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ORIGINAL ARTICLE

Genetic diversity among native isolates of rhizobia from *Phaseolus lunatus*

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Abstract The genetic diversity among native nodular rhizobia of Lima bean in soil from Piauí State, Brazil, was characterized and evaluated. The genotype UFPI-491 was used as a trap plant for rhizobia, with soil samples collected in Piaui State being used as the inoculum source. For isolation, nodules were collected at 45 days after seedling emergence—a period that presented the highest values for number and biomass of nodules evaluated in a previous experiment. In total, 50 isolates were obtained and placed into groups based on divergence from the morphological and physiological characterization. In general, the restriction patterns obtained with endonucleases *MboI*, *HaeIII* and *NheI* showed sufficient variability to discriminate between the isolates in this study. The main characteristics exhibited by isolates identified species from the genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*.

Keywords Divergence phenotypic · Biological N_2 fixation · Nodulation

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Introduction

Phaseolus lunatus, the lima bean, is the second most economically important species of *Phaseolus* and 1 of the 12 primary grain legumes (Fofana et al. 1999). This crop shows high rusticity and the capacity to resist long dry periods. These characteristics are important for success in semi-arid regions and increase the economic and social importance of the crop (Azevedo et al. 2003).

Among the legumes cultivated throughout the world, *P. lunatus* is one that has the ability to perform biological nitrogen fixation (BNF) due to symbiosis between this legume plant and nitrogen-fixing bacteria (Schultze and Kondorosi 1998). These bacteria, commonly known as rhizobia, invade root tissues and induce the formation of specialized structures known as nodules, where they differentiate and fix atmospheric nitrogen that is then supplied to the plant (Santos et al. 2007). The agronomic implications of this symbiosis have promoted research on the diversity of these bacteria, mainly in tropical soils brackets (Ormeno-Orrillo et al. 2006).

In recent years, several papers have reported the diversity of rhizobia isolated in a variety of host legumes (Giongo et al. 2008; Grossman et al. 2005; Loureiro et al. 2007; Mahdhi et al. 2008; Musiyiwa et al. 2005). The results showed a great diversity of rhizobia in soil around the world. In association with the genus *Phaseolus*, the literature has shown that studies of rhizobial diversity refer mostly to symbiosis of the common bean (*P. vulgaris*) (Martínez-Romero et al. 1991; Martínez-Romero 2003; Souza et al. 1994).

By contrast, rhizobia associated with *P. lunatus* have been far less studied. Ormeno-Orrillo et al. (2006) studied genetic diversity of native rhizobia isolated from the lima bean in Peru. They observed that lima beans are nodulated mainly by the genus *Bradyrhizobium*. However, a recent study conducted in the same location (Ormeño-Orrillo et al.

2007) observed that naturally growing species of nodulating *P. lunatus* belonged more commonly to the genus *Rhizobium* than to *Sinorhizobium*.

Zilli (2004) analyzed the diversity of *rhizobium* populations in recently clear-cut areas of the Cerrado, during a rice-cowpea and soybean rotation, using genotypic [amplified ribosomal DNA restriction analysis (ARDRA) Heyndrickx et al. 1996] and morphologic/phenotypic characterization methods, and concluded that understanding native rhizobial population diversity will ultimately contribute to the selection of inoculants for soybean and cowpea in the Brazilian Cerrado. Analysis of gene sequences coding for 16S rRNA (16S rDNA) have been used to infer phylogenetic relationships among species of rhizobia—homology between the sequences of 16S rRNA being the criterion most frequently used to estimate phylogenetic relationships among bacteria. One advantage of this approach is that DNA sequences and gene products can be compared in an evolutionary context (molecular systematics) (Van Berkun et al. 2000). Typing by DNA-based PCR can also be combined with restriction enzymes in methods such as ARDRA. The gene for 16S or 23S rDNA with or without the intergenic region is amplified by PCR with universal primers and then digested with different combinations of restriction enzymes. In contrast to other methods of analysis of fragments, such methods generate species-specific patterns (Gürtler et al. 1991) considering the conserved nature of rRNA genes.

