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Diversity of native rhizobia-nodulating *Phaseolus lunatus* in Brazil

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ABSTRACT

The studies of rhizobial diversity in the *Phaseolus* genus have focused on *Phaseolus vulgaris*. It is unclear how rhizobial diversity is associated with *Phaseolus lunatus* in areas where this legume is not native, such as Brazil. Therefore, we studied rhizobial diversity associated with *P. lunatus* bean in soils from Brazil. The study was conducted in a greenhouse, and seeds from each genotype of *P. lunatus* were sown in plastic bags containing soils originating from Northeast Brazil. The nodules used in isolation and characterization were collected at 45 days after seedling emergence. Fourteen isolates of rhizobia were obtained. DNA was extracted, and the 16S rRNA gene was sequenced using primers fD1 and rD1. More than half of the strains studied were positioned in the *Bradyrhizobium* clade (in the *B. elkani* superclade). One strain was positioned in the *Rhizobium etli*/*Rhizobium phaseoli* clade. Two strains were grouped within the *R. tropici* group. Three strains, ISOL16, ISOL21, and ISOL27, that may represent new lineages were found. According to our analysis of the partial sequence of the 16S rRNA gene of 14 rhizobia strains, there was high species diversity of rhizobia-nodulating *P. lunatus* in Northeast Brazil, including potential new species.

Key words: Biological nitrogen fixation, *Bradyrhizobium*, *Phaseolus lunatus*, *Rhizobium.*

INTRODUCION

Phaseolus lunatus, the Lima bean, is the second most economically important species of *Phaseolus* and one of the 12 primary grain legumes (Fofana *et al.*, 1997). This crop shows high rusticity and the capacity to resist long dry periods. These characteristics are important for tropical regions and increase the economic and social importance of the crop (Fofana *et al.*, 1997).

As a legume, *P. lunatus* has the ability to perform biological nitrogen fixation (BNF) through symbiosis with N_2-fixing bacteria, such as *Rhizobium* sp. (Ormeno-Orrillo *et al.*, 2006). As a prerequisite for the formation of the symbiotic association, the two partners come in contact by their cell surfaces, where the phenomenon of specificity and recognition is believed to occur (Wang *et al.*, 2012). Although some rhizobia have a restricted host range (Santamaria *et al.*, 2014), others can promiscuously enter symbiotic association with many species of legume, such as *P. lunatus*. This promiscuity has increased the diversity of these bacteria worldwide (Ormeno-Orrillo *et al.*, 2006).

Currently, studies of rhizobia have shown high diversity in soils worldwide, from tropical to temperate regions in several host legumes (Degefu *et al.*, 2013; Yang *et al.*, 2013; Aserse *et al.*, 2012; Li *et al.*, 2012). However, for the genus *Phaseolus*, studies of rhizobial diversity have focused mainly on the common bean (*P. vulgaris*) (Junier *et al.*, 2014; Aserse *et al.*, 2012; Grange *et al.*, 2007); in contrast, the knowledge of rhizobial diversity in the lima bean (*P. lunatus*) is limited to Peru and Mexico (Duran *et al.*, 2014; López-López *et al.*, 2013; Ormeno-Orrilo *et al*., 2007). Ormeño-Orrillo *et al.* (2007) observed that *P. lunatus* may be nodulated by bacteria of the genera *Rhizobium* and *Sinorhizobium.* Duran *et al.* (2014) reported that the lima bean is nodulated by *Bradyrhizobium* and found two new species of *Bradyrhizobium paxllaeri* and *Bradyrhizobium icense* in Peru.

However, the data obtained by Ormeno-Orrilo *et al.* (2007), Lopez-Lopez *et al.* (2013), and Duran *et al.* (2014) were from Peru, where *P. lunatus* is native. Peru is one of the known centers of origin and diversity of *P. lunatus.* It is unclear how rhizobial diversity is associated with *P. lunatus*

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in areas where this legume is not native, such as Brazil. Thus, we studied rhizobial diversity associated with *P. lunatus* in soils from Brazil.

