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Characteristics of nodule bacteria from *Mimosa* spp grown in soils of the Brazilian semiarid region

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The Brazilian Northeastern dry forest (Caatinga) is one of the diversification centers of *Mimosa* species. We determined the characteristics of native rhizobia isolates from nodules of *Mimosa tenuiflora* and *Mimosa paraibana* grown in pots with soils collected under Caatinga vegetation and compared the restriction ribosomal DNA profiles of the isolates with those of 16 reference strains. All plants formed abundant indeterminate nodules and all nodule isolates formed fast growing colonies. No colony altered the medium to an alkaline reaction and most of them produced low or medium amounts of extracellular polysaccharides. White and creamy colonies predominated among the isolates but orange and green colonies were present. Differences among the isolates from the *Mimosa* species tested are indicated by the greater phenotypic diversity of those obtained from *M. tenuiflora*. The analysis of the 16S rDNA gene suggests that the isolates from *M. tenuiflora* and *M. paraibana* are closely related and closer to β-rhizobia than to α-rhizobia. However, the similarity with all the tested β-rhizobia reference strains was relatively low suggesting that the isolates may belong to different bacteria species.

Key words: Biological nitrogen fixation, diversity, rhizobia, wild tree legumes.

INTRODUCTION

Legume species belonging to the genus *Mimosa* have received considerable attention in recent years because of their potential to fix large proportions of their nitrogen from the atmosphere (Freitas et al., 2010) and because of their preferential association with β-rhizobia (Chen et al., 2005; Barrett and Parker, 2006; Bontemps et al., 2010; Elliott et al., 2009; Reis Jr et al., 2010; Liu et al., 2012). *Mimosa* is one of the richest Leguminosae genera, with over 500 species, mostly neotropical, occupying diverse habitats, including lowland tropical rainforest, savanna, tropical and subtropical dry forest and thorn scrub, mid-elevation subtropical forest, desert, grassland, and wetland (Simon et al., 2011).

One of the diversification centers of *Mimosa* is the Brazilian Northeastern dry forest (Simon et al., 2011), locally called Caatinga. The Caatinga represents the largest and most isolated of the South American dry forests. It covers more than 850,000 km^2 (Albuquerque et

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al., 2012), from 02°50'S at its northern limits, to 17°20'S with a variety of different types of vegetation (Queiroz 2006). Caatinga occurs under a prevailing semi-arid climate, with a high evapotranspiration potential (1500 to 2000 mm year^{-1}) and a low precipitation (300 - 1000 mm year^{-1}) that is usually concentrated within 3 to 5 months (Queiroz 2006). The region is rich in legume species, with more than 293 speies in 77 genera, many of them endemic ones (Queiroz et al., 2009). Few of these species were studied in relation to their potential to fix nitrogen and to their microsymbionts (Freitas et al., 2010; Teixeira et al., 2010).

Mimosa paraibana Barneby and *Mimosa tenuiflora* (Willd.) Poir. are leguminous tree species with great nitrogen fixation capacity mean contributions of biological fixation for plant nitrogen reaching up to 50% in Caatinga (Freitas et al., 2010). *M. tenuiflora* is a species of wide distribution, occupying dry areas of Brazil to Mexico, Honduras and El Savador (Queiroz et al., 2009). This species is the main pioneer species in areas of caatinga with few years of regeneration (Souza et al., 2012). Its preferred symbionts are apparently β-proteobacteria, belonging to the genus *Burkholderia* (Bontemps et al., 2010; Reis Jr et al., 2010). Moreover, *M. paraiba* is a species endemic to Northeast Brazil (Queiroz et al., 2009) and there is no studies on bacteria capable of forming symbiotic nodules on their roots.

Research on *Mimosa* rhizobia have been conducted in several regions of Brazil (Chen et al., 2005; Barrett and Parker, 2006; Elliott et al., 2009; Liu et al., 2012). Isolation of rhizobia populations from Caatinga soils has been relatively rare (Bontemps et al., 2010; Reis Jr et al., 2010; Teixeira et al., 2010), mainly considering the diversity of environmental conditions in the region, that can affect the structure of rhizobia populations (Mishra et al., 2012). Moreover, most of this research centered on genetic characteristics and only Teixeira et al. (2010) described cultural characteristics of the rhizobia. Therefore, the diversity of bacteria able to nodulate *Mimosa* species in the region is little known.

Considering this scarcity of information, we characterized Caatinga native rhizobia associated to two *Mimosa* species in relation to their cultural traits and compared the restriction profiles of their amplified ribosomal DNA (16S rDNA-ARDRA) with those of 16 reference strains.

