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Identification of Rhizobia Isolated from Nodules of Mexican Commercial Soybean Varieties

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Abstract

Rhizobia, crucial for nitrogen fixation in leguminous plants, play a vital role in soybean cultivation. This study, conducted in Mexico, a major soybean importer, aimed to identify bacteria from nodules of five soybean varieties in high-production regions. Multilocus sequence analysis (MLSA) was employed for enhanced species resolution. The study identified six *Bradyrhizobium* species: *Bradyrhizobium japonicum* USDA 110, *Bradyrhizobium japonicum* USDA 6, *Bradyrhizobium elkanii* USDA 76, *Bradyrhizobium neotropicale*, *Bradyrhizobium lablabi*, and *Bradyrhizobium icense*. *Bradyrhizobium japonicum* USDA 110 predominated in the soils, displaying symbiotic preference for the Huasteca 400 variety. However, phylogenetic analysis didn't reveal a clear association between strains, soil, and soybean variety. This research sheds light on the diversity of rhizobia in Mexican soybean cultivation, contributing to the understanding of symbiotic relationships in soybean production systems.

Keywords

Nodules, Soybean, Housekeeping Genes, MLSA, Rhizobia, *Bradyrhizobium*, Nitrogen Fixation, Symbiosis, Phylogenetic Analysis

1. Introduction

Soybean [*Glycine max* (L.) Merr.] holds critical significance globally as a primary source of protein and oil [1], contributing to a production exceeding 175 million tons across approximately 80 million hectares [2]. In Mexico, soybean cultivation is primarily practiced in rainfed production systems, encompassing an

average of 164 thousand hectares over the last three years during both autumn-winter and spring-summer cycles. However, the average yield per hectare in Mexico remains modest, not surpassing 1.55 tons [3] [4]. Enhancing soybean productivity involves strategic interventions such as organic fertilization and the use of bioinoculants containing specific strains of the *Bradyrhizobium* genus [5] [6]. These measures have demonstrated effectiveness in increasing grain yield, both in rainfed and irrigated production systems [5]. The identification and selection of strains displaying high affinity and adaptation to soybean plants are crucial steps in optimizing productivity [7]. Molecular markers, particularly Multilocus Sequence Analysis (MLSA), have emerged as valuable tools for bacterial identification, enabling taxonomic assignments at the species level within the *Bradyrhizobium* genus.

Studies by Delamuta *et al.* [8] utilized MLSA, analyzing the 16S rRNA gene along with five housekeeping genes (*rec*A, *atp*D, *gln*II, *gyr*B, and *rpo*B) to reliably identify strains of the *Bradyrhizobium* genus. This methodology proved effective in phylogenetic and taxonomic analyses of nitrogen-fixing rhizobia [8]. Similarly, Li *et al.* [9], employed a polyphasic approach, combining the 16S rRNA gene and concatenated MLSA genes (*gln*II, *rec*A, *dna*K) alongside digital DNA-DNA hybridization (dDDH) in characterizing rhizobia isolates from peanut nodules (*Arachis hypogaea*). Their findings resulted in the identification of three new species: *Bradyrhizobium nanningense* sp. nov., *Bradyrhizobium guangzhouense* sp. nov., and *Bradyrhizobium zhanjiangense* sp. nov. These studies underscore the utility of MLSA in advancing our understanding of nitrogen-fixing rhizobia and optimizing symbiotic relationships for improved crop outcomes.

The MLSA method, leveraging the analysis of housekeeping genes, has proven to be a rapid and reliable approach for identifying strains at the species level within the *Bradyrhizobium* genus. This method facilitates the construction of phylogenetic and taxonomic trees, providing in-depth insights into the diversity of nitrogen-fixing rhizobia [10] [11]. Notable studies, such as the one conducted in Brazil by Kruschewsky *et al.* [12], utilized MLSA to analyze the diversity of rhizobium strains isolated from root nodules of different *Inga Mill.* species. The study employed various gene sequences, including *dna*K, *rec*A, *rpo*B, *gyr*B, *gln*II, 16S rRNA, internal transcribed spacer regions (ITS), and symbiosis-associated genes *nod*C and *nif*H. MLSA combined with the ITS region yielded the best genetic structure, revealing four main groups of *Bradyrhizobium* strains.

Recent applications of MLSA have extended to the identification and characterization of new *Bradyrhizobium* species isolated from nodules of forage legumes in Australia and South Africa [13]. Researchers have adopted a polyphasic approach in the identification of bacterial isolates, integrating MLSA with other techniques such as PCR-RFLP, phenotypic characteristics, fatty acid analysis, 16S rRNA gene sequencing, and whole-genome sequencing [10] [11] [14] [15].

The study of bacterial isolates from soybean root nodules, particularly those

capable of symbiotic nitrogen fixation, is a recurrent theme. Common molecular tools include the 16S rRNA gene, MLSA genes (*atp*D, *rec*A, *gln*II, *rpo*B, *dna*K), and *nif*H genes associated with biological nitrogen fixation [16]. In Brazil, researchers have established a database of MLSA sequences for the *Bradyrhizobium* genus, facilitating species-level studies [17]. In light of this, the primary objective of the current study is to employ MLSA for the molecular identification of strains isolated from root nodules of commercial soybean varieties cultivated in the southern region of the state of Tamaulipas, Mexico.