However, Fofana et al. (1999) obtained isolates from areas where this legume is native. Peru is one of the known centers of origin and diversity of *P. lunatus*. There have been no studies on rhizobial diversity associated with lima beans in areas where this legume is not native, such as Brazil. Thus, we studied the genetic diversity among nodulating rhizobia of lima beans in soils from Piauí State, Northwest Brazil.

Material and methods

The soil samples used in this study were collected at 0.0 to 0.2 m depth in ten areas with a history of lima bean cultivation. The selected areas are located in two regions of Piaui state (07°61′970″S and 93°43′538″W). The main soil type is an Orthic Acrisol (Typic Hapludult, US taxonomy). These soils samples were analyzed in the Soil Fertility Laboratory of the Federal University of Piauí. The values of pH ranged from 6.4 to 7.2. Soil organic matter content ranged from 24 to 30 g kg^{-1} soil. The phosphorus and potassium contents ranged from 60.2 to 75.3 and 50.1 to 62.5 mg kg^{-1} soil, respectively. The sand, silt and clay content ranged from 31.4 to 43.7, 20.1 to 30.0 and 10.4 to 25.6%, respectively.

The lima bean genotype was UFPI-491 ("Fava miúda"), obtained from the Active Germplasm Bank of Fava Bean, Universidade Federal do Piauí (BAG of fava bean of UFPI). This genotype was selected for being relatively well cultured in the States of Piauí and Maranhão. According to the data from BAG, the UFPI-491 genotype, originating from Varzea Grande City, PI, 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 Production Department of the Agricultural Sciences Center, UFPI, Campus of Socopo, from 24 March to 6 June 2007. The genotype was sown in plastic bags containing 5 kg soil originating from the selected area, following a completely randomized design with four replications. The release of free-N mineral fertilizer was applied at planting, according to the technical recommendations for the crop (Vieira 1992). At sowing, seeds were placed four per bag and thinning was performed 15 days after emergence, leaving one plant per bag. The bags were irrigated daily to maintain soil moisture close to field capacity (gravimetric method).

The nodules used for isolation and characterization of rhizobia strains were collected 45 days after seedling emergence—a period that yielded the highest values for number and biomass of nodules in a preliminary experiment. Immediately after collection, nodules were allowed to desiccate in test tubes with gel silica and a thin cotton layer screw cap-sealed.

Rhizobia isolation, as well as morphological, physiological and biochemical characterization of isolates, was performed at the Soil Biology Laboratory of Agronomical Institute of Pernambuco (IPA) according to the methodology used by Hungria (1994). The isolation was performed in Petri dishes containing YMA culture medium, pH 6.8, with Congo red indicator, and incubated at 28°C for the period necessary to highlight the colonies' growth characteristics (Vincent 1970). Morphological characteristics recorded included colony shape (CS: R round, E ellipsoid), elevation (EL: C convex, F flattened) and color (CC: WM white/milky, T transparent).

The physiological characteristics were evaluated for isolates grown in bromothymol blue medium measuring growth time (GT: F fast, 1–3 days; I intermediate, 4–5 days), formation of acids and alkalis (FAA: AC acid, AL alkaline), formation of mucus (FML: A absent, P present), volume of mucus (VM: MU much, M Medium, L little, D dry) (Melloni et al. 2006); mucus elasticity (ME: P presence of wire, A absence of wire) and production of bubbles (PB: P present, A absent). Gram tests were performed in all isolates. All isolates presented pink staining (Gram negative).

The native isolates were characterized by ARDRA having as references the type strains: *Rhizobium* sp NGR234 (ER1), *Mesorhizobium mediterraneanus* BR523 (ER2), *Rhizobium etli* CFN42 (ER3), *Ensifer fredii* USDA205 (ER4), *Bradyrhizobium japonicum* BR111 (ER5) and *Rhizobium tropici* CIAT899 (ER6). Type strains

Table 1 Morphological and physiological characteristics of nodular rhizobia isolates from fava beans, originated from soil of Piauí state, Brazil

