhizi and sojae. Syst Appl Microbiol 40:254–265. https://doi.org/10.1016/j.syapm.2017.04.005

DIEA (Dirección de Estadísticas Agropecuarias) (2020) https://descargas.mgap.gub.uy/DIEA/Anuarios/Anuario2019/Anuario2019.pdf (accessed 11 Feb 2022).

Fahräeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 16:374–438. https://doi.org/10.1099/00221287-16-2-374

Flores M, González V, Pardo MA, Leija A, Martínez E, Romero D, Pinero D, Davila G, Palacios R (1988) Genomic inestability in *Rhizobium phaseoli*. J Bacteriol 170:1191–1196. https://doi.org/10.1128/jb.170.3.1191-1196.1988

Helene LCF, O'Hara G, Hungria M (2020) Characterization of *Bradyrhizobium* strains indigenous to Western Australia and South Africa indicates remarkable genetic diversity and reveals putative new species. Syst Appl Microbiol 43:126053. https://doi.org/10.1016/j.syapm.2020.126053

Howieson JG, Dilworth MJ (Eds.) (2016) Working with rhizobia. ACIAR (Australian Center for International Agricultural Research), Canberra.

Hungria M, Boddey LH, Santos MA, Vargas MAT (1998) Nitrogen fixation capacity and nodule occupancy by *Bradyrhizobium japonicum* and *B. elkanii* strains. Biol Fertil Soils 27:393–399

Hungria M, Franchini JC, Campo RJ, Graham PH (2005) The importance of Nitrogen Fixation to Soybean Croping in South America. In: Werner D, Newton WE (eds) Nitrogen Fixation in Agriculture Forestry, Ecology, and the Environment. Springer, Dordrecht, pp 25–42. https://doi.org/10.1007/s003740050449

Iturralde ET, Covelli JM, Alvarez F, Pérez-Giménez J, Arrese-Igor C, Lodeiro AR (2019) Soybean-Nodulating Strains With Low Intrinsic Competitiveness for Nodulation, Good Symbiotic Performance, and Stress-Tolerance Isolated From Soybean-Cropped Soils in Argentina. Front Microbiol 10:1061. https://doi.org/10.3389/fmicb.2019.01061

Kaneko T, Maita H, Hirakawa H, Uchiike N, Minamisawa K, Watanabe A, Sato S (2011) Complete genome sequence of the soybean symbiont *Bradyrhizobium japonicum* strain USDA6^{T}. Genes 2:763–787. https://doi.org/10.3390/genes2040763

Lagurara PF (2018) Eficiencia simbiótica y capacidad competitiva de cepas de rizobios que nodulan soja en suelos con y sin historia

del cultivo. Academic Thesis, Montevideo, Uruguay. Facultad de Agronomía. 117 p.

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics, 1st edn. John Wiley & Sons Inc, New York, pp 115–175

Leite J, Ribeiro Passos S, Simões-Araújo JL, Gouvêa Rumjnek N, Ribeiro Xavier G, Édson Zilli J (2018) Genomic identification and characterization of the elite strains *Bradyrhizobium yuanmingense* BR 3267 and *Bradyrhizobium pachyrizi* BR 3262 recommended for cowpea inoculation in Brazil. BJM 49:703–713. https://doi.org/10.1016/j.bjm.2017.01.007

Martens M, Dawyndt P, Coopman R, Gillis M, De Vos P, Willems A (2008) Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus Ensifer (including former Sinorhizobium). Int J Syst Evol Microbiol 58:200–214. https://doi.org/10.1099/ijs.0.65392-0

Mason MLT, Matsuura S, Domingo AL, Yamamoto A, Shiro S, Sameshima-Saito R, Saeki Y (2017) Genetic diversity of indigenous soybean-nodulating *Bradyrhizobium elkanii* from southern Japan and Nueva Ecija, Philippines. Plant Soil 417:349–362. https://doi.org/10.1007/s11104-017-3263-4

Menna P, Hungria M (2011) Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. Int J Syst Evol Microbiol 61:3052–3067. https://doi.org/10.1099/ijs.0.028803-0

Menna P, Alves Pereira A, Villamil Bangel E, Hungria M (2009) rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool. Symbiosis 48:120–130. https://doi.org/10.1007/BF03179991

Okito A, Alves BRJ, Urquiaga S, Boddey RM (2004) Isotopic fractionation during N_2 fixation by four tropical legumes. Soil Biol Biochem 36:1179–1190. https://doi.org/10.1016/j.soilbio.2004.03.004

OPYPA (Oficina de Programación y Política Agropecuaria) (2020) https://descargas.mgap.gub.uy/OPYPA/Anuarios/anuario2020/anuario2020.pdf (acceded 11 February 2022).

Ribeiro Torres A, Kaschuk G (2012) Genetic variability in *Bradyrhizobium japonicum* strains nodulating soybean [*Glycine max* (L.) Merrill]. World J Microbiol Biotechnol 28:1831–1835. https://doi.org/10.1007/s11274-011-0964-3

Rivas R, Martens M, de Lajudie P, Willems A (2009) Multilocus sequence analysis of the genus Bradyrhizobium. Syst Appl Microbiol 32:101–110. https://doi.org/10.1016/j.syapm.2008.12.005

Salvagiotti F, Cassman KG, Specht JE, Walters DT, Weiss A, Dobermann A (2008) Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. Field Crops Res 108:1–13. https://doi.org/10.1016/j.fcr.2008.03.001

Somasegaran P, Hoben HJ (1994) Handbook for rhizobia: methods in legume–Rhizobium technology. Springer Verlag, New York

Stein LY, Klotz MG (2016) The nitrogen cycle. Curr Biol 26:R94–R98. https://doi.org/10.1016/j.cub.2015.12.02

Stepkowski T, Moulin L, Krzyzanska A, Mcinnes A, Law IJ, Howieson J (2005) European origin of Bradyrhizobium populations infecting lupins and serradella in soils of Western Australia and South Africa. Appl Environ Microbiol 71:7041–7052. https://doi.org/10.1016/10.1128/AEM.71.11.7041-7052.2005

Versalovic J, Schneider M, de Brujin FJ, Lupski JR (1994) Genomic fingerprint of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40

Wang T, Chen WF, Tian CF, Young JPW, Chen WX (2019) Ecology and evolution of rhizobia. Springer, Singapore

Yao Y, Sui XH, Zhang XX, Wang ET, Chen WX (2015) *Bradyrhizobium erythrophlei* sp. nov. and *Bradyrhizobium ferriligni* sp. nov., isolated form effective nodules of *Erytrophleum fordii*. Int J Syst Evol Microbiol 65:1831–1837. https://doi.org/10.1099/ijs.0.000183