Universidade de Brasília – UnB
Instituto de Ciências Biológicas
Departamento de Botânica
Programa de Pós-Graduação em Botânica

ASSOCIAÇÃO SIMBIÓTICA ENTRE ESPÉCIES DE LEGUMINOSAS DOS GÊNEROS *Mimosa* E *Stryphnodendron* E BACTÉRIAS FIXADORAS DE NITROGÊNIO

Raquel de Castro Pires

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Associação simbiótica entre espécies de leguminosas dos gêneros *Mimosa* e *Stryphnodendron* e bactérias fixadoras de nitrogênio

Raquel de Castro Pires

Orientador: Dr. Marcelo Fragomeni Simon
Co-orientador: Dr. Fábio Bueno dos Reis Junior

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Dissertação de Mestrado, autoria de Raquel de Castro Pires, intitulada: **Associação simbiótica entre espécies de leguminosas dos gêneros *Mimosa* e *Stryphnodendron* e bactérias fixadoras de nitrogênio**, apresentada como parte dos requisitos para obtenção do grau de Mestre em Botânica pela Universidade de Brasília, em 2017. Sob a orientação do Dr. Marcelo Fragomeni Simon.

Banca Examinadora:

_____

Dr. Marcelo Fragomeni Simon

Embrapa Recursos Genéticos e Biotecnologia – EMBRAPA Cenargen

_____

Dra. Ieda de Carvalho Mendes

CPAC – EMBRAPA Cerrados

_____

Dr. Thomas C. Rhys Williams

Programa de Pós-Graduação em Botânica – UNB

_____

Taciana Barbosa Cavalcanti

Embrapa Recursos Genéticos e Biotecnologia – EMBRAPA Cenargen

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SUMÁRIO

RESUMO

Grande parte das espécies da família Leguminosae se associa simbioticamente com bactérias fixadoras de nitrogênio pertencentes às classes α- e β-proteobacteria, conhecidas como rizóbios. Nesse trabalho foi estudada a associação simbiótica em dois gêneros de leguminosas, *Mimosa* L. e *Stryphnodendron* Mart.: o primeiro é notório pelo amplo sobre sua capacidade conhecimento de nodulação, ao passo que, pouco se sabe sobre as interações simbióticas envolvendo *Styphnodendron*. Os objetivos deste estudo foram avaliar a influência dos diferentes tipos de solo na associação simbiótica entre espécies do gênero *Mimosa* e bactérias fixadoras de nitrogênio, e investigar a nodulação do gênero *Stryphnodendron* em simbiose com rizóbios. No capítulo I, foi realizado um experimento com seis espécies de *Mimosa* em solos oriundos de três diferentes locais com características contrastantes. Nos dois solos mais ácidos e menos férteis as plantas de *Mimosa* se associaram com bactérias do gênero *Paraburkholderia*, enquanto que no solo mais fértil e com pH tendendo para neutro se associaram com *Rhizobium*. Fatores edáficos como pH e fertilidade, parecem favorecer a predominância de determinados tipos de rizóbio, enquanto que as espécies de plantas estudadas parecem ter baixa especificidade no estabelecimento da associação simbiótica. No capítulo II, foram selecionadas dez espécies representativas das três principais linhagens do gênero *Stryphnodendron*, a fim de revelar quais espécies são capazes de nodular e quais seriam os rizóbios associados. Sementes dessas espécies foram plantadas em seu solo de origem para avaliação da nodulação e os isolados obtidos foram identificados por meio da sequência do gene 16S rRNA. Das dez espécies avaliadas, oito mostraram-se capazes de nodular e distintas bactérias pertencentes aos gêneros *Bradyrhizobium*, *Rhizobium* e *Paraburkholderia* foram encontradas em associação simbiótica com as espécies de *Stryphnodendron*, revelando novas relações planta-bactéria e ampliando o conhecimento sobre a nodulação em espécies de leguminosas tropicais.

Palavras-chave: Interação planta-bactéria; Cerrado; nodulação; rizóbios; plantas-armadilha

ABSTRACT

Most species of the family Leguminosae are able to form a symbiotic association with nitrogen fixing bacteria belonging to the α- and β-proteobacteria classes, which are known as rhizobia. In this work the symbiotic associations in two legume genera, *Mimosa* L. and *Stryphnodendron* Mart. were investigated: the first one is notorious for the knowledge accumulated about its nodulation capacity, whereas little is known about the symbiotic interactions related to *Styphnodendron*. The goals of this study were to investigate the influence of different types of soil on the symbiotic association of *Mimosa* and rhizobia, and the nodulation of the genus *Stryphnodendron* with nitrogen-fixing bacteria. In chapter I, a greenhouse experiment was carried out with trap plants using seeds of six species of *Mimosa* and soils from three different locations with contrasting characteristics. In the two most acidic and less fertile soils, *Mimosa* plants were associated with bacteria of the genus *Paraburkholderia,* whereas in the more fertile soil with pH tending towards neutral plants were associated with *Rhizobium*. Edaphic factors such as pH and fertility influenced the presence of certain rhizobia types, while the species of plants studied appear to have low specificity in the establishment of its symbiotic relationships. In chapter II, ten species, representative of the three main lineages of the genus *Stryphnodendron,* were selected, in order to reveal which species are capable to nodulate and identify the associated rhizobia. Seeds of these species were planted in their origin soil for nodulation evaluation and the isolates obtained were identified by sequencing the 16S rRNA gene. Of ten species evaluated, eight were able to nodulate and distinct bacterias belonging to the genera *Bradyrhizobium*, *Rhizobium* e *Paraburkholderia* were found in symbiotic association with species of *Stryphnodendron*, revealing new plant-bacteria interactions and expanding the knowledge about nodulation in tropical legume species.