MATERIALS AND METHODS

Soil sampling and *Mimosa* spp. cultivation

Composite soil samples from the 0 to 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 Paraíba state; 2) Remígio, in the agreste zone of this same state; and 3) Serra Talhada, in the sertão zone of Pernambuco state. The composition and structure of the vegetation in the three areas were described by Ferraz et al. (2003), Souza (2010) and Pereira et al. (2003), respectively. Number of species and tree heights and stem diameters are higher in the agreste zone than in the sertão zone and in this last zone higher in Serra Talhada than in Santa Terezinha, probably reflecting higher water availability.

Soil subsamples were analyzed for some chemical and physical characteristics (Table 2), following the methodology described by Embrapa (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 two *Mimosa* species (*Mimosa tenuiflora* (Willd.) Poir. and *Mimosa paraibana* Barneby) were collected from a single mother tree in Remígio caatinga. The seeds were surface disinfected in etanol (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 were sown in the pots. Each legume species was sown in triplicate for each soil sample. The pots received 100 ml of nutrient solution without nitrogen (Hoagland and Arnon, 1939) every week until harvest, 120 days after seed germination. At harvest the root nodules were separated, dehydrated in silica gel and stored.

Isolation and phenotypic characterization of rhizobia

Rhizobia were isolated from the nodules in yeast mannitol agar medium (YMA, pH 6.8) (Vincent, 1970) with 25 mg kg^{-1} (w/v) of Congo red. The typical rhizobia colonies were purified and stored at -20°C, in microtubes with 1 ml YM medium(YMA without agar) plus 15% sterilized glycerol. Isolates colonies in YMA with 25 mg kg^{-1} (w/v) bromethymol blue as pH indicator (Fred and Waksman, 1958) 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) (Xavier et al., 1998). These characteristics were converted to binary data employed in a cluster analysis using the UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) algorithmand 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 (Equitability J) indices for the soils and species, where each morphological group, at 60% of the similarity (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).

Restriction analysis (ARDRA) and reference strains

DNA isolation, 16S rDNA gene amplification and restriction analysis of ribosomal DNA (ARDRA) using *Hinf*I, *Msp*I and *Dde*I endonucleases were prepared according to Teixeira et al. (2010). Twenty eight new strains were randomly selected (19 from *M. paraibana* and 9 from *M. tenuiflora*) and compared with 16 strains from Embrapa Agrobiologia bacterial diazotrophic collection: BR 7801 (*Mesorhizobium loti*), BR 527 (*Sinorhizobium terangae*), BR 7606 (*Rhizobium leguminosarum* bv *trifoli*), BR 2811 (*Bradyrhizobium elkani*), BR 114 (*Bradyrhizobium japonicum*), BR 5401 (*Azospirilum doberaneae*), BR5410 (*Azorhizobium caulinodans*), BR 2006 (*Methylobacterium nodulans*), BR 3407 (*Burkholderia sabiae*), BR 3437 (*Burkholderia nodosa*), BR 3454 (*Burkholderia mimosarum*), BR 3467 (*Burkholderia mimosarum*), BR 3471 (*Cupriavidus taiwanensis*), BR 3486 (*Burkholderia phymatum*), BR 3487 (*Burkholderia tuberum*) and BR 3498 (*Burkholderia caribensis*). The restriction fragment profiles were used to perform a cluster analysis using the Jaccard index, the UPMGA algorithm and the GelCompar II (Applied Maths) program.

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

Characteristic	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 deficit	9 - 10	4 - 5	6 - 7
Average temperature (◦C)	26	22	24

Table 2. Soil characteristics of preserved Caatinga areas in three municipalities, in the States of Paraíba (PB) and Pernambuco (PE), Brazil.

Soil characteristic	Municipality (state)		
	Santa Teresinha (PB)	Remígio (PB)	Serra Talhada (PE)
Classification	Litholic Neosol	Regolithic Neosol	Luvisol
pH (water)	8.8	4.4	6.8
P (mg dm^{-3})	7.3	8.4	4.9
N (%)	0.07	0.08	0.10
C (%)	0.73	0.96	1.09
Sand (g kg^{-1})	623	725	651
Silt (g kg^{-1})	224	122	227
Clay (g kg^{-1})	153	153	122