2. Materials and Methods

1) Soil sample and root nodules collection. Sampling locations: The bacterial isolates were obtained from soils in soybean-producing plots located in Altamira and El Mante municipalities, Tamaulipas, Mexico. Additionally, a plot without agricultural use in Reynosa, Tamaulipas, Mexico, was included (refer to **Table 1**). *The soil sampling procedure was carried out as shown followers.* A composite soil sample was created by combining five 2.5 kg subsamples. These were taken radially within one-hectare plots at a depth of 40 cm. Subsamples included one from the center and four from the plot edges. The composite sample underwent physical-chemical soil analysis following the Mexican standard NOM-021-RECNAT (2000) [18].

2) Root nodule collection. Nodules were collected from the commercial varieties Huasteca-200, Huasteca-300, Huasteca-400, Tamesi, and Vernal, adapted for production in the state of Tamaulipas, Mexico. The greenhouse experiment for nodule collection was established as follows. In the spring of 2019, soybean materials were planted in triplicate in soil collected from sterilized plastic pots. Each experimental unit comprised 12 seeds per pot per repetition. To prevent cross-contamination, seeds were washed with 1% sodium hypochlorite and

Table 1. Geographic location of commercial plots for collecting soil samples and subsequent sowing of seeds of soybean cultivars.

Sample	Code	Region	Plot	Crop rotation	Geographic location
1	MA	Altamira	Ejido Cervantes Km 40	Sorghum	22˚26'46"N; 98˚3'14"W
2	MB	Altamira	Brecha Corpus Christi Km 36 Rancho Peteron	Sorghum	22˚48'32"N; 98˚02'00"W
3	MD	Altamira	Brecha Corpus Christi Km 36 Rancho Satélite	Sorghum	22˚48'32"N; 98˚02'00"W
4	CE	Altamira	Km 55 Victoria-Tampico (Campo Experimental, INIFAP)	Soybean	22˚34'6"N; 98˚10'5"W
5	SG	Altamira	Santa Gertrudis	Safflower	22˚63'13.8"N; 98˚19'611"W
6	MF	El Mante	Rancho Humberto Alanís Km 126	Safflower	22˚48'51.7"N; 99˚00'42.4"W
7	MG	El Mante	Rancho la Pedrera Km 127	Safflower	22˚48'51.7"N; 99˚00'42.4"W
8	MH	El Mante	Ejido el Riachuelo Km 11	Safflower	22˚57'18.7"N; 99˚02'38.3"W
9	MI	El Mante	Km 102 Mante-Victoria	Sorghum	22˚48'46.3"N; 99˚00'42.8"W
10	MR	Reynosa	Brecha El Berrendo	no crop	26˚4'36.5"N; 98˚17'50.9"W

were collected at the beginning of the plant's flowering stage (R1 reproductive rinsed with sterile deionized water before planting. nodules stage), characterized by increased development and biological activity of atmospheric nitrogen fixation. Morphologically, nodules exhibited a pinkish-reddish color [19].

3) Preprocessing of nodules and isolate acquisition. Nodules were disinfected using a 0.1% sodium hypochlorite solution [19]. Subsequently, they were macerated individually in 1.5 mL Eppendorf tubes with a drop of sterile deionized water per nodule. A 50 µL aliquot of the resulting macerate, diluted to 1×10^{-5}, was evenly spread onto Extract Yeast-Mannitol-Agar (ELMA) modified with soil extract. The medium was supplemented with Bromothymol Blue (ABT) (0.5 g/100mL ethanol) as a pH indicator and dispensed into Petri dishes in triplicate [20]. Strain selection was based on the medium's coloration. Yellow indicated fast-growing rhizobia (1 - 3 days), typically genus *Rhizobium*, while blue indicated slow-growing rhizobia (5 - 10 days), generally genus *Bradyrhizobium*. Purity was confirmed on yeast extract-mannitol agar (YEMA) medium. Gram staining was performed to exclude Gram-positive motile bacilli. Out of the 287 isolated strains, 81 were chosen based on macroscopic and microscopic characteristics, including color, appearance, shape, and texture [21]. Additionally, the infectivity rate of each strain in greenhouse conditions was assessed by the number of nodules formed in the roots. Interestingly, the CE soil exhibited a higher abundance of strains with characteristics typical of the *Bradyrhizobium* genus.