Isolate	CS[a]	EL[b]	CC[c]	GT[d]	FAA[e]	FM[f]	VM[g]	ME[h]	PB[i]
ISOL1	R	C	WM	I	Ac	A	D	A	P
ISOL2	R	C	T	F	Ac	P	L	P	A
ISOL3	R	C	T	F	Ac	P	MU	P	P
ISOL4	R	C	WM	F	Ac	P	M	A	P
ISOL5	R	C	WM	F	Ac	P	MU	A	P
ISOL6	R	C	WM	I	Al	P	M	A	P
ISOL7	R	C	WM	I	Ac	P	M	P	P
ISOL8	R	C	WM	F	Al	P	L	P	P
ISOL9	R	C	WM	F	Ac	P	L	P	P
ISOL10	R	C	WM	I	Ac	P	MU	A	P
ISOL11	R	C	T	F	Ac	P	M	P	P
ISOL12	R	C	WM	I	Al	P	L	P	P
ISOL13	R	C	T	F	Al	P	L	A	P
ISOL14	R	C	WM	I	Ac	P	MU	A	P
ISOL15	E	C	WM	I	Ac	P	MU	A	P
ISOL16	R	C	T	I	Ac	P	MU	P	P
ISOL17	R	C	WM	I	Al	P	L	A	P
ISOL18	R	C	T	I	Al	P	MU	A	P
ISOL19	R	C	WM	I	Al	P	M	A	P
ISOL20	R	C	T	I	Al	P	M	A	P
ISOL21	R	C	WM	F	Ac	P	MU	P	P
ISOL22	R	C	WM	I	Ac	P	MU	A	P
ISOL23	R	C	WM	I	Al	P	MU	A	P
ISOL24	R	C	WM	I	Al	P	M	A	P
ISOL25	R	C	WM	I	Al	P	MU	A	P
ISOL26	R	C	WM	F	Al	P	L	A	P
ISOL27	R	C	WM	I	Ac	P	MU	A	P
ISOL28	R	C	WM	I	Al	P	M	A	P
ISOL29	R	C	WM	I	Al	P	M	A	P
ISOL30	R	A	WM	F	Al	P	M	A	P
ISOL31	R	A	WM	F	Al	P	M	A	P
ISOL32	R	C	WM	I	Al	P	MU	A	P
ISOL33	R	C	WM	I	Ac	P	MU	A	P
ISOL34	R	C	WM	I	Ac	P	M	A	P
ISOL35	R	C	WM	I	Al	P	MU	A	P
ISOL36	R	C	WM	F	Ac	P	M	A	P
ISOL37	R	C	WM	I	Al	P	MU	A	P
ISOL38	R	A	WM	F	Ac	P	MU	A	P
ISOL39	R	C	WM	F	Ac	P	MU	A	P
ISOL40	R	A	WM	F	Ac	P	MU	A	P
ISOL41	R	C	WM	F	Ac	P	MU	A	P
ISOL42	R	C	WM	F	Al	P	MU	A	P
ISOL43	R	C	WM	F	Al	P	L	A	P
ISOL44	R	C	WM	F	Ac	P	MU	A	P
ISOL45	R	A	WM	I	Ac	P	MU	A	P
ISOL46	R	C	WM	I	Al	P	M	A	P
ISOL47	R	C	WM	I	Ac	P	MU	A	A
ISOL48	R	C	WM	F	Ac	P	MU	A	P
ISOL49	R	A	WM	I	Ac	P	MU	A	A
ISOL50	R	C	WM	I	Ac	P	MU	A	P

[a] Morphological forms (*R* rounded, *AE* ellipsoid)

[b] Elevation (*C* convex, *A* flattened)

[c] Color (*WM* white/milky, *T* transparent)

[d] Physiological growth time (*F* fast, *I* intermediate)

[e] Formation of acid and alkalis (*Ac* acidic, *Al* alkaline)

[f] Formation of mucus (*A* absent, *P* present)

[g] Volume of mucus (*D* dry, *L* low, *M* medium, *MU* much)

[h] Mucus elasticity (P presence of wire, *A* absence wire)

[i] Production of bubbles (*A* absent, *P* present)

