Diversity of rhizobia isolated from nodules of indigenous tree legumes from the Brazilian dry forest

Diversidad de rizobios aislados de nódulos de leguminosas arbóreas nativas del Bosque Seco Tropical en Brasil

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Abstract

The diversity of rhizobial isolates in tropical dry forests (TDF) has been studied due to the great importance of finding new species of bacteria capable of fixing nitrogen in tree legumes. In the Brazilian TDF (caatinga), Leguminosae is the most important family of plants, so the knowledge of microbial communities that make symbioses with native plants can help in the understanding of the interaction plant-microorganisms, as well as in the optimization of biological processes that can improve the cultivation system in an area so rich and understudied. In this study was determined the characteristics of rhizobia isolated from nodules of *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Mimosa caesalpiniifolia* Benth, grown in soils collected under caatinga vegetation. The phenotypic and molecular characterization of the isolates, with use of fingerprint markers such as BOX, REP, ERIC and BOX-PCR were performed. Amplification technique by duplex PCR with the *nif*H and *nod*C genes was used for the authentication of isolates. The results showed that given the variation found in the amplification of the *nif*H and *nod*C genes, the duplex PCR technique can show false-positive results, as these genes have a very large polymorphism. The lack of knowledge of isolates that make symbioses with these plants further help to conclude that this authentication technique cannot yet be applied to all legume-nodulating isolates. Regarding the analyses made with fingerprint markers, it was observed that all were very efficient in the ability to distinguish species and that the greater the number of markers used, the safer the knowledge on the taxonomy and diversity of rhizobia.

Keywords: diversity Index; Duplex-PCR; fingerprint markers; *nif*H gene; *nod*C gene; polyphasic approach.

Resumen

La diversidad de aislamientos rizobianos en el Bosque Seco Tropical de Brasil (BST) (Caatinga) ha sido ampliamente estudiada debido a la gran importancia de encontrar nuevas especies de bacterias con capacidad para fijar nitrógeno en leguminosas arbóreas, entre ellas, *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke y *Mimosa caesalpiniifolia* Benth. El bioma del BST es importante en Brasil, por tanto, el conocimiento de comunidades microbianas que hacen simbiosis con plantas autóctonas ayuda en la comprensión de la interacción planta-microorganismos, así como en la optimización de procesos biológicos que pueden mejorar el sistema de cultivo en un área como ésta, rica en diversidad y que ha sido poco estudiada. Este estudio se realizó en tres municipios del Sertão de Pernambuco y Paraíba, con las especies antes citadas. Para el efecto, se realizaron las mediciones siguientes: aislamiento de rizobios y su caracterización fenotípica y molecular con el uso de marcadores de fingerprint como BOX, REP, ERIC y BOX-PCR; y confirmación de los aislamientos, utilizando la técnica de amplificación por dúplex-PCR con los genes *nif*H y *nod*C. Los resultados mostraron que dada la variación encontrada en la amplificación de los genes *nif*H y *nod*C, la técnica duplex-PCR puede mostrar resultados falsos positivos, ya que estos genes tienen un polimorfismo muy grande. La falta de conocimiento de los aislamientos que hacen simbiosis con estas plantas ayuda, aún más, a concluir que esta técnica de autenticación todavía no puede ser aplicada a todos los aislados nodulantes de leguminosas. En cuanto a los análisis realizados con

marcadores de *fingerprint* (huellas digitales), se observó que todos eran muy eficientes en la capacidad de distinguir especies y que cuanto mayor es el número de marcadores utilizados, más seguro es el conocimiento sobre la taxonomía y diversidad de rizobios.

Palabras Clave: abordaje polifásico; PCR-Duplex; índice de diversidad; huella genética; *nif*h gen; *nod*c gen.

Introduction

Tropical dry forests cover more than 40% of the tropical vegetation which corresponds to 1,048,700 km^2, mainly distributed between America, Africa and Eurasia, but also in a small proportion (3.8%) in Australasia and southeast Asia (Miles *et al.*, 2006). Floristically, the Leguminosae represent the dominant family in these forests, which may lead to believe that these forests are presumed a hotspot of symbiotic N fixation.

This high diversity of species of legumes in Caatinga may be providing the maintenance of the diverse population of rhizobia in the soil. These soils represent natural repositories of new potential elite strains of rhizobia for inoculation of legumes (Boakye *et al.*, 2016).

Rhizobia are classified into two major types according to Gyaneshwar *et al.* (2011), including α-Proteobacteria and β-proteobacteria. Liu *et al.* (2007) thoroughly studied rhizobia obtained from native leguminous trees through phenotypic characteristics and genotypic and molecular classification of isolates within the following genera: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium* and *Azorhizobium*, and Liu *et al.* (2012) proposed a new reclassification of strains with new genera: *Burkholderia*, *Azorhizobium, Methylobacterium, Devosia, Burkholderia, Cupriavidus* and *Shinella*, respectively.