MATERIALS AND METHODS

The soil samples used in this study were collected at 0.0 to 0.2 m depth in two regions (Nova Esperança and Santa Rita) with a history of *P. lunatus* cultivation. The *P. lunatus* variety was UFPI-491 ("Fava miúda"), obtained from the Active Germplasm Bank (AGB) from the Federal University of Piauí (UFPI). This variety was selected for being widely grown in the states of Piauí and Maranhão, Brazil. According to the data from AGB, the UFPI-491 accession is originated from Varzea Grande, PI, Brazil, and has seeds with white skin and average length and width of 17.53 and 18.18 mm, respectively.

The experiment was conducted in a greenhouse at the Plant Science Department of the Agricultural Science Center - UFPI, Campus of Socopo, from March 24 to June 06, 2013. Seeds were sown in plastic bags containing 5 kg soil originating from the selected area, following a completely randomized design with four replications. At sowing, seeds were placed four per bag; fifteen days after emergence, the thinning was performed, leaving one plant per bag. The bags were irrigated daily to maintain soil moisture close to field capacity (gravimetric method).

The nodules used in isolation and characterization were collected at 45 days after seedling emergence, when the number and biomass of nodules were the highest in a preliminary experiment. Immediately after harvest, nodules were desiccated in test tubes with silica gel, overlayed with a thin cotton layer, and stored in screw-cap vials.

Rhizobia isolation was performed at the Soil Biology Laboratory of Agronomical Institute of Pernambuco (IPA) according to the methodology used by Hungria *et al.* (2001). The isolates were kept in 20% (v/v) glycerol at "80°C for long-term storage and cultured in 10 mL YMB at 28°C for 4–5 days. DNA from isolates was extracted using the Invitek invisorb kit from GmbH bacteria as recommended by the manufacturer.

The 16S rRNA gene was amplified using the primers fD1 and rD1, which are able to amplify full-length 16S rDNA sequences in most bacterial genera (Weisburg *et al.*, 1991). Polymerase chain reaction (PCR) amplification was performed in a 25 μL volume mixing the template DNA (5 ng/μL) with polymerase reaction buffer (100 mM Tris–HCl, 15 mM MgCl2, 500 mM KCl, pH 8.3), 25 μM (each) deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate, 0.1 μM of each primer fD1 and rD1, and 2.5 U of *Taq* DNA polymerase (Promega). The following temperature profile was used for DNA amplification: an initial denaturation step of 94°C for 3 min, followed by 30 cycles of 94°C for 50 sec, 57°C for 50 sec, and 72°C for 1 min, and a final extension step of 72°C for 7 min. A negative control containing 1μL of water instead of DNA was included in every PCR run. PCR products were separated by electrophoresis in a Tris–Borate–EDTA (TBE) 0.5% agarose gel (80 V), and the gels were documented through the LabImagem 1D of Loccus photographed using LPIX-HE.

The 16S rRNA gene of the strains was purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The purified products were sequenced in both the forward and reverse direction using primers fD1 and rD1 and DYEnamicTM Terminator Cycle Sequencing Kit (Amersham Biosciences). Reaction products were resuspended in formamide and sequenced using a MegaBACE 1000 (Amersham Biosciences) capillary array DNA sequencing instrument. The 14 16s rRNA gene sequences determined in this study were deposited in the GenBank database (Table 1).

The nucleotide sequences were initially submitted to a BLAST search for preliminary species assignment (www.ncbi.nlm.nih.gov/blast). Additional pairwise comparisons were made with DNAMan version 4.0 (Lynnon Biosoft), using the Optimal Alignment option with the following parameters: k-tuple = 2, gap penalty = 7, gap open = 10, and gap extension = 5. Nucleotide sequences were aligned using the Clustal W program (www.ebi.ac.uk/clustalw). Phylogenetic analysis was performed using MEGA v. 6.0 (www.megasoftware.net) (Tamura *et al.*, 2013). The phylogenetic trees based on sequences of 16S rRNA genes were constructed using the neighbor-joining (NJ) algorithm (Saito and Nei, 1987) and maximum likelihood (ML) methods in MEGA v. 6.0 using the Kimura 2-parameter distance correction model (Kimura, 1980). Bootstrap support for each node was evaluated with 1000 replicates.