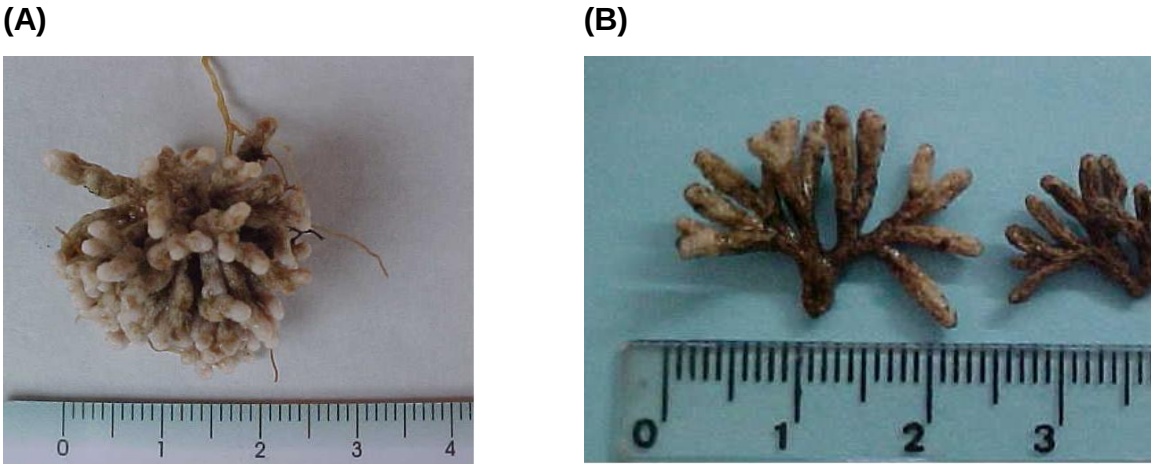

Figure 1. Shape of nodules found in *Mimosa paraibana* (A) and *M. tenuiflora* (B) roots from plants grown in soils collected under mature Caatinga vegetation.

RESULTS

Mimosa spp. nodulation

All harvested plants, cultivated in all three soils, had nodules of indeterminate growth with dark red interior and sizes up to 3 cm in diameter (Figure 1). Sixty one isolates were obtained from the nodules of *M. paraibana* and 62 from the nodules of *M. tenuiflora*. All isolates developed very fast, in less than 24 h, and formed circular colonies in the YMA medium.

Phenotypic characteristics of rhizobia isolates

No isolate changed the medium pH to an alkaline reaction. Most of the isolates obtained from *M. tenuiflora* changed the medium pH to an acid reaction: 96% when