Keywords: Interaction plant-bacterial; Cerrado; nodulation; rhizobia; trap plants

INTRODUÇÃO GERAL

Os membros de Leguminosae Juss. ocorrem em grande diversidade de hábitats, sendo encontrados em quase todas as áreas do mundo, exceto em mar aberto e na Antártica (Sprent 2009). Compreendem aproximadamente 19.300 espécies distribuídas pelo mundo (Lewis et al. 2005), sendo que cerca de 220 gêneros e 2800 espécies ocorrem no Brasil, abrangendo todo o território nacional (Lima et al. 2015). Essa é a terceira maior família de Angiospermas, sendo superada apenas por famílias Orchidaceae e Asteraceae, representando ainda a segunda maior família em importância agrícola e econômica depois de Poaceae (Lewis et al. 2005). As leguminosas possuem grande importância para a produção de alimentos (feijão, soja, amendoim, dentre outras), no setor industrial, no setor médico (plantas medicinais) e suas madeiras têm diversos usos que vão de matéria-prima para construções pesadas à fabricação de papel, além de serem usadas como forrageiras e na ornamentação de jardins (LPWG 2013; Yahara et al. 2013).

A maioria das leguminosas é conhecida por sua habilidade em formar simbiose com bactérias fixadoras de nitrogênio, conhecidas como bactérias diazotróficas, obtendo assim, parte ou todo o nitrogênio necessário para seu crescimento (Franco et al. 1992). No caso das leguminosas, o termo genérico utilizado para o grupo de bactérias diazotróficas que forma nódulos em suas raízes é rizóbio. O nódulo é um órgão especializado formado pela planta nos pelos radiculares (raramente nos caules), onde, os rizóbios são alojados (Sprent 2017).

Leguminosae é tradicionalmente dividida em três subfamílias: Caesalpinioideae, Faboideae/Papilionoideae e Mimosoideae (Lewis et al. 2005). Essas três subfamílias têm grandes diferenças em relação à forma de crescimento e associação simbiótica com bactérias fixadoras de nitrogênio. Apesar de a capacidade simbiótica estar presente na maioria das leguminosas, nem todas as espécies se associam com rizóbios. A maioria das espécies da subfamília Caesalpinioideae, por exemplo, não fazem associações simbióticas. Por outro lado, a maioria das espécies das subfamílias Papilionoideae e Mimosoideae se associa com essas bactérias (Moreira e Siqueira 2006). Avanços recentes na classificação das leguminosas com base em filogenias moleculares têm levado a atualizações na classificação ao nível de subfamília (LPWG 2017), mas ao mesmo tempo corroborado as diferentes capacidades de nodulação entre as diversas linhagens dessa família (Doyle 2016; Andrews e Andrews 2017; Sprent et al. 2017).

A habilidade que as leguminosas têm de se associar com rizóbios representa uma importante fonte de nitrogênio para a agricultura e para os ecossistemas naturais (Yahara et al. 2013). O processo de fixação biológica de nitrogênio (FBN) fornece um suprimento contínuo de nitrogênio para o crescimento da planta e acúmulo de matéria orgânica no solo (Franco e Faria 1997).

O nitrogênio atmosférico (N_2) é encontrado em grande quantidade no ar atmosférico (78%), inclusive no espaço poroso do solo. Porém, nenhuma planta consegue utilizá-lo diretamente como nutriente, pois essa forma atmosférica não é assimilada, principalmente pela tripla ligação covalente (N≡N) que é estável entre os dois átomos em questão. Apenas alguns microrganismos (diazotróficos) são capazes de transformar o N_2 em amônia (NH_3), composto químico utilizado pelas plantas (Reis Junior et al. 2006). A reação da transformação do N_2 em NH_3 é catalisada pela enzima nitrogenase (Young 1992).

Nas leguminosas, antes da formação do nódulo, um "diálogo" molecular entre a planta hospedeira e a bactéria é iniciado, com a liberação de flavonoides secretados pelas raízes das plantas. Leguminosas usam esses compostos para se "comunicarem" com os rizóbios e estimular a expressão de genes que implicam na síntese de fatores de nodulação nas bactérias (fatores Nod). Dessa forma, a planta percebe o sinal mediado pelos fatores Nod e permite a entrada do rizóbio, que penetra nos pelos das raízes e inicia o estabelecimento de uma relação simbiótica (Sprent 2009; Robledo et al. 2010; Wang et al. 2012; Remigi et al. 2016). Assim, os rizóbios induzem à formação de nódulos nas raízes, onde eles disponibilizam o N_2, reduzido à NH_3, em troca de compostos de carbono da planta. Durante o processo de FBN o interior dos nódulos apresenta uma coloração rósea, indicando que a leg-hemoglobina está presente. Essa proteína é importante para manutenção dos níveis de oxigênio, que são mantidos baixos no interior do nódulo, permitindo o processo respiratório do rizóbio e ao mesmo tempo o funcionamento da nitrogenase, enzima sensível ao oxigênio. (Moreira e Siqueira 2006; Robledo et al. 2010).