Lafay and Burdon (2007) studying the molecular diversity of legume-nodulating bacteria, observed that, in Australia tropical areas are centers of biodiversity and biodiversification to root-nodulating legumes. In other words, the biodiversification aims to spread the risk, minimize the ecological loss and maximize the overall performance of the ecosystem, while biodiversity refers to the variety and variability of microorganisms in the environment.

Polyphasic studies are being increasingly applied in microbial diversity, considering many morpho-physiological parameters of colonies to help group the native strains, e.g.: growth time, consistency, change in pH (acid and alkali production), mucus, edge of the colony, shape, color and diameter of the colonies; besides the use of fingerprint molecular markers such as BOX, ERIC (Enterobacterial Repetitive Intergenic Consensus) and REP (Repetitive Extragenic Palindromic Sequences) (Versalovic *et al.*, 1994). It is also observed the use of specific genes such as 16S rRNA (Kim *et al.*, 2014) and *nif*H and *nod*C (Fernandes Júnior *et al.*, 2013; Languere *et al.*, 2001) for studies on the diversity and phylogeny of microorganisms.

The most important factor for greater understanding of the diversity of soil microflora and the optimization of biological processes which increase the sustainability of agricultural systems is the concept of species diversity, assessed by two components: species richness, based on the total number of species and uniformity, which is based on the relative abundance of species and the degree of dominance, according to Simpson, Margalef, Wiener and Shannon index (2015). The application of these indices reveals the extent of diversity of an ecosystem, as well as its stability.

A study by Freitas et al. (2014), with native isolates from nodules of *Mimosa* spp. in the Caatinga, using molecular techniques, found that the isolates are more related to β-Rhizobia than to α-Rhizobia and all have fast growth habit. Mishra *et al.* (2012) demonstrated that *Mimosa* spp. forms symbioses with both types of rhizobia in their native environments in South and Central America, including *Burkholderia*, *Cupriavidus* and *Rhizobium* species. This study aimed to assess the diversity of microorganisms in isolates from nodules of three native tree legumes of the dry forest.

Materials and methods

Soil sampling and tree legume cultivation

Soil samples from the 0 – 20 cm superficial layer were collected in areas of preserved Caatinga vegetation in three municipalities, with different climate conditions (Table 1): (1) Santa Terezinha, in the Sertão Zone of the state of Paraíba; (2) Remígio, in the Agreste zone of this same state; and (3) Serra Talhada, in the Sertão Zone of the state of Pernambuco. The composition and structure of the vegetation in the three areas were described by Souza (2011). Number of species and tree heights and stem diameters are higher

in the Agreste zone than in the Sertão Zone and, regarding this last zone, the values were higher in Serra Talhada than in Santa Terezinha, probably reflecting higher water availability.

Table 1. General characteristics of preserved Caatinga areas in three municipalities, in the States of Paraíba (PB) and Pernambuco (PE), from the Brazilian Dry Forest.

Characteristics	Municipality (state)		
	Santa Teresinha (PB)	Remígio (PB)	Serra Talhada (PE)
Coordinates	07º03'S and 37º29'W	6°52'S and 35°47'W	07º59'S and 38º18'W
Altitude (m)	380	596	500
Annual rainfall (mm)	824	700	768
Months with water déficit	9 – 10	4 – 5	6 - 7
Average temperature (°C)	26	22	24
Soil type	Litholic Neosol	Regolithic Neosol	Luvisol
Soil characteristics			
pH (water)	8.8	4.4	6.8
P (mg dm^{-3})	7.3	8.4	4.9
Sand (g kg^{-1})	623	725	651
Silt (g kg^{-1})	224	122	227
Clay (g kg^{-1})	153	153	122

Soil subsamples were analyzed for some chemical and physical characteristics (Table 1) following the methodology described by Solos (1997). The samples were dried, passed through a 6 mm mesh sieve, and portions of 1 kg were placed in pots maintained under greenhouse conditions. Seeds of *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Mimosa caesalpiniifolia* Benth. were collected from single mother trees in Remígio and Serra Talhada. The seeds were surface-disinfected in ethanol (70% v/v - 3 min) and sodium hypochlorite (1 % v/v - 3 min), rinsed five times with sterile distilled water, rolled onto YMA plates to test for surface sterility and then sown in the pots. The pots received 100 ml of nutrient solution without nitrogen very week until harvest, 120 days after seed germination. At harvest, the root nodules were separated, dehydrated in silica gel and stored.

Rhizobia isolation and phenotypic characterization

Rhizobia were isolated from the nodules in yeast mannitol agar (YMA, pH 6.8) medium [18] with 25 mg kg^{-1} (w/v) of congo red. The typical rhizobial colonies were purified and stored at -20 °C, in microtubes with 1 ml YM medium (YMA without agar) plus 15% sterilized glycerol. The colonies isolated in YMA with 25 mg kg^{-1} (w/v) bromo-

thymol blue as pH indicator were observed for the following characteristics: growth period, pH alteration of growth medium, colony morphology (shape, size, border, transparency, surface) and amount of extracellular polysaccharides (EPS). These characteristics were converted to binary data employed in a cluster analysis using the UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) algorithm and the Jaccard similarity index.