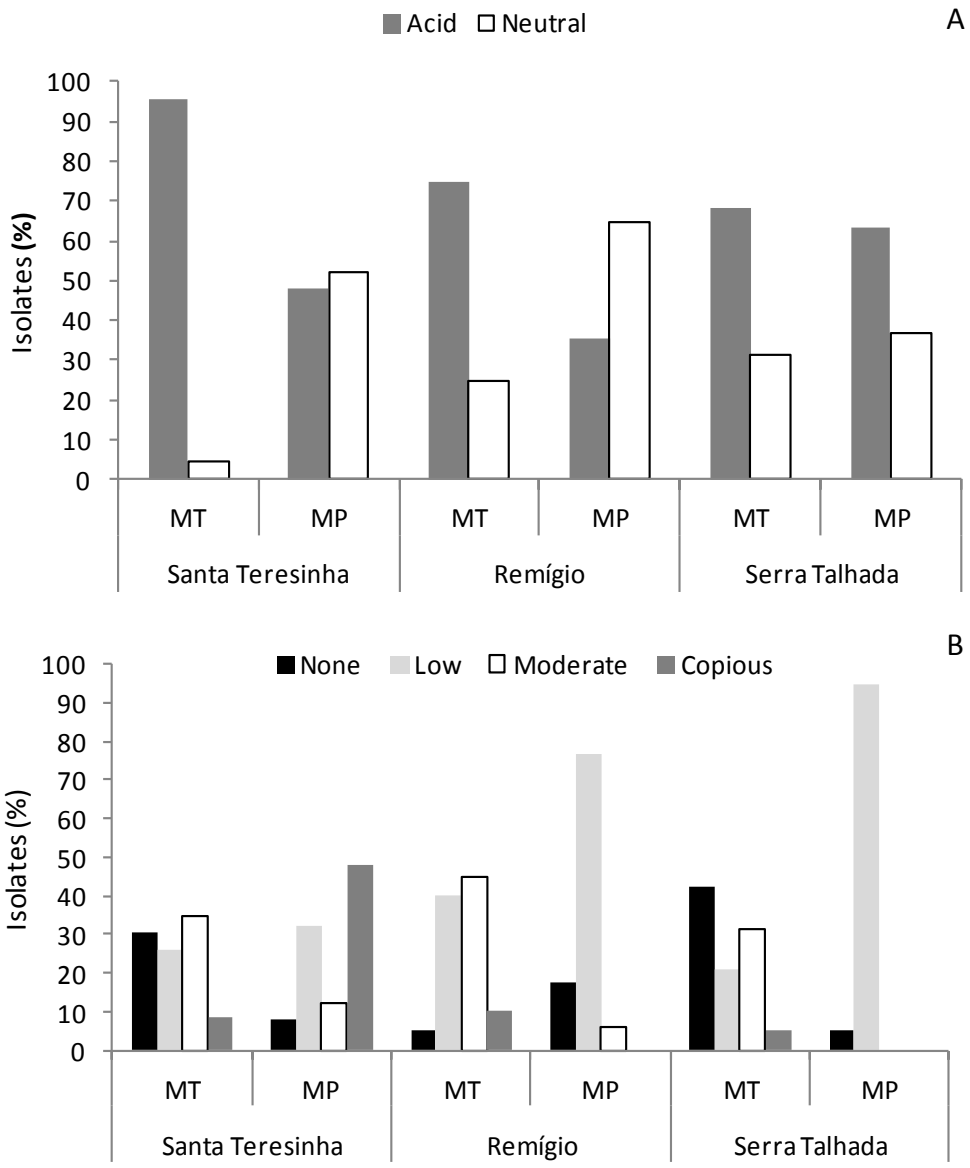

Figure 2. pH change (A) and amount of extracellular polysaccharides (EPS) production (B) in YMA medium of bacterial nodule isolates from *Mimosa tenuiflora* (MT) and *M. paraibana* (MP) grown in soils collected under mature Caatinga vegetation.

when grown in the soil from Santa Terezinha, 75% in the soil from Remígio and 68% in the soil from Serra Talhada (Figure 2). Most of the isolates from *M. paraibana* grown in the soil from Serra Talhada (62%) also changed the pH to an acid reaction but the proportions were lower in the soils from Santa Terezinha (48%) and Remígio (35%).

Most of the colonies had a cream color (74% of those from *M. tenuiflora* and 67% from *M. paraibana*) but there were also white, orange and green colonies. At 48 h in YMA, the most common diameters of colonies from *M. tenuiflora* were punctiform (29%), 3 mm (19%) and 4 mm (16%) while those from *M. paraibana* were 2 mm (31%), punctiform (23%) and 1 mm (21%). Among the isolates from *M. tenuiflora*, the highest proportion (30 to 40% in the three soils) produced colonies with moderate amounts of extracellular polysaccharides (EPS), 26% were dry colonies and only in the colonies originating from the Santa Terezinha soil the proportion of high EPS producers (46%) surpassed the proportion of moderate producers (Figure 2). Among the isolates from *M. paraibana* most (30 to 95%) produced low amounts of EPS, except in the Santa Terezinha soil where the proportion of colonies with copious amounts of EPS was also high (48%).

The isolates from *M. tenuiflora* were classified into 19 phenotypic groups and those of *M. paraibana* into 16