Todas as bactérias conhecidas por nodular leguminosas pertencem ao filo das proteobactérias gram-negativas. Até o início do século XXI, bactérias da ordem Rhizobiales, dentro da classe α-proteobacteria, eram consideradas as únicas que nodulavam com as leguminosas. No entanto, a partir do ano 2001, surgiram relatos que comprovaram que membros da classe ß-proteobacteria também nodulam e podem fixar nitrogênio em associação com leguminosas (Chen et al. 2001, 2003; Moulin et al. 2001;

Vandamme et al. 2002; Elliot et al. 2007, 2009; Reis Junior et al. 2010; Angus et al. 2012; Bontemps et al. 2010; Liu et al. 2014; Lemaire et al. 2015). Em particular, estudos realizados no gênero pantropical *Mimosa* L., foram fundamentais para demonstrar que grupos de ß-proteobacteria desempenham papel essencial na fixação de nitrogênio em associação com leguminosas. Apenas as classes de α-bactéria e ß-bactéria têm membros comprovados que formam nódulos em leguminosas (Sprent 2009). Dentre as α-bactérias, estão compreendidos os gêneros *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium* e *Devosia*, entre outros. E dentre as ß-bactérias, os gêneros *Paraburkholderia* e *Cupriavidus* (Sprent 2009; Andrew e Andrew 2016).

A capacidade de formar simbiose com essas bactérias, α e/ou ß-rizóbios, é conhecida para diversos grupos de plantas de Leguminosae, sobretudo para aqueles de importância para agricultura. Porém, para alguns outros gêneros, praticamente não existem estudos nesse sentido, como no caso de *Stryphnodendron* Mart. (Bournaud et al. 2013).

A presente dissertação de Mestrado está dividida em duas partes, que correspondem a dois capítulos, preparados no formato de artigos. O primeiro capítulo teve como foco a nodulação de seis espécies de *Mimosa*, em diferentes tipos de solos, com o intuito de observar a especificidade entre as plantas hospedeiras e as bactérias em relação às diferentes características dos solos. O segundo capítulo aborda a nodulação em espécies de *Stryphnodendron*, onde foram investigadas bactérias que formam simbiose com as espécies desse gênero.

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CAPÍTULO I

Soil characteristics determine the rhizobia in association with different species of *Mimosa* in central Brazil

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Soil characteristics determine the rhizobia in association with different species of *Mimosa* in central Brazil

Raquel C. Pires[1], Fábio B. dos Reis Junior[2*], Jerri E. Zilli[3], Doreen Fischer[3], Andreas Hofmann[3], Euan K. James[4], Marcelo F. Simon[5*]

1 Instituto de Ciências Biológicas. Departamento de Botânica. Universidade de Brasília, Campus Universitário Darcy Ribeiro, 70910-970, Brasília-DF, Brazil

2 Embrapa Cerrados, 73301-970, Brasília-DF, Brazil

3 Embrapa Agrobiologia, 23891-000, Seropédica-RJ, Brazil

4 The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

5 Embrapa Recursos Genéticos e Biotecnologia, 70297-400, Brasília-DF, Brazil

* Corresponding authors

Abstract

Background and Aims To evaluate the influence of soil type on the symbiosis between *Mimosa* spp. and rhizobia.

Methods A greenhouse experiment was carried out with trap plants using seeds of six species of *Mimosa* and soils from three different locations in central Brazil: Posse, Brasília and Cavalcante. Plant dry biomass and number of nodules were measured after four months. Symbiotic bacteria were isolated from nodules and their molecular identification was performed. Three housekeeping genes (16S rRNA, *recA* and *gyrB*) plus the *nodC* and *nifH* symbiotic genes were used to determine the identity of the symbionts and to reconstruct the phylogenetic relationships among the isolated nitrogen-fixing bacteria.

Results Rhizobia from the Betaproteobacterial genus *Paraburkholderia* (former *Burkholderia*) and the Alphaproteobacterial genus *Rhizobium* were isolated from different species of *Mimosa*. As in previous studies, the phylogenies of their symbiosis-essential genes, *nodC* and *nifH*, were broadly congruent with their core housekeeping genes (16S rRNA, *recA* and *gyrB*), which suggests limited or no horizontal gene transfer. Edaphic factors such as pH and fertility influenced the occurrence of these unrelated rhizobial types in the nodules on these *Mimosa* spp.

Conclusions Mimosa species have the ability to associate with different types of rhizobia (α- and β-proteobacteria), suggesting low specificity between host and bacterium in experimental conditions. Soil factors such as pH, nitrogen and fertility

seem to favour the predominance of certain types of rhizobia, thus influencing the establishment of symbiotic relationships.