4) Multilocus Sequence Analysis (MLSA). Pure bacterial cultures were grown in Extract Yeast-Mannitol-Agar (ELMA) broth at a density of 10^9 cells per mL. For each 15 µL of bacterial culture, 3 µL of 0.01% Tween was added and incubated at 65˚C for 20 minutes. The MLSA housekeeping genes (*rec*A, *gln*II, *dna*K, *gyr*B, and *atp*D) were amplified according to the protocol outlined in **Table 2**. Each amplification reaction consisted of a 14 µL mixture, including 7 µL of PCR Master Mix 2× (Promega™, USA), 2 µL of nuclease-free water (Promega™, USA), 1 µL of each primer pair, and 3 µL of template DNA. Amplifications were performed using a thermal cycler (C1000 Touch BIO-RAD, USA) under the conditions specified in **Table 1**. Amplicons were visualized through 1% agarose gel electrophoresis (Promega™, USA) and confirmed using the DNA Ladder Marker 100 bp (Promega™, USA) on a BIO-RAD Molecular Imegen®-GEL DOC™ XR image documenter (Bio-Rad Laboratories Inc.). The amplicons of each gene were purified using the commercial ExoSAP-IT® kit (AFFYMETRIX; Cleveland, USA). Sequencing reactions were carried out at Eurofins MWG from Operon USA (Thermo Fisher Scientific).

5) Bioinformatic analysis and diversity. The MLSA gene sequences were concatenated and subjected to taxonomic analysis using the database for the taxonomic and phylogenetic identification of the genus *Bradyrhizobium* (http://mlsa.cnpso.embrapa.br/). The platform employs sequence analysis multilocus for taxonomic assignment, following the methodology outlined by Azevedo *et al.* [17]. Phylogenetic analysis was conducted using the MEGA Ver 6.0

Table 2. Primers and amplification conditions of the housekeeping genes *rec*A, *gln*II, *dna*K, *gyr*B and *atp*D.

Primer	Sequence * (5' a 3')	Fragment size (pb)	PCR conditions	Reference
TSrecAf	CAACTGCMYTGCGTATCGTCGAAGG	525	2 min 94˚C, 32 × (45 s 94˚C, 30 s 58˚C, 1.5 min 72˚C) and 7 min 72˚C.	[22]
TSrecAr	CGGATCTGGTTGATGAAGATCACCATG			
TSatpDf	TCTGGTCCGYGGCCAGGAAG	574	2 min 94˚C, 32 × (45 s 94˚C, 30 s 58˚C, 90 s 72˚C) and 7 min 72˚C.	[22]
TSatpDr	CGACACTTCCGARCCSGCCTG			
TSglnIIf	AAGCTCGAGTACATCTGGCTCGACGG	602	2 min 95˚C, 35 × (45 s 95˚C, 30 s 58˚C, 1.5 min 72˚C) and 7 min 72˚C.	[22]
TSglnIIr	SGAGCCGTTCCAGTCGGTGTCG			
gyrB1043f	TTCGACCAGAAYTCCTAYAAGG	800	2 min 94˚C, 5 × (2 min 94˚C, 2 min 58˚C, 1 min 72˚C) 28 × (30 s 94˚C, 1 min 58˚C, 1 min 72˚C) and 5 min 72˚C.	[23]
gyrB1043r	AGCTTGTCCTTSGTCTGCG			
dnaK1777f	AAGGARCANCAGATCCGCATCCA	370	2 min 94˚C, 32 × (1 min 94˚C, 1 min 62˚C, 40 s 72˚C), 5 min 72˚	[24] [25]
dnaK1777r	TASATSGCCTSRCCRAGCTTCAT			

(Molecular Evolutionary Genetics Analysis) program. The sequences of the 81 strains were aligned with four reference sequences (*Bj*_USDA110, *Bj*_USDA6, *Bradyrhizobium*_sp., and *Rhizobium*_sp.) using MUSCLE [26]. Phylogenetic trees were constructed using the Kimura 2-parameter method for calculating evolutionary distances [27]. The maximum likelihood method was applied with resampling of 10,000 replicates for robustness [28].

3. Results and Discussion

3.1. The Analysis of Physical and Chemical Properties

The collected soils revealed several key characteristics (**Table 3**). Most of the soils displayed alkaline pH and belonged to the clayey textural class. Soil organic matter content varied, with some soils showing deficiency (e.g., MA soil with 0.96%) and others having a high organic matter content (e.g., MF sample with 8%). Most soils exhibited low electrical conductivity (below 1.0 dS/m), indicating a lack of salts, except for the MR sample, which had a moderately high value of 3.5 dS/m. Nitrogen concentration ($N\text{-}NO_3$, ppm) was generally low across all samples, with values below 10.6 ppm. Available phosphorus content ranged from low (4 ppm in the MD sample) to intermediate (13.30 ppm in the MB sample). Extractable potassium showed moderate to high percentages in most samples, except for the MB sample, which had a low value of 3 ppm. In summary, the soil samples had low carbonate values (below 2.72%), low sodium values (below 24.52 meq/l), and moderately alkaline pH. Soil physical properties

Table 3. Physical and chemical properties of soils collected for the isolation of Mexican commercial soybean nodules.