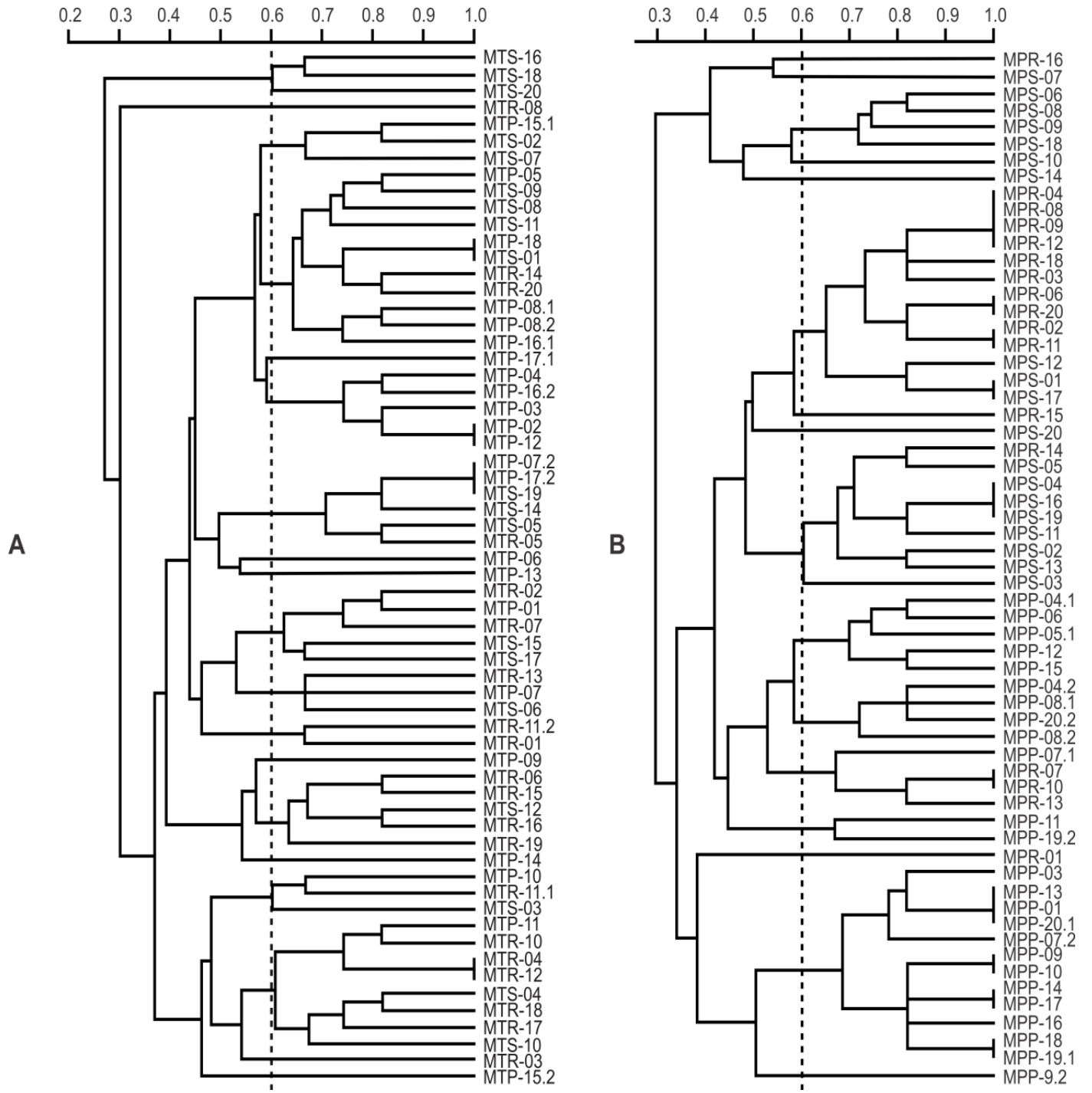

Figure 3. Phenotipic similarity dendrogram among *M. tenuiflora* (A) and *M. paraibana* (B) nodule isolates froms oils of preserved Caatinga. The letters MT and MP indicates the isolates from *M. Tenuiflora* and *M. Paraibana* respectively. The letters M P, R and S indicates isolates native from soils of Santa Terezinha, Remígio and Serra Talhada (municipalities in the States of Paraíba (PB) and Pernambuco (PE), Brazil.), respectively.

groups (Figure 3). Eight groups from each plant species were composed of a single isolate which can be considered to belong to different or rare types. The isolates from *M. paraibana* had a tendency to group according to the soil, mainly the isolates originating from the soil of Santa Terezinha, 23 of them clustering into four groups exclusive of isolates from the soil of this area (Figure 3B). On the other hand, the isolates from *M. tenuiflora* had no clear tendency to group according to the soil.