Keywords: Biological nitrogen fixation; nodulation; rhizobia; β-rhizobia; host-specificity; Cerrado.

Introduction

Leguminous plants are important for natural and agricultural ecosystems because of their ability to fix atmospheric N_2 in nodules formed on their roots via symbioses with diazotrophic bacteria (rhizobia). In this association, which is critical for plant nutrition and global N cycling, rhizobia provide nitrogen in exchange for carbon compounds supplied by the plant (Sprent 2009; Robledo et al. 2010).

All bacteria currently known to nodulate leguminous plants belong to the group of gram-negative proteobacteria. Until the beginning of the XXI century, bacteria of the order Rhizobiales, within the α-proteobacteria class, were considered the only ones that nodulated Leguminosae. However, since 2001, several reports have demonstrated that members of the β-proteobacteria class also nodulate and fix nitrogen in association with these plants (Chen et al. 2001; Vandamme et al. 2002; Chen et al. 2003; Elliott et al. 2007a, b, 2009; Bournaud et al. 2013; Liu et al. 2014; Lemaire et al. 2015). In particular, studies carried out with the pantropical *Mimosa* genus have been used to demonstrate that bacteria from the β-proteobacteria play a key role in nitrogen fixation in association with leguminous plants (Chen et al. 2005a, b; Elliott et al. 2009; Gyaneshwar et al. 2011; Bontemps et al. 2010; Reis Junior et al. 2010; Lammel et al. 2013; Platero et al. 2016). Indeed, *Mimosa* is now used as a model for studies involving these symbiotic relationships in natural ecosystems.

Previous studies have shown that bacteria from the genus *Paraburkholderia* (former *Burkholderia*) are the main symbionts of *Mimosa* species in the central region of Brazil (Chen et al. 2005a; Elliott et al. 2007a, Bontemps et al. 2010; Reis Junior et al. 2010), French Guiana, Costa Rica, and in areas where such plants were introduced such as Asia and Australia (Chen et al. 2005b; Barret and Parker 2006; Parker et al. 2007; Liu et al. 2012; Mishra et al. 2012; Gehlot et al. 2013; Melkonian et al. 2014). A division of the genus *Burkholderia* was recently proposed, involving the creation of a novel genus *Paraburkholderia* containing the primarily environmental and plant-associated species which broadly corresponds to the "Plant Beneficial and Environmental (PBE)" clade of Suárez-Moreno et al. (2012), and which separates them

from *Burkholderia sensu stricto*, which encompasses some environmental strains, but is particularly known for its human clinical and phytopathogenic species (Sawana et al. 2014; Beukes et al. 2017). All known nodulating *Burkholderia* species are currently placed in the genus *Paraburkholderia*, the exception being *B. symbiotica* which, on the basis of whole genome comparisons, is most likely to be placed in a new genus along with some other members of the "Transition Group I" defined by Estrada-de los Santos et al. (2016), such as *B. caryophylli*, *B. soli* and *B. rhizoxinica* (Estrada-de los Santos, Venter, Hirsch, James and Steenkamp, unpublished).

In Uruguay, the main symbionts associated with *Mimosa* belong to the genus *Cupriavidus* (Platero et al. 2016), which is related to *Paraburkholderia* in the β-proteobacteria class. In Mexico, an important center of *Mimosa* diversity, species of this genus are predominantly associated with α-proteobacteria from the genera *Rhizobium* and *Ensifer* (Bontemps et al. 2016). In India, the genus *Ensifer* is also predominant in two endemic species of *Mimosa* (Gehlot et al. 2013).

The preference of *Mimosa* for association with some distinct groups of rhizobia in different regions may be related to soil characteristics, such as pH and fertility, which influence symbiont selection (Elliott et al. 2009; Garau et al. 2009; Thrall et al. 2011; Liu et al. 2012, 2014; Mishra et al. 2012; Lammel et al. 2015; Stopnisek et al. 2014; Lemaire et al. 2015). For example, sites with high levels of heavy metals (zinc, copper and nickel) in soil tend to favour symbiosis with *Cupriavidus*, as verified in Uruguay (Platero et al. 2016) and New Caledonia (Klonowska et al. 2012). In central Brazil, where soils are generally acidic, *Paraburkholderia* spp. are the main *Mimosa* symbionts (Bontemps et al. 2010; Reis Junior et al. 2010). On the other hand, the predominance of α-proteobacteria in *Mimosa* species endemic to Mexico may be related to the presence of more fertile and pH neutral soils (Bontemps et al. 2016).

Symbiotic interactions generally depend on the expression of lipo-chitoligosaccharides (LCOs) or "Nod factors" produced by bacteria in response to plant secreted flavonoids, proteins and polysaccharides from the root surface. Any changes in the structures of these Nod factors can alter the specificity between plants and bacteria (Wang et al. 2012). In addition, this specificity varies among legumes, as some species are associated only with bacteria from one group (α- or β-proteobacteria), or with a few species of rhizobia, while others, more promiscuous, are associated with several types of rhizobia (Peix et al. 2015, Dall'Agnol et al. 2016). Factors that can influence the choice of symbionts by a plant species include the composition and diversity of the

microbial community, soil characteristics, such as pH, salinity, nitrogen and nutrient levels, and altitude (Thrall et al. 2011; Lemaire et al. 2015, 2016).