The results of the cluster analysis were used to calculate richness (Taxa S and Margalef), diversity (Shannon H), dominance (Simpson 1-D) and uniformity (Evenness, J) indices for the soils and species, where each morphological group, at 60% of the similarity (de Conceição Jesus et al., 2005), was considered as one operational taxonomic unit. The PAST (Palaeontological Statistics) program was used to perform cluster analysis and diversity indices calculation (Hammer *et al*, 2001).

Genetic characterization

Confirmation of isolates: *nif*H- and *nod*C-PCR amplification. The *nif*H amplification was performed using the *nif*HKD primer (Fernandes Jr. et al, 2013), whose sequences were PolF (TGC-GAYCCSAARGCBGACTC) and PolR (ATSGCCAT-CATYTCRCCGGA). For amplification of the *nod*C gene, it was used the *nod*Cr (AYGTHGTYGAYGAC-GGTTC) and *nod*Cr (I) (CGYGACAGCCANTCKC-TATTG) primers, according to Laguerre et al. (2001). The composition of the duplex PCR reaction mixture was as follows: reaction buffer 1X Taq Polymerase Invitrogen, MgCl$_2$ (2.5 mM), dNTPs (1.2 mM), Taq Polymerase (0,3Ü), primers (1 μM *nif*H + 0.6 μM *nod*C, template DNA (40 ng). The amplification conditions in the thermal cycler Biometra® were an initial cycle of denaturation (94 °C for 5 min); 35 cycles of denaturation (1 min at 94 °C), annealing (45 sec at 55 ºC) and extension (1 min at 72 °C); and a final extension cycle at 72 °C for 1 min.

PCR amplification with REP, ERIC and BOX-PCR. The isolates from the nodules of the three species and the three soils and six reference strains (ER1 – *Rhizobium* SP NGR234, ER2 – *Mesorhizobium mediterraneanus* BR 523, ER3 – *R. etli* CFN42, ER4 – *Ensifer fredii* USDA205, ER5 – *Bradyrhizobium japonicum* BR111 and ER6 – *R. tropici* CIAT899), provided by EMBRAPA Agrobiology, were placed to grow in liquid TY. After 48 hours of growth, 1.5 uL aliquots of the cultures were transferred to microcentrifuge tubes, being centrifuged for 3 min. at 13,000 rpm. Each Isolates received the following legend: we use the initial two letters of the common name of the plant and the last one of the place collected for example: (Jurema Branca - Serra Talhada – JBS), (Jurema

Preta – Patos - JPP), (Jurema Branca – Remigio – JBR) and (Sabiá – Remigio –SBR).

The genomic DNA extraction was performed using the Wizard Genomic DNA kit (PROMEGA) as described by the manufacturer. The DNA was diluted in the ratio 1: 500, leaving a final concentration of about 20 to 40 ng.μL^{-1}.

Isolates were amplified by repetitive element polymerase chain reaction (rep-PCR), using BOX-A1R primer (5'-CTACGGCAAGGCGAC-GCTGACG-3'), and by ERIC-PCR, using ERIC-1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers, to assess genetic diversity. The BOX-PCR reaction containing 5 µL GoTaq Colorless polymerase (PROMEGA), 1.0 µM BOX-A1R primer and 1 uL template (60 ng), supplemented with water to a final volume of 10 µL. The cycling was performed as follows: initial denaturation at 95 °C for 7 min; 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C) and extension (8 min at 65 °C); and a final extension cycle at 72 °C for 16 min (Freitas *et al.* 2013).

Products obtained by using BOX-PCR were analyzed using horizontal gel electrophoresis in a 1.5% agarose gel containing SybrGold. A 10,000 bp molecular marker (INVITROGEN) was loaded alongside BOX-PCR products to estimate the DNA fragment size. The gel was run at 150 V for 1 h, with final visualization under UV radiation.

Regarding ERIC-PCR reaction, it contained 5.0 µL GoTaq Colorless Master mix (PROMEGA), 0.4 mM each primer, 2 µL free of ultrapure water and 100 ng of genomic DNA with a final volume of 10 µL. The amplification cycle was performed in a thermocycler Biometra® in the following conditions: 95 °C for 7 min, 30 cycles (1 min at 95 °C, 1 min at 55 °C; 2 min at 72 °C) and a final extension cycle of 72 °C for 16 min. The amplified fragments were separated by electrophoresis at 80 V, for three hours, in agarose gel at 1.5%, stained and photographed in a photodocumenter Loccus.