Richness, diversity and equitability were higher among

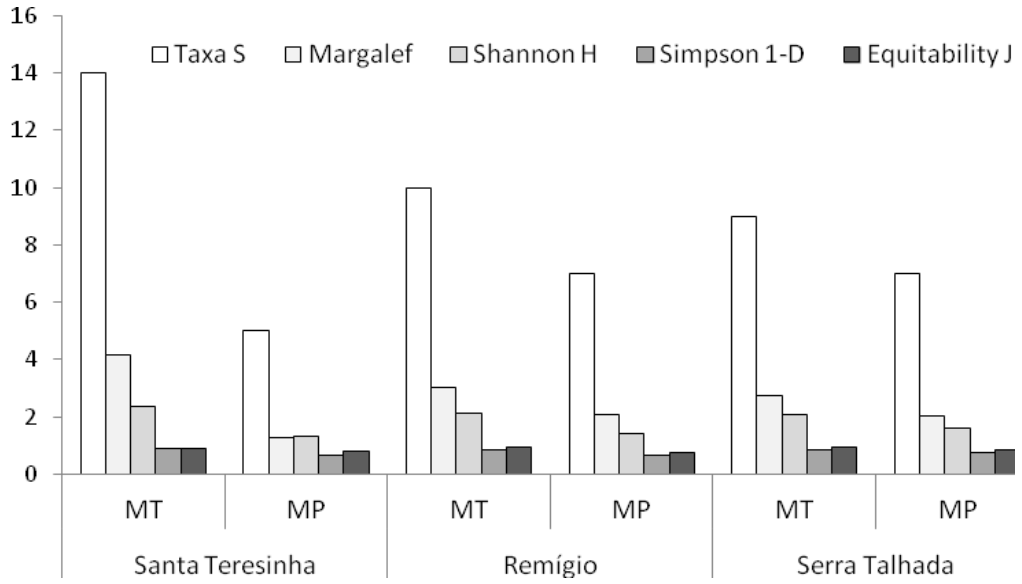

Figure 4. Taxa S**,** Margalef, Shannon H, Simpson 1-D and Equitability J indices for bacterial nodule isolates from *Mimosa tenuiflora* (MT) and *M. paraibana* (MP) grown in soils collected under Mature Caatinga vegetation.

the isolates from *M. tenuiflora* than from *M. paraibana* (Figure 4). For the first species, the order of isolate diversity for the soils was Santa Terezinha, Remígio and Serra Talhada while for the second species it was the inverse order.

Restriction analysis (ARDRA) cluster

Four large groups (Figure 5) were formed in the cluster analysis based on the restriction analysis of ribosomal DNA (ARDRA). One group was composed of 26 out of 28 of the new isolates, both from *M. paraibana* (17 out of 19 total new isolates) and from *M. tenuiflora* (9 new isolates). The second group was somewhat related to the first group and was composed mostly of strains typical of β-rhizobia genera. The third group was composed of strains of the type typical of α-rhizobia genera. The fourth group included only two isolates from *M. paraibana* (MPS5 and MPS10) and had a low similarity with both the β-rhizobia and the α-rhizobia groups. Two pairs of isolates (MPS 19 and MTP 16-2; MPS 17 and MPS 12) and one groups of five isolates (MPS1, MPS6, MPS7, MPS8 and MPS9) from *M. paraibana* had 100% similarity.

DISCUSSION

Mimosa spp. nodulation

The spontaneous nodulation of *M. paraibana* and *M. tenuiflora* indicates the presence, in the three soils, of native populations of bacteria able to colonize the roots of both plant species. Ample populations of nodulating bacteria are common in the soils of the regions where the legume species are native. On the other hand, nodulation frequently fails when a legume species is planted outside its original region (Bala et al., 2003; Souza et al., 2007). *M. tenuiflora* has a large distribution in tropical dry forests, from Brazil to Mexico (Queiroz et al., 2009) and naturally occurs in the three Caatinga fragments where the soils were collected (Ferraz et al., 2003; Pereira et al., 2003; Souza, 2010). *M. paraibana* has a more restricted distribution, being endemic to the Caatinga, and spontaneously occurs only in the Caatinga fragment of Remígio (Pereira et al., 2003). In spite of that, it also nodulated when planted in the soil of the two other areas. There is no information on the natural occurrence of rhizobia populations able to form symbiosis with the several *Mimosa* species growing in soils of the Brazilian semiarid, but the spontaneous nodulation of *M. paraibana* indicates that this occurrence may be quite general. These species may also be very promiscuous, nodulating with a large spectrum of microsymbionts, as observed for *M. pudica* (Bontemps et al., 2010).

The nodules of *M. tenuiflora* and *M. paraibana* were indeterminate, as has been described for those of other Mimosoideae legumes. Indeterminate nodules, with a wide range of size, formats and ramifications are usually attributed to species of Mimosoideae (Sprent et al., 2007), without influence of the microsymbionts (Lammel et al., 2007).However, some of the nodules grew more than usually reported (Patreze and Cordeiro, 2004), reaching more than 20 mm in their longest axis (Figure 1). *M. paraibana* is one of the endemic species of