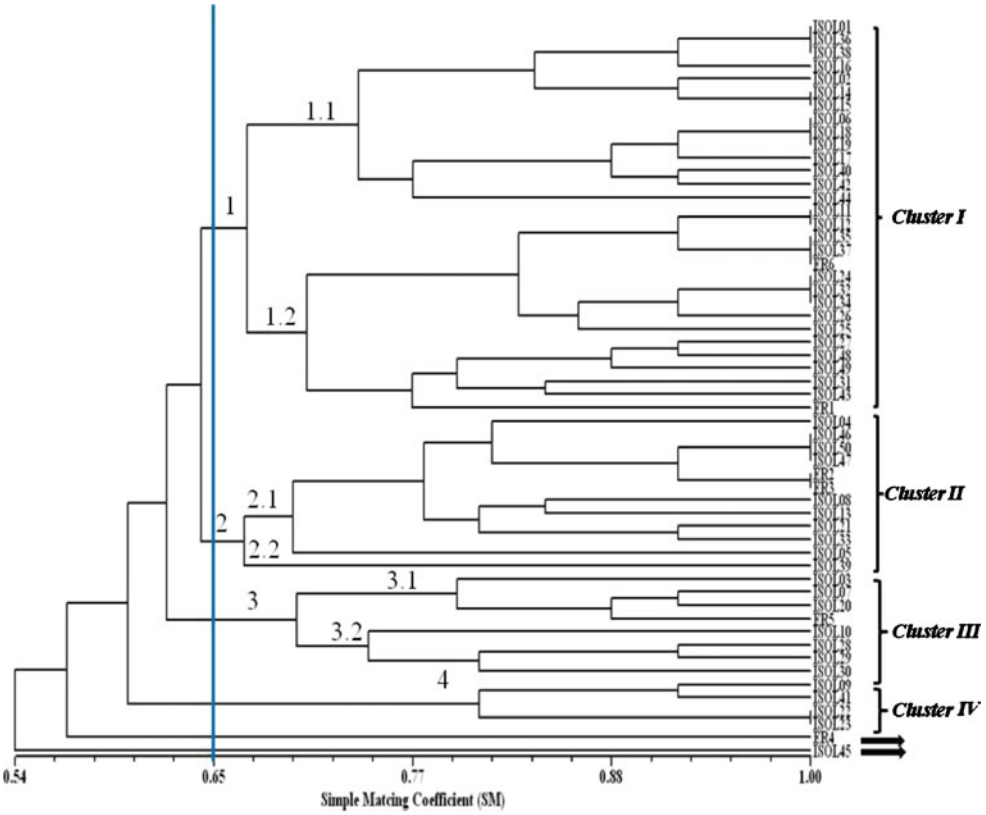

Fig. 1 Dendrogram of genetic similarity compiled from 50 isolates obtained by unweighted pair group method with arithmetic mean (UPGMA) based on the amplified ribosomal DNA restriction analysis (ARDRA) patterns with the enzyme *Mbo*I