RESULTS AND DISCUSSION

Based on the similarity of partial 16S rRNA gene sequences (length 1376–1383 bp), 14 isolates were identified (Table 1), suggesting a high level of species diversity. The overall topologies of the phylogenetic trees obtained with the NJ and ML methods were similar (data not shown). Most isolates were divided into two main groups, *Bradyrhizobium* and *Rhizobium*, with a bootstrap confidence of 100% and 71%, respectively (Fig. 1). More than half of the strains studied were positioned in the *Bradyrhizobium* clade (in the *B. elkani* superclade), with a bootstrap confidence of 98%.

TABLE 1: List of rhizobial isolated from root nodules of *Phaseolus lunatus* L. (Lima bean) in Brazil and their identities and phylogenetic position

Isolate and reference strains	Geographic origin[a]	Identity based on partial 16S rRNA gene sequence			Phylogenetic assignments and Groups
		Closest species (accession number)	Identity (%)	Length (bp)[b]	
ISOL 6	NE	*Bradyrhizobium* sp. (KC677617)	99	1376	*Bradyrhizobium*
ISOL 14	SR	*Rhizobium* sp. (HQ906957)	99	1377	*Allorhizobium*
ISOL 15	SR	*Rhizobium tumefaciens* (KF875446)	99	1380	*Rhizobium*
ISOL 16	SR	*Rhizobium* sp. (AF510388)	99	1376	*Rhizobium etli*
ISOL 17	SR	*Bradyrhizobium* sp. (FJ390941)	100	1379	*Bradyrhizobium*
ISOL 18	SR	*Uncultured Bradyrhizobium* sp. (FJ193430)	99	1379	*Bradyrhizobium*
ISOL 19	SR	*Uncultured Bradyrhizobium* sp. (FJ193273)	99	1380	*Bradyrhizobium*
ISOL 20	SR	*Bradyrhizobium* sp. (KC677617)	100	1379	*Bradyrhizobium*
ISOL 21	SR	*Rhizobium* sp. (JF722653)	99	1378	*Rhizobium tropici*
ISOL 26	SR	*Bradyrhizobium* sp. (FJ390941)	100	1379	*Bradyrhizobium*
ISOL 27	SR	*Rhizobium miluonense* (JN896360)	99	1377	*Rhizobium tropici*
ISOL 35	NE	*Bradyrhizobium* sp. (FJ390941)	99	1379	*Bradyrhizobium*
ISOL 37	NE	*Bradyrhizobium* sp. (KC677617)	100	1379	*Bradyrhizobium*
ISOL 38	NE	*Rhizobium* sp. (JX566578)	99	1383	*Rhizobium*

[a] Relevant characteristic of the isolates used in this study (NE – Nova Esperança; SR – Santa Rita).
[b] The length of 16s rRNA gene sequences of the test strains used for identification of them using Genbank database (NCBI) blast program.

B. elkanii is a species used as inoculant on soybean in Brazil (Torres *et al.*, 2012). The *Bradyrhizobium* strains grouped distantly from *B. icense* and *B. paxllaeri*, the only species of *Bradyrhizobium* obtained from *P. lunatus*, in Peru (Durán *et al.*, 2014).

One strain was positioned in the *R. etli*/*R. phaseoli* clade (from now on called the *R. etli* group). Two strains were grouped within the *R. tropici* group. *R etli* and *R. tropici* are natural, rizhobia-nodulating, common bean (*P. vulgaris* L.) in Brazil (Grange *et al.*, 2007). Two strains were placed in the *R. radiobacter* (formerly *Agrobacterium radiobacter*) phylogenetic branch. The remaining strain was positioned in the *Allorhizobium* clades.

Considering that a level of sequence identity of 98.65% for the 16S rRNA genes may represent species demarcation (Kim *et al.* 2014) or even 97% sequence identity as species limit, all strains appear to be species of *Rhizobium* and *Bradyrhizobium*, associated with *P. lunatus*, with minimum identities of 99% (Table 1). Furthermore, the strains ISOL16, ISOL21, and ISOL27, which were grouped distantly from the reference strains of the *R. etli* and *R. tropici* groups with bootstrap confidences of 99%, may represent new lineages.