The profiles of BOX and ERIC bands were analyzed for the presence (1) or absence (0) of each amplicon gel using the Simple Matching coefficient. The program used to perform cluster analysis was NTSYS-PC 2.1. The dendrograms were constructed using UPGMA (Unweighted Pair Group Method Using Arithmetic Averages), which is a SAHN (sequential, agglomerative, hierarchical, non-overlapping) clustering technique, and graphics were made using tree plot (Sokal, 1986)

Results and discussion

The diversity indices of Shannon-Wiener, Margalef, Menhinick, Simpson and Pielou were applied to assess the richness, dominance and evenness of the isolates. It is observed for the Shannon-Wiener index, which expresses the richness and evenness of the species, that the isolates of *M. tenuiflora* have greater richness compared to *M. caesalpiniifolia* and *P. stipulacea*. Consequently, it has a lower dominance, when observing the Simpson index for the isolates of the three species. The evenness index of Pielou showed the lowest abundance for the studied isolates of *M. caesalpiniifolia*, while the isolates of *P. stipulacea* showed the greatest abundance. That is, the evenness is the inverse of dominance, as demonstrated in Figure 1.
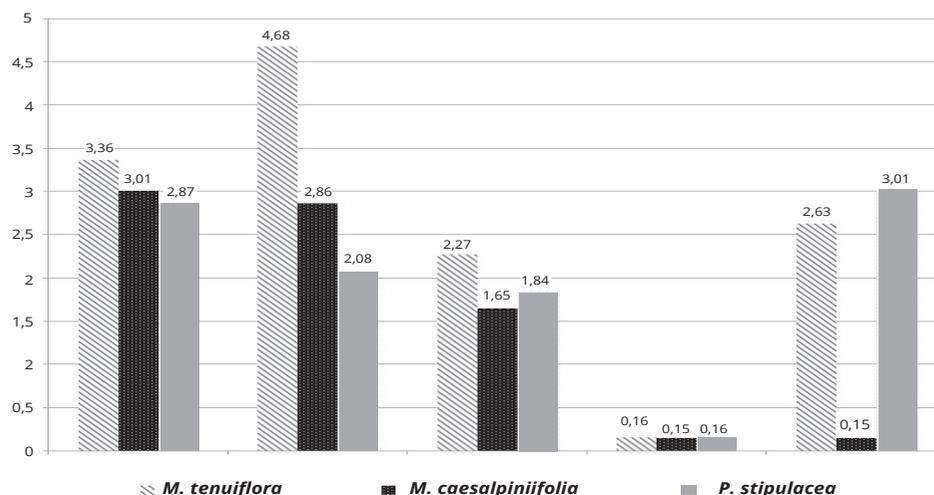
Figure 1: Diversity index of Shannon-Wiener, Margalef, Menhinick, Simpson and Evenness of isolated the Indigenous Tree Legumes: *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Mimosa caesalpiniifolia* Benth. from the Brazilian Dry Forest.

Margalef and Menhinick indices are also used to measure the richness of species and both of them showed similar results to those obtained by Shannon index, only differing in values. The studied isolates have a high diversity that includes a wide variety and relative abundance of species.

To molecularly authenticate the 27 studied isolates (Figure 2) amplification with the *nif*H and *nod*C genes was performed, being observed that three JBR (from *P. stipulacea*) isolates showed no amplification with any of the genes: JBR02, JBR09 and JBR18. The isolates JBR13, JBR06 and JBR12 showed identical profiles with an amplicon of 500 bp and 700 bp, except for the isolate JBR13, which did not show the band of 700 bp. The isolates JBR16, JBR05, JBR11, JBR14, JBR42 proved to be extremely polymorphic in duplex PCR, with fragments that could be considered for the two genes.
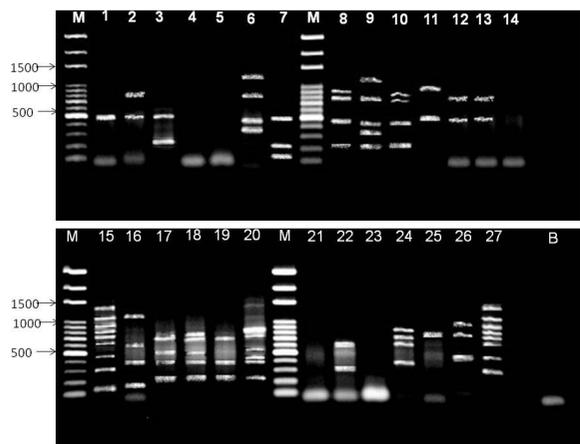
Figure 2: Duplex-PCR Simultaneous duplex amplification of the *nif*H and *nod*C genes in legume isolates: *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Mimosa caesalpiniifolia* Benth. from the Brazilian Dry Forest for molecular authentication. Legend: M: Amresco 100 pb, 1:JBR13, 2: SBR15, 3:JBS06, 4:JBR02, 5: SBR10, 6:JPP18, 7:SBR01, 8:SBR20, 9:JPP13, 10:SBR02, 11: SBR06, 12:JBR06, 13:JBR12, 14:JBR09, 15: SBR19, 16:JBR16, 17:JBR05, 18:JBR11, 19:JBR14, 20:JBR42, 21:JBR18, 22:JPP08.2, 23:JPP14, 24:SBR04, 25:JBS02, 26:SBR14, 27:JBS05, B: Branco.