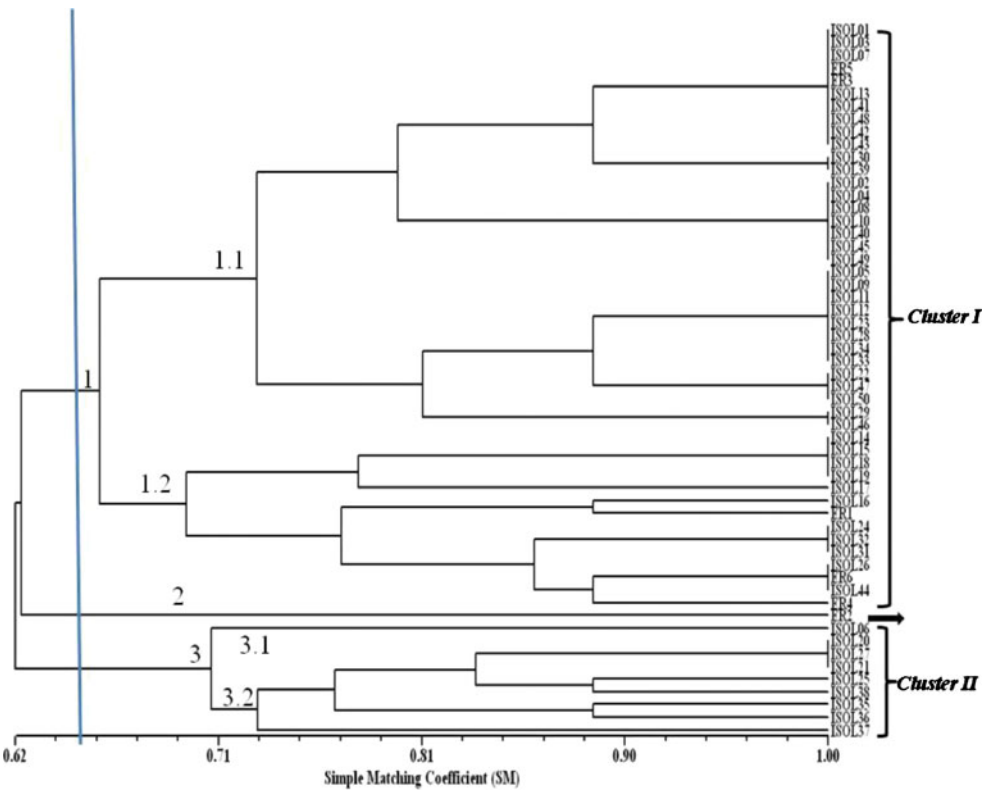

Fig. 2 Dendrogram of genetic similarity compiled from 50 isolates obtained by UPGMA based on the amplified ribosomal DNA restriction analysis (ARDRA) patterns with the enzyme *Nhe*I

were provided by EMBRAPA-Agrobiologia, RJ, Brazil (Dra. Rosa Pittar) and were preserved at −80°C in the Laboratório de Genoma (IPA). The DNA from isolates was extracted using the Invitek Invisorb kit (http://www.invitek.de) as recommended by the manufacturer. 16S rDNA genes were amplified using primers fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rD1 (5′-AAG GAG GTG ATC CAG CC-3′) and subsequently digested with endonucleases *Mbo*I, *Nhe*I and *Hae*III.

After digestion, cleavage was confirmed by visualizing the banding pattern obtained upon agarose gel electrophoresis (2.5%) in 0.5X TBE buffer for 4 h at 80 V with dye SybrGold (Invitrogen; http://www.invitrogen.com) and photographed using a Loccus LPIX-HE (http://www.loccus.com.b). The restriction patterns (ribotypes) were characterized by the number and size of the fragments obtained. Dendrograms were constructed with the program NTSYS-pc v.2.1 (http://www.exetersoftware.com), the similarity matrices used the correlation coefficient Simple Matching (SM), and the unweighted pair group method with arithmetic mean (UPGMA) algorithm was used according to the electrophoretic profiles obtained.

Results and discussion

Upon cultivation of the *P. lunatus* UFPI-491 genotype used as a trap plant, 50 pure isolates with phenotypic characteristics different from bacterial colonies were obtained (Table 1). The morphological characteristics, in general, showed low variation in colony form among the isolates. All isolates presented with a rounded shape, except ISOL15 and ISOL8, which presented with ellipsoid shapes. The isolates obtained yielded convex and flattened colonies. Most isolates showed white colonies with milky appearance, while a few isolates showed transparent colonies. According to Jordan (1984), colors found in colonies of rhizobia include white, yellow or pink, although it is unusual to find rhizobia forming yellow or pink colonies (Hungria 1994; Santos et al. 2007; Soares et al. 2006).

Results indicate morphological diversity between native nodular rhizobia of fava in Piauí soils. Morphological diversity among other groups of rhizobia was also found by Silva et al. (2007) and Martins et al. (1997) for isolates obtained in soils from Pernambuco and Mato Grosso do Sul, respectively.

The isolates showed rapid and intermediate growth. The growth patterns observed indicate that the isolates may be more adapted to the edaphoclimatic conditions of the region, since rapid and intermediate growth favor greater survival in the soil as compared to slow-growing isolates (Medeiros et al. 2007).

Concerning the production of acid and alkali compounds, a prevalence of acid reactions was observed, although some isolates presented alkaline reactions. The presence of intermediate-growth isolates with production of alkali,