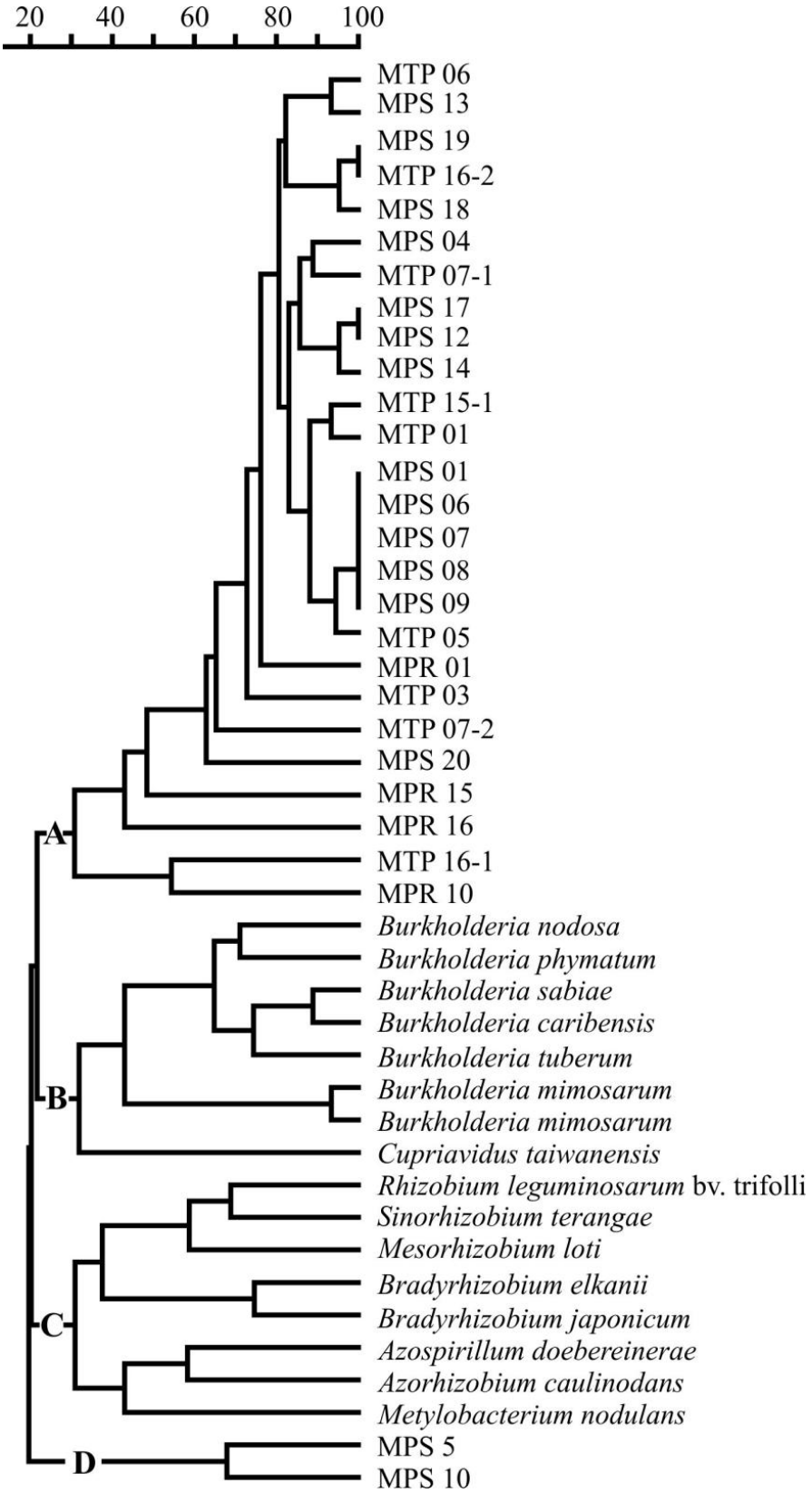

Figure 5. Genetic similarity dendrogram among 28 bacterial nodule isolates from *Mimosa tenuiflora* (MT) and *M. paraibana* (MP) grown in soils collected under mature Caatinga vegetation and 16 α- and β-reference rhizobia strains based on PCR-ARDRA of the 16S rDNA gene.

caatinga that only recently was identified as capable of fixing nitrogen (Freitas et al., 2010) and the description of its nodules is being reported for the first time.