Soils	% OM[a]	E.K (ppm)	pH	EC (dS/m)	% Sand	% Clay	% Silt	Textural Class	N-NO_3 (ppm)	P (ppm)	Ca (meq/l)
MA	0.96	356	8.1	0.35	43.8	41.80	14.3	Clayey	3.85	3.60	1.35
MB	1.34	3	8.0	0.67	53.4	36.16	10.3	Sandy clay	3.65	13.3	1.68
MD	1.66	168	8.4	0.76	35.4	47.80	16.7	Clayey	2.3	3.00	1.88
CE	1.54	440	8.1	0.97	32.2	47.80	20.0	Clayey	10.6	6.70	1.66
SG	0.83	519	8.0	1.12	29.4	50.16	20.3	Clayey	6.45	7.00	2.21
MF	8.00	628	8.0	0.69	45.4	42.16	12.3	Clayey	3.67	5.60	2.36
MG	2.75	208	8.2	0.62	17.4	58.16	24.3	Clayey	4.45	1.80	2.03
MH	3.01	454	8.2	0.79	42.2	33.80	24.0	Clay loam	5.55	6.20	2.10
MI	2.24	270	8.1	0.30	23.4	52.16	24.3	Clayey	4.15	8.20	1.57
MR	2.95	152	7.7	3.59	45.4	26.16	28.3	Loam	3.23	7.70	11.97
	Mg[b] (meq/l)	Sodio (meq/l)	K (meq/l)	Carbonates	Bi Carbonates	Chlorides	Sulfates	Cu	Zn	Mn	Fe
MA	2.06	7.20	0.16	1.63	1.09	1.60	2.02	1.817	0.546	0.78	0.37
MB	5.07	12.62	0.38	1.09	1.63	3.20	2.94	2.352	2.028	1.08	0.46
MD	13.9	0.56	1.09	1.63	4.20	2.41	0.800	0.421	0.605	0.47	13.9
CE	16.2	2.78	1.09	2.17	4.80	3.29	1.534	6.910	7.780	1.15	16.2
SG	19.4	2.44	2.72	0.54	4.80	5.47	2.122	2.543	2.208	0.92	19.4
MF	9.25	0.43	1.09	1.09	2.60	2.19	1.839	1.011	0.817	0.50	9.2
MG	13.5	0.48	2.17	1.09	2.60	2.85	1.241	0.640	1.609	0.73	13.5
MH	11.5	0.64	2.72	1.09	2.40	2.24	1.184	0.865	3.119	0.74	11.5
MI	7.34	0.18	2.17	1.09	1.20	2.11	1.273	0.251	1.007	0.53	7.3
MR	24.5	0.47	2.17	3.26	8.40	10.60	0.735	3.219	0.587	0.51	24.5

a, %OM = % organic matter, E.K = extractable potassium, pH = hydrogen potential, EC = electric conductivity, P = phosphorus, Ca =, P = calcium; b, Mg = magnesium, Na = sodium, K = potassium, Cu = copper, Zn = zinc, Mn = manganese, Fe = iron.

such as compaction and erosion and chemical properties such as alkalinity, acidification and sodication, as well as low amounts of microbiota, are main factors that affect soil organic matter [29]. Therefore, soil correction with the application of gypsum or agricultural lime might not be necessary in the analyzed samples. However, for some agricultural soils with low or moderate organic matter (MA, SG, MB, CE, MD) and low available nitrogen (MA, MB, MD, MF), agricultural interventions are recommended to enhance the bioavailability of nitrogen and increase soil organic matter. This could involve practices such as organic fertilization or the use of biofertilizers to improve soil fertility.

An interesting case is presented by the CE soil sample. The CE sample exhibits the highest amount of available nitrogen and a moderate level of organic matter

(Table 3). This soil is used experimentally, undergoing soybean cultivation twice annually with different genotypes. Soybeans, in association with soil bacteria of the *Bradyrhizobium* genus, form a symbiotic relationship where these bacteria fix atmospheric nitrogen to the soil, leading to an accumulation of nitrate reserves in the CE soil. One potential strategy to enhance agricultural soils, exemplified by the CE soil, is agricultural rotation involving soybeans and the use of biofertilizers containing beneficial microorganisms. This approach can contribute to improving soil microbiota, increasing organic matter, and maintaining a more neutral pH. Sun *et al.* [30] suggest intercropping soybeans rather than continuous sowing to prevent soil quality deterioration. Continuous soybean cultivation may lead to soil quality issues, including the accumulation of nitrogen reserves, potentially inhibiting soybean growth and reducing grain production. The inclusion of cereal crops in the rotation with soybean increases the resource available to heterotrophic soil microbial communities, which in turn increases carbon and nitrogen cycling and therefore higher crop productivity compared with continuous soybeans [31]. However, in this work, a greater amount of organic matter and available nitrate was observed in soils collected in rotation with the safflower crop compared to soils collected where the crop was sorghum (Table 3), which may indicate a greater demand for nitrogen and organic matter in sorghum cultivation. Agricultural practices significantly influence soil properties and microbial diversity, particularly regarding alterations in pH, organic matter, total nitrogen, and phosphorus [32]. Therefore, adopting sustainable practices, such as crop rotation and biofertilizer application, becomes crucial for maintaining soil health and productivity in agricultural systems.