In the case of *Mimosa*, it is also possible that the plants of each country or continent may have coevolved with their respective symbionts over millions of years resulting in increased specificity. For example, after the ancestors of the main lineages of *Mimosa* from Brazil and Mexico diverged, their descendants may have coevolved with the rhizobia of the local rhizosphere, *Paraburkholderia* in the case of the Cerrado and Caatinga biomes in Brazil, and *Rhizobium* and *Ensifer* in the central highlands of Mexico (Bontemps et al. 2010; Moulin et al. 2015; Bontemps et al. 2016; Sprent et al. 2017), which may have resulted in high rhizobia host-specificity. This might be particularly relevant for endemic plant species, which are restricted to very particular sites and narrow environmental conditions, and might therefore tend to restrict their symbiotic ability to a very limited range of rhizobia.

Despite the growing knowledge on the symbionts associated with leguminous plants in several ecosystems, the rhizobia host-specificity in natural environments and how different types of soil can affect this interaction are still poorly understood. In this study, we conducted an experiment to compare the nodulation of six native Brazilian species of the genus *Mimosa* in three soil types from central Brazil with distinct physicochemical characteristics. We hypothesized that endemic host legume species are expected to exhibit greater specificity in relation to their association with rhizobia, while widely distributed species may associate with a wide variety of symbionts. Regarding soil characteristics, according to recent literature (e.g. Elliott et al. 2009; Bontemps et al. 2016), it is expected that more fertile soils with high pH would favour association with α-proteobacteria, whereas more acidic and nutrient poor soils would result in nodulation with β-proteobacteria.

The aims of the present study were to verify the influence of soil type on nodulation and the composition of associated rhizobial species. Three housekeeping genes (16S rRNA, *recA* and *gyrB*) plus the *nodC* and *nifH* symbiosis-essential genes were used to determine the identity of the symbionts and to reconstruct the phylogenetic relationships among the isolated nitrogen-fixing bacteria.

Materials and Methods

Collecting sites and plant species

In order to perform the nodulation tests, seeds of *Mimosa* species and soil samples containing their associated rhizosphere from three different sites in central

Brazil with contrasting edaphic and vegetation characteristics, were used (Table 1). Soil samples from each locality were collected (0 to 20 cm depth) and analysed according to the protocols of Embrapa (1997).

A deciduous seasonal forest predominates in the collection site at Posse, with soil derived from limestone with high fertility and pH tending to neutral. The Brasília site is a typical woody savanna (cerrado) in a deep clay Oxisol, with low fertility and low pH. The Cavalcante site comprises an open savanna shrubby vegetation (cerrado rupestre) on sandy-rocky soil with low fertility and low pH (Table 1). Soil analysis shows high cation exchange capacity with elevated levels of Ca, Mg, K, P, nitrogen and organic matter in Posse, whereas the Brasília and Cavalcante sites have lower levels of cations, nitrogen and organic matter, but high concentrations of Al (Table 2).

Mature seeds of two *Mimosa* species per site were collected in the field. Plant species used in the study (Table S1) vary according to the extent of their geographic distribution: *M. xanthocentra* is a ruderal species of wide distribution in South America; *M. acutistipula* occurs in the Cerrado and Caatinga regions in Brazil often associated with fertile soils; *M. claussenii* and *M. radula* are widely distributed within the Cerrado region; *M. kalunga* and the undescribed *Mimosa* sp. are endemic to the region of Cavalcante in northern Goiás (Barneby 1991; Simon et al. 2010). The identity of the so far unidentified *Mimosa* sp. could not be determined as it might represent a new taxon. These species were chosen because of their abundance in each locality. They represent different lineages distributed throughout the phylogeny of the genus, with *M. claussenii*, *M. kalunga* and the *Mimosa* sp. belonging to the same clade (Simon et al. 2011).

Experiments with trap plants

Experiments with trap plants (Bontemps et al. 2016; Mishra et al. 2012) were performed to evaluate nodulation and growth of the *Mimosa* species cultivated in three different soil types, including the origin of the soil where the seeds were collected. Seeds of six species of *Mimosa* were used (Table S1): two from Posse (*M. acutistipula* and *M. xanthocentra*), two from Brasília (*M. claussenii* and *M. radula*) and two from Cavalcante (*M. kalunga* and *Mimosa* sp.). Seeds were immersed in 70% alcohol for 30 seconds and sodium hypochlorite solution (2.5% active chlorine) for five minutes for surface sterilization. Subsequently, seeds were placed in a 23 mm mesh sieve to be washed five times with sterile distilled water. The dormancy was broken by scratching the testa of the seeds under sterile conditions. The seeds were then placed in Petri dishes with moistened filter paper, and after ten days, seedlings were transferred to 300 ml pots

filled with soil (no fertilizer added), and cultivated for four months. Seedlings cultivated in sterilized sand were used as a negative control. Nodules from four to seven plants of each species were used for isolating rhizobia.