Only one of the SBR isolates from the bait plant *M. caesalpiniifolia* was not amplified (SBR10) with any of the two primers. The SBR15 isolates presented two amplicons of 500 and 700 bp (*nod*C); SBR01 (3 amplicons; 500 bp - *nod*C, 200 bp and 100 bp - *nif*H); SBR20 and SBR02 (amplicons of 200, 480 bp - *nif*H; 700, 900 bp - *nod*C). The isolate SBR06 showed two bands with sizes of 500 and 1000 bp, which may be related to the *nod*C gene. The most interesting fact was that the amplification of these genes in isolate SBR19 proved to be a real fingerprint, what is quite intriguing because it would be necessary to clone each fragment of it and make a sequence to know to which fragment it belongs. This same behavior was observed with the isolate JBS. The

isolates SBR04 and SBR14 presented bands in the following sizes: 380, 600, 700 and 800 bp; and 480, 700 and 800 bp, respectively, we consider that both amplified the *nif* and *nod* genes. JBS isolates showed two fragments: JBS06 - 500 bp and 250 bp; and JBS02 - 480 and 600 bp, we consider that both genes were amplified. Of the three isolates of JPP, only JPP08.2 amplified three band sizes: 500, 400 and 300 bp. Interestingly, these same primers were used in type strains of *R. tropici*, *Ensifer* sp., *Bradyrhizobium* sp., *B. japonicum*, *Azospirillum brasilense*, *Herbaspirillum seropedicae* in duplex PCR, and generate fragments of 360 bp for the *nif*H gene and 980 bp for the *nod*C gene (Fernandes Jr. et al., 2013). The phylogeny of the *nod*C and *nif*H genes are similar, however, cases of incongruity are detected, suggesting that a genetic rearrangement is occurring in the course of evolution.

Given the variation found for the *nif*H and *nod*C genes in this study, we observed that these isolates have distinct characteristics for these two genes compared to the type strains mentioned above, since it was found a pattern of bands with much variation forming a fingerprint profile. Many features of the *nif*H gene, based on the phylogenetic tree, are entirely consistent with the phylogeny of the 16S rRNA of nitrogen-fixing bacteria. Notwithstanding, the diversity of the *nif*H genes allows a wider representation of the taxonomic diversity of fixing bacteria, showing that they can be used for studies on the diversity of the nitrogen-fixing bacterial community. Studies comparing the phylogeny of the 16S rRNA, *nif* and *nod* genes support the idea of lateral transfer of symbiotic genes, and that genetic rearrangements may be involved in the acquisition and development of rhizobial symbiotic functions.

Multiplex PCR has been widely used in soil and environmental microbiology, especially in the rhizobiology, according to Fernandes Jr. *et al.* (2013). In the work reported by these authors, it was found a 360 bp amplicon relative to the *nif*H gene and a 980 bp amplicon relative to the *nod*C gene when amplified alone. But when amplified in duplex PCR, the amplicon of the *nod*C gene was shown to be much greater than 980 bp. Languere *et al.* (2001), studying the *nod*C and *nif*H genes of six bacterial genera, observed that the amplicon of the *nod*C gene can range between 930-1300 bp, and the amplicon of the *nif*H gene ranges between 780-890 bp. The same authors studying combined data of each gene through RFLP analysis observed that combination of analyses of the *nif* and *nod* genes revealed 50 symbiotic genotypes (*nod-nif*).

It can be concluded that the authentication with the *nif*H and *nod*C genes can result in false-positive data, since both genes are highly