3.2. Taxonomic Assignment of Strains Isolated from Soybean Nodules

The taxonomic assignment of the 81 isolates from nodules of Mexican soybean varieties, based on the concatenation of the MLSA genes and submission to the exclusive sequences database of strains of the genus *Bradyrhizobium* at EMBRAPA Brazil, demonstrated an average precision above 93% in taxonomy assignment at the species level. Notably, all isolates (100%) were classified within the Bradyrhizobium genus. The 81 isolates were further categorized into six different species within the genus *Bradyrhizobium*, with the following distribution, *Bradyrhizobium japonicum* USDA 110: 53 isolates, *Bradyrhizobium japonicum* USDA 6: 19 isolates, *Bradyrhizobium elkanii* USDA 76: 4 isolates, *Bradyrhizobium neotropicale*: 2 isolates, *Bradyrhizobium icense*: 2 isolates, *Bradyrhizobium lablabi*: 1 isolate (Table 4).

This underscores the dominance of the *Bradyrhizobium* genus in the symbiotic association with soybean plants [32] [33] [34]. The specific identification of strains within this genus is crucial for understanding their potential contributions to nitrogen fixation and their symbiotic relationships with soybeans. The species identified in this study from *Bradyrhizobium* genus, are well-established soybean symbionts. *B. japonicum*, is widely recognized as the most universal

Table 4. Taxonomic assignment of 81 bacterial isolates based on concatenation of five MLSA genes.

ID	Description	Soil[b]	Nodule origin[c]	Genera	Identity	Accession number
1	*Bradyrhizobium japonicum* USDA 110	SG	H400	*Bradyrhizobium*	96%	BjU110
2	*Bradyrhizobium japonicum* USDA 110	MH	H400	*Bradyrhizobium*	95%	BjU110
4	*Bradyrhizobium japonicum* USDA 110	MH	H400	*Bradyrhizobium*	94%	BjU110
5	*Bradyrhizobium elkanii* USDA 76	MH	H400	*Bradyrhizobium*	91%	BjU76
7	*Bradyrhizobium japonicum* USDA 110	SG	H400	*Bradyrhizobium*	97%	BjU110
8	*Bradyrhizobium elkanii* USDA 76	SG	H400	*Bradyrhizobium*	95%	BjU76
9	*Bradyrhizobium japonicum* USDA 110	SG	H400	*Bradyrhizobium*	94%	BjU110
10	*Bradyrhizobium japonicum* USDA 110	SG	H400	*Bradyrhizobium*	94%	BjU110
12	*Bradyrhizobium japonicum* USDA 110	SG	H400	*Bradyrhizobium*	95%	BjU110
16	*Bradyrhizobium japonicum* USDA 6	SG	H400	*Bradyrhizobium*	94%	BjU6
18	*Bradyrhizobium japonicum* USDA 110	SG	H400	*Bradyrhizobium*	91%	BjU110
19	*Bradyrhizobium japonicum* USDA 110	MH	H400	*Bradyrhizobium*	91%	BjU110
20	*Bradyrhizobium japonicum* USDA 110	MH	H400	*Bradyrhizobium*	91%	BjU110
21	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	95%	BjU110
22	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	95%	BjU110
23	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	95%	BjU110
28	*Bradyrhizobium japonicum* USDA 6	SG	H400	*Bradyrhizobium*	96%	BjU6
30	*Bradyrhizobium japonicum* USDA 110	MH	H400	*Bradyrhizobium*	95%	BjU110
31	*Bradyrhizobium japonicum* USDA 110	MH	H400	*Bradyrhizobium*	95%	BjU110
32	*Bradyrhizobium japonicum* USDA 110	CE	Vernal	*Bradyrhizobium*	94%	BjU110
33	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	92%	BjU110
37	*Bradyrhizobium japonicum* USDA 6	CE	Tamesi	*Bradyrhizobium*	97%	BjU6
41	*Bradyrhizobium neotropicale*	SG	H400	*Bradyrhizobium*	98%	Bn
42	*Bradyrhizobium japonicum* USDA 6	MH	H400	*Bradyrhizobium*	91%	BjU6
46	*Bradyrhizobium lablabi*	MH	H400	*Bradyrhizobium*	97%	Bl
47	*Bradyrhizobium japonicum* USDA 6	MH	H400	*Bradyrhizobium*	96%	BjU6
50	*Bradyrhizobium japonicum* USDA 6	MB	H200	*Bradyrhizobium*	93%	BjU6
53	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	92%	BjU110
54	*Bradyrhizobium japonicum* USDA 110	CE	Tamesi	*Bradyrhizobium*	91%	BjU110
56	*Bradyrhizobium japonicum* USDA 6	CE	Vernal	*Bradyrhizobium*	91%	BjU6
58	*Bradyrhizobium japonicum* USDA 110	MB	H200	*Bradyrhizobium*	92%	BjU110
59	*Bradyrhizobium japonicum* USDA 110	CE	Tamesi	*Bradyrhizobium*	92%	BjU110
62	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	91%	BjU110