For comparison of plant growth (dry biomass) and nodulation among different types of soil an analysis of variance (ANOVA) was conducted and significant differences between means were assessed by Duncan's test at the 5% level of significance using the MSTAT-C software (Michigan State University). Means and standard errors were calculated from values of three replicates of each *Mimosa* spp. in each soil.

Rhizobia isolation

We randomly harvested one to six nodules per treatment (species/soil type), depending on their availability. Collected nodules were rehydrated in sterile distilled water for 2.5 hours. Superficial sterilization of nodules was performed by soaking them in ethanol (95%) for 30 seconds, followed by immersion in 2.5% sodium hypochlorite solution for five minutes and then five washes in sterile water. After this procedure, 500 µl of sterile saline solution was added to 2 ml Eppendorf tubes and the nodules were crushed in this solution with sterile forceps. The resulting solution was serially diluted to 10^{-3} in sterile saline solution. Thereafter, 100 µl of the last two dilutions (10^{-2} and 10^{-3}) were plated on Medium 79 of Fred & Waksman (1928), otherwise known as YMA medium, with Congo red (Vincent 1970), using two replicates for each dilution. The plates were incubated for two to seven days at 30°C and, after this, individual colonies were collected to obtain a pure culture of each bacterial isolate. Some nodules did not allow for the isolation of potentially symbiotic bacteria.

DNA extraction, amplification and sequencing

Bacteria isolated from nodules formed during the experiment with trap plants were cultured in YMA solid medium with Congo red for 72 hours at 28° C. Thereafter, a purified colony was transferred into YMA liquid medium for 24 hours at 28°C. Subsequently, bacterial DNA was extracted using Pure Link Genomic DNA Kits (Invitrogen), following manufacturer's instructions.

The extracted DNA from each isolate was used as template for PCR reactions and the sequencing of five genes: 16S rRNA, *recA, gyrB, nodC* and *nifH*, which are widely used in phylogenetic studies with symbiotic bacteria (e.g. Peix et al. 2015). The PCR products were generated and sequenced in both directions. Primers used in gene

amplification are listed in Table S2. The sequences obtained were compared to sequences deposited in the GenBank database (Table S3).

Phylogenetic analyses

To characterize the isolates at taxonomic level, representative sequences of several bacterial strains were obtained from GenBank and aligned with the sequences generated in this work using ClustalW, imported into BioEdit 4.8.4 (Hall 1999) and manually corrected. Phylogenetic analysis based on the 16S rRNA, *recA*, *gyrB*, *nodC* and *nifH* genes was performed following a maximum likelihood analysis (ML) implemented by the RAxML-HPC v.8 program using the GTR-CAT nucleotide substitution model (Stamatakis 2006) from the CIPRES portal (Miller et al. 2010). To obtain support values, data sets were retested a thousand times using the bootstrap method (Felsenstein 1985). Individual phylogenetic trees were constructed with the sequences aligned for each of the previously cited genomic DNA regions, inferring genetic distance and similarity among the studied bacteria. Sequences from reference strains closest to the genera *Paraburkholderia*, *Cupriavidus*, *Burkholderia*, *Rhizobium*, *Bradyrhizobium*, *Ensifer* and *Mesorhizobium* were included in the phylograms of the five genes. Whenever possible, sequences from the same reference species were used for all five genes. In addition, a phylogeny based on concatenated sequences of 16S rDNA, *recA* and *gyrB* was generated.

Evaluation of nodulation capacity

Confirmation of nodulation capacity was verified by means of an authentication experiment. The species *M. pudica* L. was chosen as a "model host" because it is a fast-growing species and has an ability to nodulate with a wide range of rhizobia, mainly β-proteobacteria (Chen et al. 2005a; Bontemps et al. 2010; Mishra et al. 2012), but also some *Rhizobium* species (Elliott et al. 2009; Baraúna et al. 2016). Seeds were sterilized and their dormancy was broken as cited before. Subsequently these seeds were pre-germinated on a cotton tray soaked with sterile dH_2O and incubated for 48 h at 27°C.

Seedlings were tested in a perlite substrate according to Elliott et al. (2009). Glass tubes (50 ml volume) were half-filled with perlite and then autoclaved at 121°C and 1 atm for 30 min. Sterilized Hoagland nutrient solution (Hoagland and Arnon 1938) without N was applied to the perlite until the saturation point. The seedlings were inoculated with one ml culture of the isolates grown in YMA liquid medium for 48 h. The possibility of cross-contamination was investigated using a uninoculated negative

control randomly inserted among treatments. All plants remained in a growth chamber with controlled temperature (26°C) and 16 h light / 8 h dark cycle. After one month the plants were collected and the presence / absence of nodules was recorded. Because the *Rhizobium* isolates did not nodulate effectively with *M. pudica*, we also used Siratro (*Macroptilium atropurpureum* Moc. & Sessé ex DC.) as an additional host, since this is a promiscuous papilionoid species with high capacity for nodulating with α-proteobacteria (Elliott et al. 2007b; Mishra et al. 2012). Comparisons between the vigour of inoculated and control plants, as well as observation of effective nodules, were used as qualitative evidence for the symbiotic capacity of rhizobial isolates.