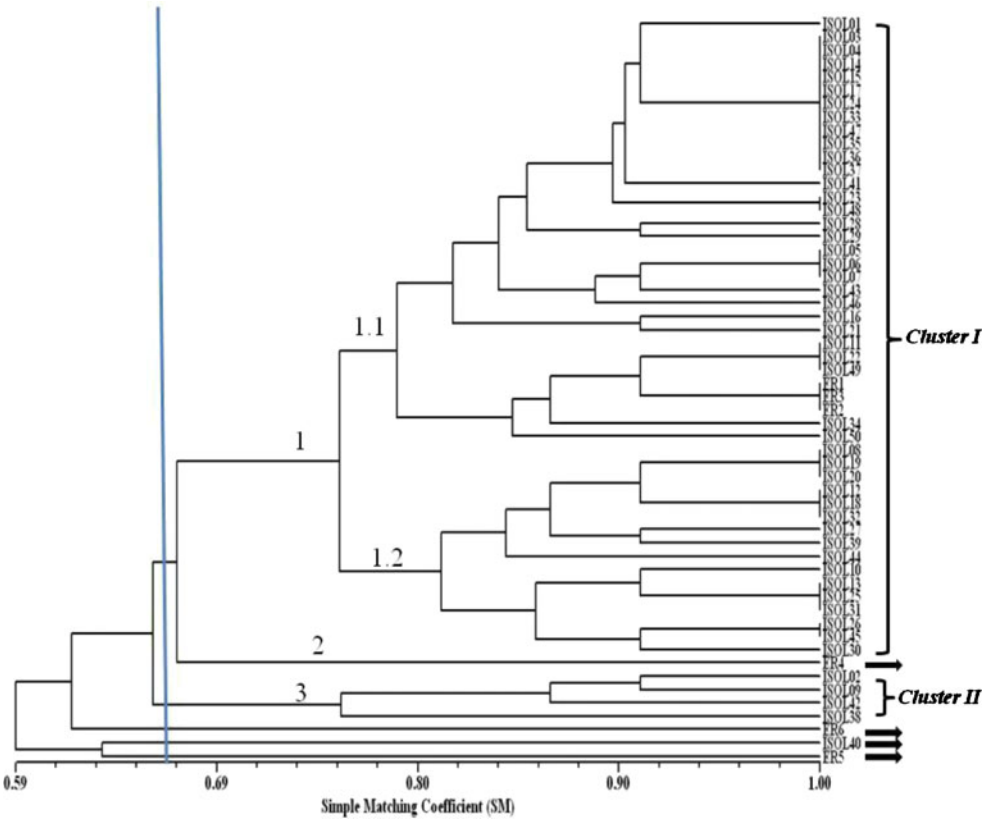

Fig. 3 Dendrogram of genetic similarity compiled from 50 isolates obtained by UPGMA based on the amplified ribosomal DNA restriction analysis (ARDRA) patterns with the enzyme *Hae*I