Continued

63	*Bradyrhizobium japonicum* USDA 110	CE	Vernal	*Bradyrhizobium*	91%	BjU110
66	*Bradyrhizobium japonicum* USDA 6	CE	H400	*Bradyrhizobium*	91%	BjU6
68	*Bradyrhizobium japonicum* USDA 110	SG	Tamesi	*Bradyrhizobium*	92%	BjU110
70	*Bradyrhizobium icense*	CE	H400	*Bradyrhizobium*	97%	Bi
75	*Bradyrhizobium japonicum* USDA 6	CE	Vernal	*Bradyrhizobium*	92%	BjU6
77	*Bradyrhizobium japonicum* USDA 110	SG	H400	*Bradyrhizobium*	94%	BjU110
79	*Bradyrhizobium japonicum* USDA 110	MF	Tamesi	*Bradyrhizobium*	92%	BjU110
80	*Bradyrhizobium japonicum* USDA 6	MF	Tamesi	*Bradyrhizobium*	92%	BjU6
83	*Bradyrhizobium elkanii* USDA 76	CE	H400	*Bradyrhizobium*	91%	BjU76
85	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	92%	BjU110
86	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	92%	BjU110
87	*Bradyrhizobium japonicum* USDA 110	MB	H200	*Bradyrhizobium*	91%	BjU110
88	*Bradyrhizobium japonicum* USDA 110	MB	H200	*Bradyrhizobium*	91%	BjU110
89	*Bradyrhizobium japonicum* USDA 6	MG	Tamesi	*Bradyrhizobium*	92%	BjU6
90	*Bradyrhizobium japonicum* USDA 110	MG	Tamesi	*Bradyrhizobium*	91%	BjU110
91	*Bradyrhizobium neotropicale*	CE	Tamesi	*Bradyrhizobium*	90%	Bn
92	*Bradyrhizobium elkanii* USDA 76	SG	Tamesi	*Bradyrhizobium*	91%	BjU76
93	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	91%	BjU110
95	*Bradyrhizobium japonicum* USDA 6	CE	Vernal	*Bradyrhizobium*	97%	BjU6
96	*Bradyrhizobium japonicum* USDA 6	CE	Vernal	*Bradyrhizobium*	95%	BjU6
97	*Bradyrhizobium japonicum* USDA 110	MH	H400	*Bradyrhizobium*	92%	BjU110
99	*Bradyrhizobium japonicum* USDA 110	CE	H400	*Bradyrhizobium*	91%	BjU110
101	*Bradyrhizobium japonicum* USDA 110	MF	Tamesi	*Bradyrhizobium*	91%	BjU110
105	*Bradyrhizobium japonicum* USDA 110	SG	Tamesi	*Bradyrhizobium*	91%	BjU110
108	*Bradyrhizobium japonicum* USDA 110	CE	H300	*Bradyrhizobium*	91%	BjU110
109	*Bradyrhizobium japonicum* USDA 110	CE	H300	*Bradyrhizobium*	91%	BjU110
111	*Bradyrhizobium japonicum* USDA 110	MG	H400	*Bradyrhizobium*	91%	BjU110
114	*Bradyrhizobium icense*	MG	H300	*Bradyrhizobium*	95%	Bi
117	*Bradyrhizobium japonicum* USDA 110	SG	Vernal	*Bradyrhizobium*	91%	BjU110
119	*Bradyrhizobium japonicum* USDA 110	MH	H200	*Bradyrhizobium*	91%	BjU110
120	*Bradyrhizobium japonicum* USDA 110	CE	Vernal	*Bradyrhizobium*	92%	BjU110
122	*Bradyrhizobium japonicum* USDA 110	SG	H200	*Bradyrhizobium*	92%	BjU110
126	*Bradyrhizobium japonicum* USDA 110	CE	Vernal	*Bradyrhizobium*	92%	BjU110
134	*Bradyrhizobium japonicum* USDA 110	MF	H400	*Bradyrhizobium*	92%	BjU110
139	*Bradyrhizobium japonicum* USDA 110	CE	H200	*Bradyrhizobium*	92%	BjU110

Continued

141	*Bradyrhizobium japonicum* USDA 6	MA	H200	*Bradyrhizobium*	92%	BjU6
143	*Bradyrhizobium japonicum* USDA 6	MD	Vernal	*Bradyrhizobium*	92%	BjU6
144	*Bradyrhizobium japonicum* USDA 6	MD	Vernal	*Bradyrhizobium*	92%	BjU6
145	*Bradyrhizobium japonicum* USDA 110	MR	H200	*Bradyrhizobium*	91%	BjU110
146	*Bradyrhizobium japonicum* USDA 110	MI	H200	*Bradyrhizobium*	91%	BjU110
149	*Bradyrhizobium japonicum* USDA 110	MR	H400	*Bradyrhizobium*	92%	BjU110
151	*Bradyrhizobium japonicum* USDA 110	CE	H300	*Bradyrhizobium*	92%	BjU110
152	*Bradyrhizobium japonicum* USDA 110	MR	H200	*Bradyrhizobium*	91%	BjU110
153	*Bradyrhizobium japonicum* USDA 6	MR	H200	*Bradyrhizobium*	96%	BjU6
156	*Bradyrhizobium japonicum* USDA 110	MR	H300	*Bradyrhizobium*	91%	BjU110
162	*Bradyrhizobium japonicum* USDA 6	MG	Tamesi	*Bradyrhizobium*	92%	BjU6
163	*Bradyrhizobium japonicum* USDA 110	MR	Vernal	*Bradyrhizobium*	90%	BjU110
233[a]	*Bradyrhizobium japonicum USDA* 6			*Bradyrhizobium*	86%	BjU6

a = strain isolated from commercial biofertilizer; b = See **Table 1**; c = varieties (Huasteca 200, Huasteca 300, Huasteca 400).