Results

Plant biomass and number of nodules

All *Mimosa* species grew in their original and in the other tested soils (Figure 1). *M. acutistipula* and *M. xanthocentra*, from Posse, produced higher biomass in their soil of origin compared to the biomass obtained in other soils (Table 3). *Mimosa kalunga* and the *Mimosa* sp. from Cavalcante, and *M. claussenii* and *M. radula* from Brasília, also produced slightly more biomass in the soil from Posse, the most fertile of all soils, but these differences, with the exception of *M. kalunga*, were not statistically significant (Table 3).

Most *Mimosa* species were able to nodulate in the three soil types, the exceptions being *M. claussenii* and *M. xanthocentra*, which did not nodulate in soils from Posse and Cavalcante, respectively. The species from Posse, *M. acutistipula* and *M. xanthocentra*, showed higher number of nodules in their original soil than the other species growing with this same substrate. These two species also had higher nodulation in their original soil when compared to their nodulation in soils from Brasília and Cavalcante. The other species, with the exception of *M. claussenii*, did not have a notable nodulation difference when the three soils were compared (Table 3). There was no nodulation on plants grown in the control with sterilized sand.

Identification of rhizobia using sequences of the housekeeping genes 16S rRNA, *recA* and *gyrB*

The 54 isolates obtained in this study were identified at least to genus level by comparing their 16S rRNA and *recA* gene sequences with those in the GenBank database using the BLASTN algorithm (Altschul et al. 1990). The BLASTN results classified them in the α- and β-classes of Proteobacteria and showed that they shared a

high similarity (97% - 100%) with already known species of bacteria (data not shown). Nine isolates were assigned to the genus *Rhizobium*, while the other 45 isolates belonged to the genus *Paraburkholderia*. All bacteria isolated from nodules of plants growing on soils from Brasília and Cavalcante were identified as *Paraburkholderia* spp. On the other hand, all isolates from Posse grouped within *Rhizobium*, except isolate POS_MSP2 that grouped with *Paraburkholderia* (Table 4).

Individual phylogenies based on housekeeping genes (16S rRNA, *recA* and *gyrB*) were highly congruent, although the 16S rRNA tree was less resolved (Figures S1-S3). A phylogeny based on the concatenation of these three genes is also shown (Figure 2). Phylogenetic analyses of the sequences of the three genes separated the isolates between the α- and β- proteobacteria, and confirmed that all tested *Mimosa* species associated with bacteria from both classes, except for *M. claussenii* which did not nodulate with *Rhizobium* (Figure 2).

The α-proteobacteria isolates grouped in a clade containing three *Rhizobium* strains from the study of Bontemps et al. (2016) and with *R. altiplani*, a newly-described species of *Rhizobium* associated with *M. pudica* in central Brazil (Baraúna et al. 2016) (Figure 2). Isolates identified as β-proteobacteria grouped in *Paraburkholderia*, forming two distinct clades: one of them containing *P. nodosa*, and the other with *P. tuberum* and *P. sprentiae*. The first clade contains mostly isolates from Brasília and the second from Cavalcante (Figures 2, S1-S3).

Phylogenetic analysis of the symbiotic *nodC* and *nifH* genes

Sequences of the 54 isolates were obtained for *nodC*, but *nifH* sequences were obtained for only 46 of them due to problems with PCR amplification. The tree topologies based on these two genes involved in nodulation (*nodC*) and nitrogen fixation (*nifH*) were similar to that of the other housekeeping genes, with the isolates also distributed within their respective α- and β-rhizobial groups (Figures 3 and 4).

In the *nodC* and *nifH* phylogenies the isolates that grouped with α-proteobacteria were contained in a clade closest to *Rhizobium* sp. strain JPY479 which was isolated from *M. xanthocentra* by Bontemps et al. (2010). In general, the isolates identified as β-proteobacteria were grouped into two *nodC* clades, one closest to *P. tuberum* sv. mimosae and another to *P. nodosa*. For *nifH* two clades were also formed, one closest to *P. mimosarum* and *P. nodosa* and the other with strain JPY306 (Bontemps et al. 2010), which was subsequently placed in *P. tuberum* sv. mimosae by Mishra et al. (2012) along with several other strains from central Brazil.

Evaluation of nodulation capacity

The nodulation ability of all rhizobia isolated in the trap experiment was tested in *M. pudica*, a species known to be promiscuous. In this experiment all rhizobia confirmed their nodulation capacity, but the nine *Rhizobium* isolates (all obtained from Posse soil) formed small nodules with white coloration in their interior indicating ineffective nodulation. On the other hand, five of these *Rhizobium* isolates were tested on the alternative host Siratro and showed effective nodulation of it. The 20 isolates from Cavalcante and the 24 isolates from Brasília soil, all belonging to the genus *Paraburkholderia*, resulted in effective nodulation in *M. pudica*, with nodules showing pinkish-red coloration in their interior, a sign of the presence of the symbiosis-essential protein leghemoglobin (Table 4). The strains of *Paraburkholderia* that nodulated *M. pudica* and those of *Rhizobium* that nodulated Siratro resulted in healthier (green leaves) and more vigorous plants compared to non-inoculated controls (which had no nodules and reduced growth; Fig. S4); taken together, these data provide compelling qualitative evidence for the symbiotic capacity of most of the isolates from this study.