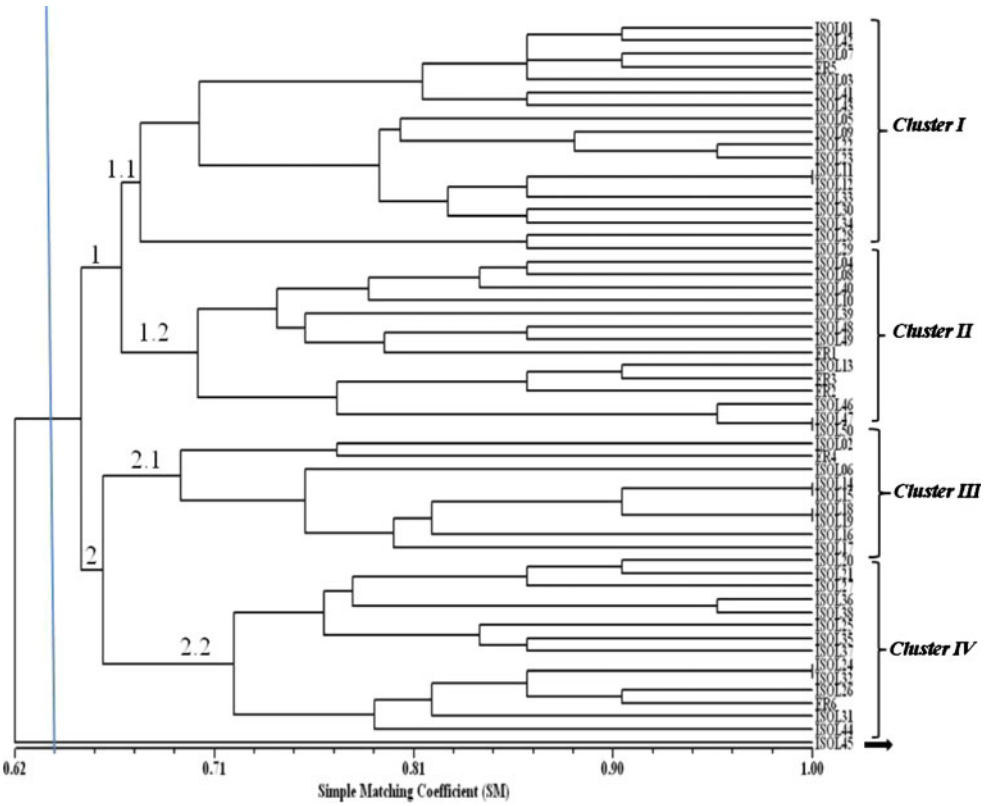

Fig. 4 Dendrogram of genetic similarity compiled from 50 isolates obtained by UPGMA based on the amplified ribosomal DNA restriction analysis (ARDRA) patterns with enzymes *Mbo*I and *Nhe*I

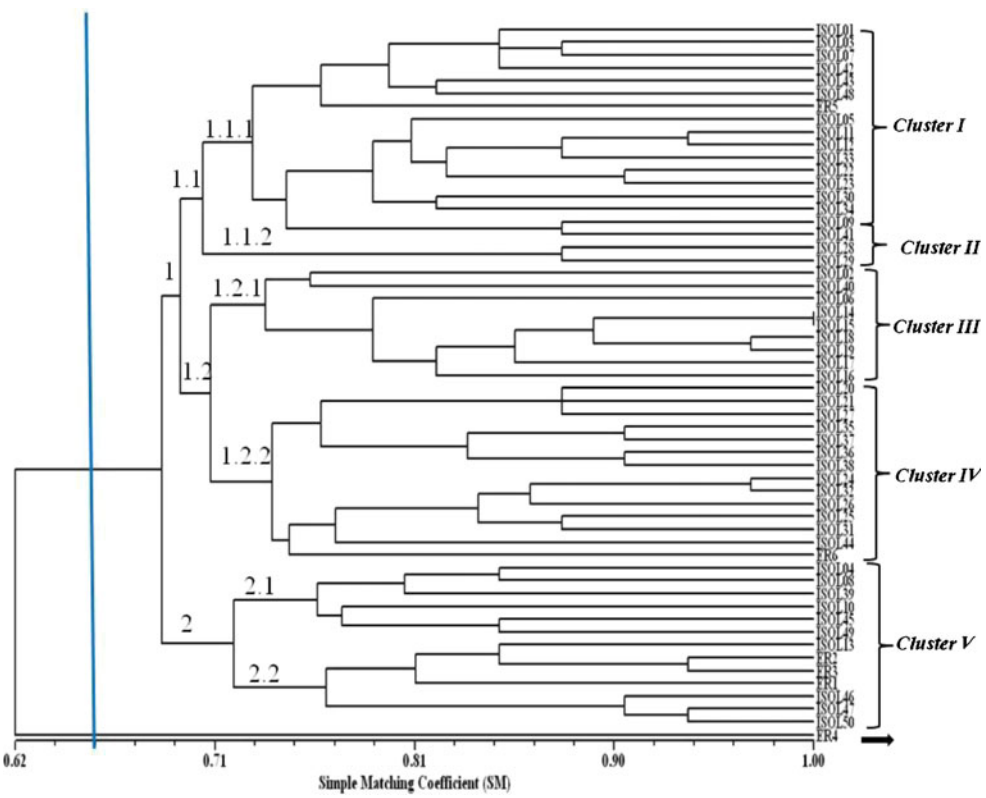

Fig. 5 Dendrogram of genetic similarity compiled from 50 isolates obtained by UPGMA based on the amplified ribosomal DNA restriction analysis (ARDRA) patterns with the enzymes *Mbo*I, *Nhe*I and *Hae*III

characteristics that exemplify the genus *Bradyrhizobium* (Vargas et al. 2007) suggests that this genus performs symbiosis with *P. lunatus*. Similar results were observed by Ormeno-Orrillo et al. (2006) in isolates obtained from lima bean nodules grown in the soil of Peru, where the genus *Bradyrhizobium* is the predominant symbiont. The bacteria from the genus *Bradyrhizobium* are able to form symbiosis with several species, such as *Phaseolus vulgaris* (Parker 2002), *Glycine max* (Hungria 1993), *Vigna unguiculata* (Zilli et al. 2006) and *Arachis hypogaea* (Gerin et al. 1996).