bacterial isolate within the genus *Bradyrhizobium*. Studies, such as the one conducted by Shao *et al.* [35], utilizing the MLSA method with concatenated housekeeping genes and IGS sequences, have concluded that soil characteristics and the competitive capacity of rhizobia play significant roles in shaping symbiotic rhizobial communities. This provides insights into community formation and the biogeographic properties of rhizobia.

Microbial populations are subject to diverse biotic and abiotic factors, including soil properties, climate, humidity, temperature, pathogens, crop rotation, plant variety, plant parts (organs) and field location [36] [37] [38] [39]. Bacterial diversity, as determined by various studies, is profoundly influenced by the degree of habitat alteration resulting from variations in land-use management practices, especially those affecting the properties of semiarid soils. Understanding the complex interplay between these factors is crucial for devising effective agricultural practices that enhance symbiotic relationships between rhizobia and soybeans, leading to improved soil health and crop productivity [40].

3.3. Phylogenetic Analysis

The phylogenetic analysis of the 81 isolates, based on the concatenation of housekeeping genes, revealed four main groups (**Figure 1**): Group one: comprising 12 isolates, with eight from the species *Bradyrhizobium japonicum* USDA110, three from *Bradyrhizobium japonicum* USDA6, and one from *Bradyrhizobium elkanii* USDA76. The reference strain in this group was *Bradyrhizobium japonicum* USDA6 (*Bj*_USDA6). Group two: consisting of 18 strains, with 12 from the species *Bradyrhizobium japonicum* USDA110, three from *Bradyrhizobium japonicum* USDA6, one isolate from *Bradyrhizobium icense*,