Prior to this study, there were no studies of rhizobial species diversity of nodulating *P. lunatus* in Brazil. Rhizobia associated with *P. lunatus* have not been well studied. Their taxonomic position is unclear, although some studies have been conducted already with *P. lunatus* (Durán *et al*. 2001; López-López *et al*. 2013; Ormeño-Orrillo *et al*. 2006). Studies focusing on rhizobial diversity are focused usually on symbionts in centers of legume diversity (Lie *et al.*, 1987). Because Brazil is not considered a center of *P. lunatus* diversity, the genetic diversity found among the strains may be considered high as compared with previous studies of

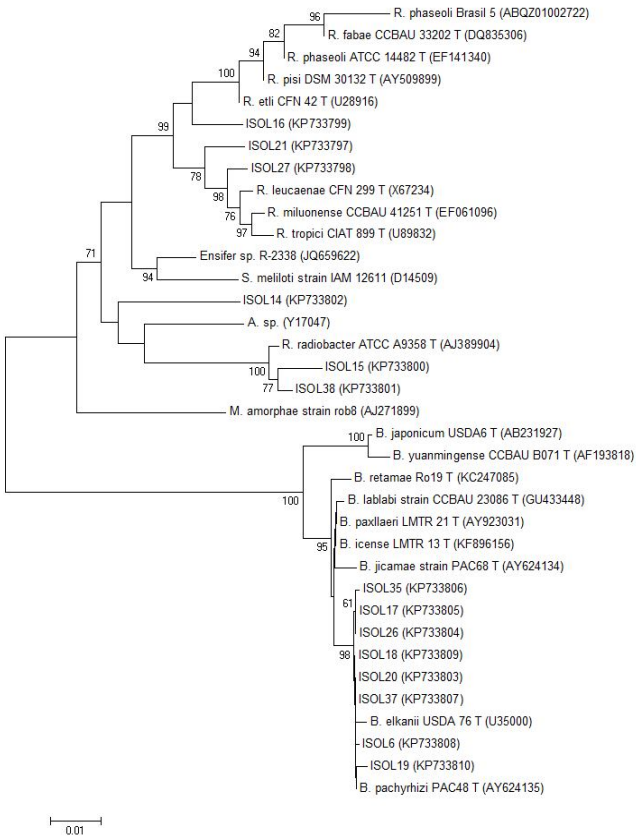

FIG 1: Neighbor-joining phylogeny of 16S rRNA gene sequences (1129 aligned positions). Accession numbers are indicated within parenthesis. Numbers above the branches are bootstrap percentages (for clarity, only values of 60% are shown). GenBank accession numbers are given after the strain names. The scale bar indicates the number of substitutions per site.

rhizobia isolated from legumes and their sites of origin, such as *P. vulgaris* (Souza *et al.*, 1994) and the *P. lunatus* (Ormeno-Orrilo *et al.*, 2006). Previously, Santos *et al.* (2011) found high morphological and physiological diversity among native nodular rhizobia of *P. lunatus* in Northeast Brazil. Also, Santos *et al.* (2011) found isolates with rapid and intermediate growth, as found in the genera *Sinorhizobium*, *Rhizobium*, and *Bradyrhizobium*. Our results showed rhizobia associated with *P. lunatus* that were different from those found by Ormeno-Orrillo *et al.* (2006) in the soil of Peru, a center of domestication of *P. lunatus*, where the genus *Bradyrhizobium* is the predominant symbiont.

Previously, the symbiotic effectiveness of these rhizobial isolates found in this study was evaluated by Antunes *et al.* (2011) which compared the isolates with two reference *Rhizobium* strains CIAT 899 and NGR 234. They found eight isolates with higher N accumulation and N_2-fixation efficiency compared with the reference strains CIAT 899 and NGR 234. It shows that these isolates present strong potential to improve N fixation in *P. lunatus* and also to be selected for practical utility as inoculant.

Finally, according to the analysis of the partial sequence of the 16S rRNA genes of 16 rhizobia strains, there is high species diversity of rhizobia-nodulating *P. lunatus* in Northeast Brazil, including potential new species.

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