polymorphic between species and biovars. Since the isolates are originating from Caatinga and the lack of knowledge studied plants and kind of isolate, this technique is not advisable.
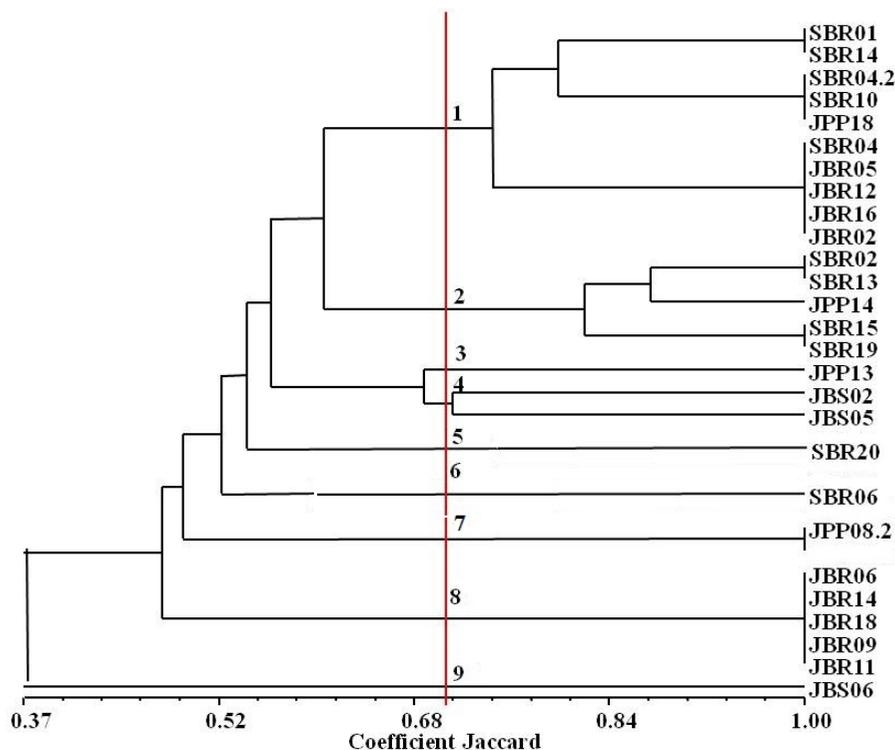
The results of the morpho-physiological characteristics of the isolates originated a dendrogram where ten clusters were formed (Figure 3). 70% similarity was stipulated as a basis to compare the clusters. Cluster 1 was comprised of 10 isolates, namely: SBR01, SBR14, SBR04.2, SBR10 and JPP18 (with similarity of approximately 80%), and SBR04, JBR05, JBR12, JBR16, JBR02 (with similarity of 75%). It is noteworthy that in this Cluster are three of the four isolates that did not amplify with the *nod* and *nif* genes. Cluster 2 was formed by the isolates SBR02 and JPP14, with 86% similarity between them, and the isolates SBR15 and SBR19, identical with similarity of 82%.

Clusters 3 to 10, with the exception of Cluster 9, were basically constituted of 1 or 2 isolates, yet Cluster 9 a larger cluster of isolates was formed *P. stipulacea* from Remígio. However, the similarity was very low, less than 52%. And among these isolates, one (JBR09) was not amplified with the *nod* and *nif* genes. It can be concluded that the morpho-physiological characteristics are incipient data to determine the diversity of an isolate, given that these isolates were shown to be very

similar but with a similarity rather lower than expected. Chagas Jr. *et al.* (2010) verified that the morpho-physiological characteristics show a high diversity among populations and that this diversity may be related to molecular studies.

Figure 4 shows the dendrogram formed by the BOX-PCR marker. In this analysis, the type strains were inserted for comparison. Interestingly, the type strains of rhizobia gathered almost in the middle of the dendrogram, with a few isolates of this study grouped to the same. Only the isolate JBS02 showed a similarity of 72% with *Ensifer fredii* USDA205, a rhizobium of rapid growth. Cluster 7, formed by the isolates SBR14, SBR15, SBR19 and JBR13, showed a similarity of 71% with *Rhizobium* sp NGR234, also a rhizobium of rapid growth and fairly promiscuous. The remaining isolates studied with BOX marker formed distinct groups proving to be new isolates, many of them being identical among themselves.

In Figure 5 it can be seen that the dendrogram formed by the ERIC-PCR marker showed a great diversity among the isolates. With 11 clusters formed, the type strains grouped in the lower part of the dendrogram, with a similarity ranging from 65%, with *Rhizobium* sp. NGR234 and *M. mediterraneanus* BR523, to 89%, with *R. etli* CFN42 and *B. japonicum* BR111. The most interesting in was that eighteen of the twenty-seven isolates