Following the classification proposed by Melloni et al. (2006), 50% of isolates presented colonies with high production of mucus. However, isolated colonies varying among moderate, low and no production of mucus (ISOL1) were observed. The production of mucus (exopolysaccharides) in most isolates is an important feature related to the initial infection of roots (Freitas et al. 2007). Thus, isolates that show this characteristic possess a greater competitive advantage in infection, colonization and formation of nodules.

The phenotypic characteristics observed for isolates suggest that they were subject to different selective environmental pressure. Additionally, according to Hartwig (1998), in addition to edaphoclimatic conditions, genotypic characteristics of the macrosymbiont influence symbiotic specificity. Some studies in Brazil have shown variability in nodulation by rhizobial strains in soybeans (Bohrer and Hungria 1998), common-bean (Franco et al. 2002) and cowpea (Xavier et al. 2006).

According to the classification scheme proposed by Wang et al. (2008), to date six genera have been proposed: *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium*. Based on the sequences of 16S rRNA genes, all defined rhizobia are members of the subdivision of the Proteobacteria. This indicates that, like other tropical legumes (Santos et al. (2007), *P. lunatus* also exhibits symbiosis with a wide range of rhizobia. Generally, the restriction patterns obtained with endonucleases *Mbo*I (Fig. 1), *Nhe*I (Fig. 2) and *Hae*III (Fig. 3) showed sufficient variability to discriminate between the isolates in this study. The enzyme *Mbo*I showed the most discrimination among isolates, giving a banding pattern very different between each. The simple matching coefficient (SM) was set to 65% genetic similarity in all the dendrograms formed, and we observed four clusters for this enzyme. Cluster 1 was composed of 28 strains grouping with reference strains ER6 (*Rhizobium tropici* CIAT899) and ER1 (*Rhizobium* sp NGR234). In cluster 2, ten isolates clustered with strains ER2 (*Mesorhizobium mediterraneus* BR523) and ER3 (*Rhizobium etli* CFN42). In cluster 3, seven isolates clustered with ER5 (*Bradyrhizobium japonicum* BR11). Finally, in cluster 4, only four isolates grouped and the ISOL45 and ER4 (*Ensifer fredii* USDA205) formed monophyletic branches.

With endonucleases *Nhe*I and *Hae*III, the variability was lower, showing that these enzymes are not sufficient to detect the genetic variability in these isolates. When we compiled the data matrix with two enzymes, *Mbo*I + *Nhe*I (Fig. 4), and *Nhe*I + *Hae*III showed that genetic variability was greater among isolates in all dendrograms compiled, and that the ER4 reference behaved as an outgroup. The phylogenetic tree constructed from the compilation of the matrices of the three endonucleases *Mbo*I, *Nhe*I and *Hae*III showed three major clusters and strain ER4 with the same behavior mentioned above (Fig. 5). In cluster 1 and 2, 18 isolates were grouped together with *B. japonicum* BR11). In cluster 3 and 4, 21 isolates were clustered and almost all seem to be different isolates, except isol14 and isol15, which were identical and appear raw, monophyletic to the ER6 reference strain of *Rhizobium tropici* CIAT899. In cluster 5, the three reference strains *M. mediterraneus* BR523, *R. etli* CFN42, and *Rhizobium* sp NGR234 were grouped with ten isolates. As in this study, the fragment used for amplification and subsequent ARDRA analysis of 16S rDNA was isolated and showed a great diversity and complexity of trees.

Conclusion

We can conclude that most of the isolates may be bacteria forming symbiosis with *P. lunatus* and have not been characterized genetically since, according Rosado et al. (1999), phylogenetically more distant species are the most isolated and using the 16S rDNA fragment may be useful for classification. The complexity of the phylogenetic trees indicates that the 16S rDNA gene studied in isolates proved to have genetic characteristics quite distinct from the bean culture. This may indicate that different soils and different geographic regions may have interfered with this diversity.

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