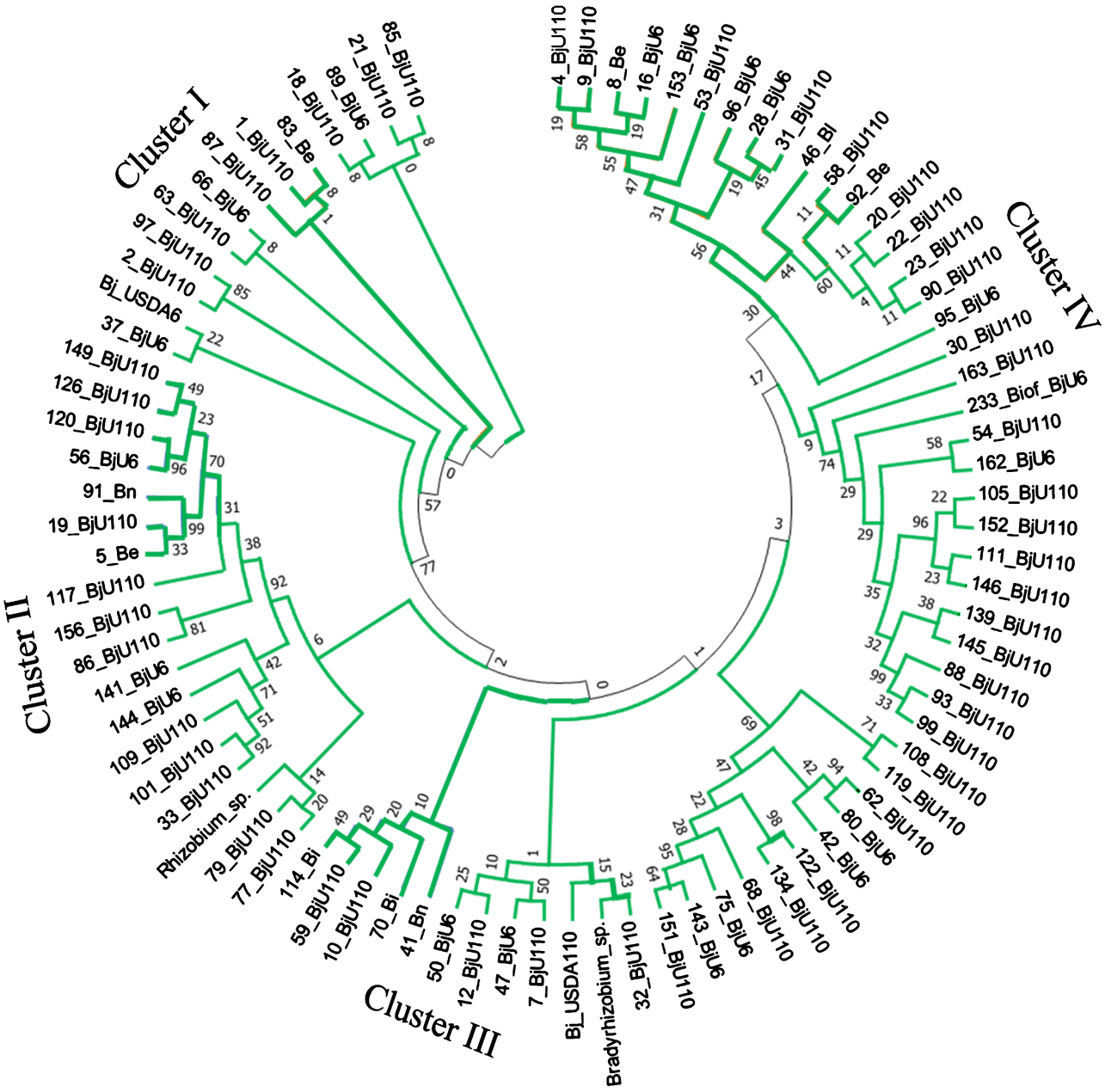

Figure 1. Phylogenetic analysis of 81 bacterial strains based on the concatenation of five MLSA genes, the maximum likelihood method and the Kimura 2-parameter evolutionary model. The strength of the consensus of the phylogenetic tree was determined by inferred resampling of 10,000 replicates.

one from *Bradyrhizobium neotropicale*, and the reference strain *Rhizobium*_sp. Group three: comprising three subgroups with a total of 23 isolates. The subgroups included 12 isolates of *Bradyrhizobium japonicum* USDA110, six isolates of *Bradyrhizobium japonicum* USDA6, two strains of *Bradyrhizobium icense*, one strain of *Bradyrhizobium neotropicale*, and two reference strains, *Bradyrhizobium*_sp, and *Bradyrhizobium japonicum* USDA110 (*Bj*_USDA110). Group four**:** made up of 31 isolates, with the highest presence of the species *Bradyrhizobium japonicum* USDA110 (21 isolates), followed by *Bradyrhizobium japoni-*

cum USDA6 (seven isolates), two isolates of *Bradyrhizobium elkanii* USDA76, and one strain of the species *Bradyrhizobium lablabi*.

This phylogenetic clustering provides insights into the genetic relationships among the isolates, indicating distinct groups and patterns within the soybean rhizobial community. The diversity observed underscores the complexity of the soybean-rhizobia symbiotic relationship and highlights the adaptability of different *Bradyrhizobium* species to the soybean varieties and soil conditions in the studied region. The prevalence of *Bradyrhizobium japonicum* USDA110 among the isolates from commercial soybean nodules in the southern region of the state of Tamaulipas, Mexico, indicates its significant representation in the studied agricultural soils. This particular species demonstrated a higher affinity, with 53 out of the 81 analyzed isolates belonging to *Bradyrhizobium japonicum* USDA110. The variety Huasteca 400, in particular, exhibited a notable association with this species, with 27 strains identified. These were predominantly isolated from the CE soils (10 isolates), SG (7 isolates), and MH (7 isolates). Additionally, by infectivity tests of the 81 strains isolated and identified in the five soybean materials, it was proven that 27 isolates were capable of being infective and nodular in the five soybean varieties (data not shown), of these 27 isolates, 16 belong to the species Bradyrhizobium japonicum USDA110 isolated from different soils and a different variety of soybean, this species generating a greater number of nodules in the root [41] (in the process of publication).

Interestingly, despite the diversity observed in the phylogenetic analysis, there wasn't a clear association between the identified species, soybean variety, and the agricultural soil of origin. This suggests a rich diversity of isolates in the region. The lack of a clear pattern in the association could be attributed to various factors, including the complex interactions between soybean varieties, soil conditions, and rhizobial populations. Such diversity is not uncommon in tropical environments, as reported in Mexico and Brazil, where symbiotic strains of the *Bradyrhizobium* genus have been isolated from various legumes, emphasizing the importance of cross-infectivity between legumes and its agricultural implications [42] [43]. The ability of certain isolates to nodulate soybean from legumes other than soybean underlines the potential for diverse rhizobial populations to contribute to soil fertility and plant growth. This cross-infectivity has implications for agricultural practices, indicating the importance of considering the broader legume-rhizobium interactions in crop rotation and symbiotic nitrogen fixation strategies [44].

4. Conclusion

The conclusion highlights the efficacy of MLSA as a valuable tool for investigating taxonomic relationships among bacterial species in the genus *Bradyrhizobium* by concatenating housekeeping genes, this method provided a high level of resolution for interspecific differences and divergence, enabling precise identification. In the context of this study, it revealed a significant presence of the spe-

cies *Bradyrhizobium japonicum* USDA110 in soils of soybean-producing plots. Importantly, the analysis also showcased diversity at the strain level, with no clear association observed between identified species and the soil of origin. This underscores the richness and complexity of rhizobial populations in the studied agricultural soils. In practical terms, understanding the diversity and prevalence of specific *Bradyrhizobium* species, such as *Bradyrhizobium japonicum* USDA110, has implications for soybean cultivation. The absence of a clear association between species and soil collection suggests that various factors, including soybean varieties and local soil conditions, contribute to the diversity of rhizobial populations. This complexity should be considered in agricultural practices, especially in regions where soybean is a vital crop.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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