Figure 3: Dendrogram of similarity resulting from the morphological and physiological characteristics of the nodule isolates of *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Duce and *Mimosa caesalpiniifolia* Benth. from the Brazilian Dry Forest.
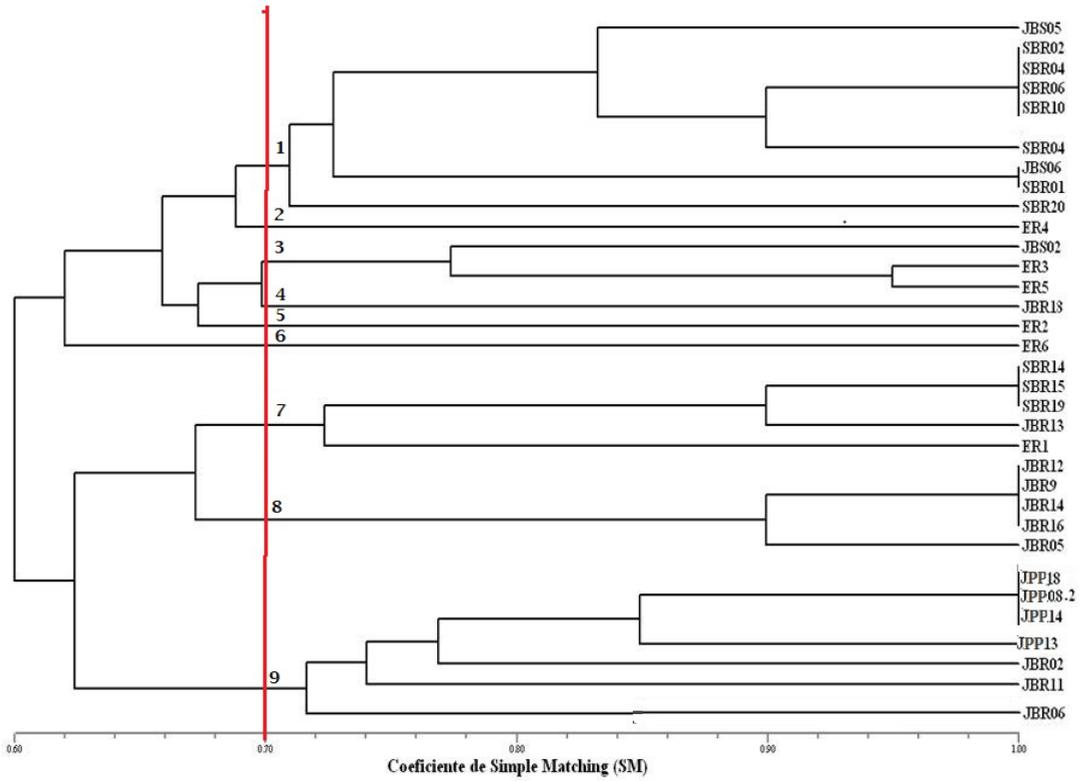
Figure 4: Similarity dendrogram resulting from the BOX-PCR fingerprinting marker of species nodule isolates of *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Mimosa caesalpiniifolia* Benth. from the Brazilian Dry Forest.
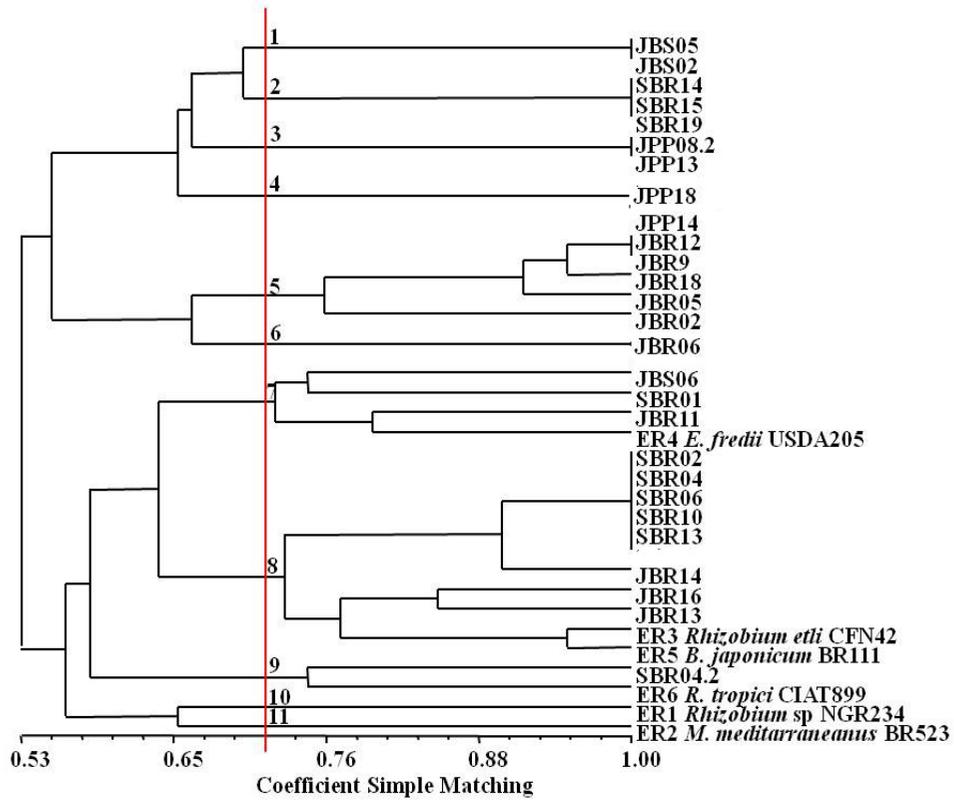
Figure 5: Similarity dendrogram resulting from the ERIC-PCR fingerprinting marker of species nodule isolates of *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Mimosa caesalpiniifolia* Benth. from the Brazilian Dry Forest.

were grouped in the upper part of the dendrogram without any type strain studied. In subcluster 7, the *Ensifer fredii* USDA205 ensifer - ER4 strain was grouped with three isolates: JBS6, SBR01, JBR11.