Phenotypic characteristics of rhizobia isolates

The growing interest on microsymbionts associated to *Mimosa* spp. has resulted in a large number of articles on the subject (Chen et al., 2005; Barrett and Parker, 2006; Bontemps et al., 2010; Elliott et al., 2009; Reis Jr et al., 2010; Liu et al., 2012). However, few of these articles describe the characteristics of the culture colonies formed by these symbionts. Recently, Teixeira et al. (2010) observed that all the isolates obtained from nodules of *M. tenuiflora* grown in a soil from a Caatinga area (Petrolina, Pernambuco State, Brazil) had a rapid development and acidified the medium and that most of them produced a large quantity of EPS. Fast-growing acid-producing rhizobia are the most common symbionts of several African and Asian wild tree legumes (Wolde-Meskel et al., 2004; Shetta et al., 2011). The isolates obtained from *M. paraibana* and *M. tenuiflora* cultivated in the soils from Serra Talhada, Santa Terezinha and Remígio also had rapid development but, contrasting with the African isolates, some of them did not modify the YMA culture medium (51% of the *M. paraibana* isolates and 19% of the *M. tenuiflora* isolates). Large production of EPS was only observed in 17 isolates (20% of all the *M. paraibana* isolates and 8% of the *M. tenuiflora* isolates). Rapid growth is a common characteristic of native isolates from the Brazilian semiarid region obtained from nodules of several species (Teixeira et al., 2010; Medeiros et al., 2009). Usually, isolates of rapid development do not form dry colonies (Teixeira et al., 2010) but this was the case in 16% of the isolates from *M. tenuiflora* and 6% of the isolates from *M. paraibana* (Figure 2).

White, creamy or translucent colonies are commonly formed by bacteria associated with wild tree legumes such as *Acacia* spp. (Wolde-Meskel et al., 2004), *Millettia pinatta* (Rasul et al., 2012) and *Mimosa tenuiflora* (Teixeira et al., 2010). White and creamy colonies also predominated among the isolates from *M. tenuiflora* and *M. paraibana* but orange and green colonies were also present. Considering that descriptions of colonies formed by rhizobia associated to *Mimosa* spp. are scarce (Chen et al., 2005; Bontemps et al., 2010; Reis Jr et al., 2010; Teixeira et al., 2010), it is difficult to evaluate the frequency of these phenotypes.

Medeiros et al. (2009) reported that punctiform colonies are commonly formed by isolates from nodules of *Vigna unguiculata* cultivated in soils from Caatinga areas. Cowpea associates with a large diversity of rhizobia and due to this characteristic it is frequently used as a trap culture (Melloni et al., 2006; Medeiros et al., 2009). However, only 29 and 23% of the isolates from the nodules of *M. tenuiflora* and *M. paraibana* formed this type of colony. Therefore, the bacteria associated with *M. paraibana* and *M. tenuiflora* seem to differ, from a certain extent, from those described in native rhizobia collections obtained from soils of the region using cowpea as a trap culture. Mishra et al. (2012) demonstrated that different legume species can form associations with different rhizobia populations, in spite of being cultivated in the same soils. The wide phylogenetic distance from cowpea and *Mimosa* species, belonging to two distinct legume subfamilies (Papilionoidea and Mimosoidea), may explain part of the difference in their microsymbionts.

Differences among the isolates from the two *Mimosa* species tested are indicated by the greater phenotypic diversity of those obtained from *M. tenuiflora* (Figure 4). This species may establish symbiosis with a larger array of rhizobia species, and this could be explained by its larger spatial distribution which may determine different patterns of co-evolution with the microsymbionts.

Analysis of the 16S rDNA gene

The analysis of the 16S rDNA gene suggests that the isolates from *M. tenuiflora* and *M. paraibana* are closely related, independently from the soil of cultivation, clustering in the first group of the dendrogram (Figure 5). All isolates show the same phenotypic characteristics of mucus production, acid or neutral reaction and homogeneous and circular colonies. Other characteristics such as differences in color of colonies and amount of mucus generated clustering on phenotypic dendrograms that cannot be observed in the genetic analysis. They were also closer related to β-rhizobia than to α-rhizobia, corroborating previous reports of prevalence of this group among species of the Mimosoideae subfamily (Chen et al., 2005; Reis Jr et al., 2010). However, the similarity with all the tested β-rhizobia reference strains was relatively low.

Two isolates (MPS5 and MPS10) are clustered apart these two groups. According to their position they could be alpha that were not well resolved by the analysis, or another class of proteobacteria.

Conclusion

The results demonstrate that the bacteria populations from the nodules of *Mimosa* spp. species native from the soils of the semiarid Brazilian region have cultural characteristics different from those obtained from the same soils but using other legume species as trap plants. In spite of fast growth, the isolates can form from dry colonies to colonies with large production of EPS. The isolates are more related to β-rhizobia than to α-rhizobia, but the low similarity with the strains from the Embrapa collection suggests that the rhizobia isolated from the nodules of *M. tenuiflora* and *M. paraibana* are different bacteria species.

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