Moreover, the isolates of this study were shown to be different from the type strains studied here, and may be new species not yet known. When compiling the data of BOX-PCR and ERIC-PCR markers (Figure 6) little difference was observed. Only a few changes in the position of isolates were verified.

But the real conclusion is that these isolates are distinct and need more studies to be characterized as new species originating from leguminous plants of Caatinga. Several authors, such as Versalovic et al. (1994) believe that these markers, for being associated with high levels of polymorphism, showing the gene region amplified by the REP, ERIC and BOX primers, may have a role in the adaptive evolution process, helping in the interaction of microorganisms with hostile environments such as high temperature, high aluminum content, salinity, among others. Fre-

itas *et al.* (2014), working with native isolates of *Mimosa* spp. in soils of the Brazilian semi-arid region, found that these isolates have different cultural characteristics from those obtained in the same soil but using other species of legumes as bait plants. Based on these results, it can be seen a great diversity among the isolates, leading us to conclude that there is a great variability in the studied isolates and that a polyphasic study considering other known markers to obtain a more accurate taxonomy is necessary.

Conclusion

The morpho-physiological and molecular characterization of the isolates originating from three tree legumes, *M. tenuiflora*, *P. stipulacea* and *M. caesalpiniifolia*, revealed a wide variety of bacteria colonizing this plant.

The authentication method of the *nod*C and *nif*H genes with rhizobia isolates still needs to be better adapted, once the primers used produce a very high polymorphism.
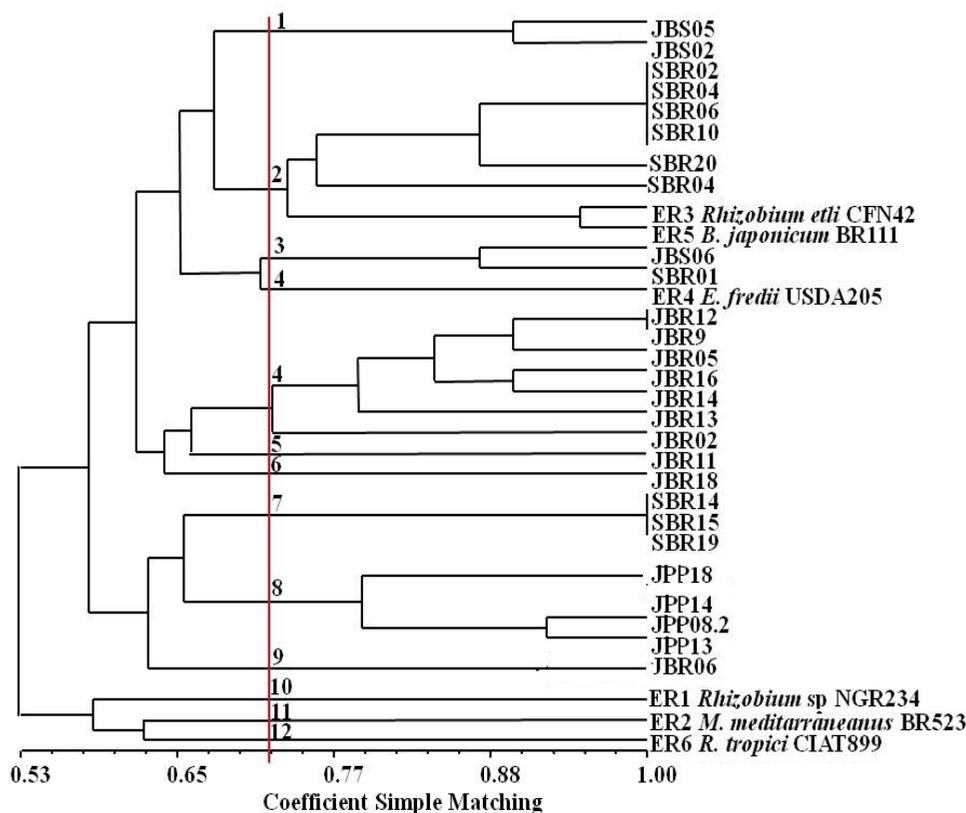
Figure 6: Dendrogram of similarity resulting from the compilation of BOX-PCR and ERIC-PCR fingerprinting markers of species nodule isolates of *Mimosa tenuiflora* (Willd.) Poir., *Piptadenia stipulacea* (Benth.) Ducke and *Mimosa caesalpiniifolia* Benth. from the Brazilian Dry Forest

For this study, we accepted that the isolates showing amplicons greater than 500 bp would have amplified the *nod*C gene, and those which amplified fragments smaller than 500 bp would have amplified the *nif*H.

The analyses with molecular markers proved to be quite efficient, especially when these data were compiled. Thus showing that the larger the number of molecular markers used, the greater the capacity of distinction between species.

Acknowledgements

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