Discussion

We observed a clear difference in the development of some species of *Mimosa* grown in different types of soil from central Brazil. Plants growing in the soil from Posse, which was the most fertile, tended to produce more biomass, which was statistically significant for the local species *M. acutistipula* and *M. xanthocentra*, as well as *M. kalunga* (Cavalcante endemic). Neither *M. acutistipula* nor *M. xanthocentra* performed well in the lower fertility Brasília and Cavalcante soils, even in the presence of potential symbiotic associations, suggesting that these plants are not well adapted to poor soils. On the other hand, most species native to Brasília and Cavalcante did not perform better when grown in richer soil (Posse), suggesting that they are better adapted to low fertility soils. Regarding the number of nodules, the mimosas did not show differences related to the soil types, except for the species from Posse, *M. acutistipula* and *M. xanthocentra*, which produced more nodules when growing in their original soil. Most likely, this large number of nodules found in an environment with high N and organic matter content may be related to the adaptation of both symbiotic partners (plant and rhizobia) to this environment. There are several studies supporting the concept that biological nitrogen fixation (BNF) is often (but not invariably) more active where the N supply is low (Vitousek et al. 2013). On the other hand, even in environments

considered rich in N, BNF may be important to compensate for losses of this nutrient, thus avoiding its depletion in these soils (Pons et al. 2007).

The analysis of the 16S rRNA, *recA* and *gyrB* genes demonstrated that α- and β-rhizobia were found in this study. Overall, the rhizobia found here are in accordance with the high incidence of both α- and β-proteobacteria in the soil microbiota as detected in metagenomics studies carried out in different sites across the Cerrado region (Quirino et al. 2009; Araújo et al. 2012; Castro et al. 2016).

Rhizobium altiplani, the predominant species found in Posse, was first recorded associated with *M. pudica* growing in an anthropogenic neutral to alkaline soil (pH 7.7) in the Distrito Federal (DF) state of Brazil (Baraúna et al. 2016). The occurrence of *R. altiplani* in the soil from Posse reinforces its preference for less acidic and more fertile soils. It is interesting to note that *Rhizobium* sp. strains from the study of Bontemps et al. (2016) also grouped with *R. altiplani*. These strains were obtained from nodules of *Mimosa* spp. growing in relatively fertile soils with neutral-alkaline pH in Mexico.

The isolates of *Paraburkholderia* obtained in our experiment were closest to *P. nodosa*, *P. tuberum*, and *P. sprentiae*, and hence were similar to rhizobia isolated from other studies that sampled nodules from *Mimosa* in different countries, including many localities in Brazil (Chen et al. 2005a, 2006, 2007). For example, several strains of *P. nodosa* and *P. tuberum*-like bacteria, together with *P. mimosarum*, *P. diazotrophica*, and other unidentified *Paraburkholderia* isolates were obtained from different sites in the Cerrado and Caatinga biomes in central and north-eastern Brazil (Bontemps et al. 2010) where acidic soils with low amounts of available nutrients occur, such as those found in the Brasília and Cavalcante sites in the present study.

Paraburkholderia nodosa has *M. bimucronata* and *M. scabrella*, from south Brazil as its original hosts (Chen et al. 2005a, 2007; Lammel et al. 2013), but it is also capable of nodulating with many different *Mimosa* species (Bontemps et al. 2010), and is very widespread in South America. Indeed, *P. nodosa* was also the main species found in nodules of common bean (*Phaseolus vulgaris*) when it was used as trap plants, showing its ability to associate with other legumes in Cerrado soils (Dall'Agnol et al. 2016).

Along with *P. nodosa*, bacteria related to *P. tuberum* are among the most commonly found β-rhizobia associated with different *Mimosa* species in Brazil and elsewhere in South America and Mexico (Bontemps et al. 2010, 2016; Mishra et al. 2012; Lammel et al. 2013). Closely related bacteria belonging to *P. tuberum sensu stricto* were originally isolated from the papilionoid legume *Aspalathus carnosa* L. in

South Africa (Vandamme et al. 2002), and the type strain of this species (STM678^T) is capable of nodulating many native South African papilionoid legumes (Elliott et al. 2007b; Beukes et al. 2013; Lemaire et al. 2015, 2016). Indeed, it has been proposed that *P. tuberum* has two symbiovars (sv. mimosae and sv. papilionoideae) in terms of host, geographical distribution and nod gene phylogeny. The sv. papilionoideae strains from South Africa cannot nodulate with *Mimosa* (Elliott et al. 2007b; Mishra et al. 2012; Estrada-de los Santos et al. 2015; Lemaire et al. 2016; de Meyer et al. 2016).

Paraburkholderia sprentiae was originally isolated from root nodules of *Lebeckia ambigua* E. Mey. growing in the Western Cape of South Africa; it is most closely related to *P. tuberum* (De Meyer et al. 2013), and it has not previously been isolated from any legume outside South Africa. It should be noted, however, that the large species complex represented by *P. tuberum* and *P. sprentiae* is currently being revised (Venter, Steenkamp, James & de Meyer, unpublished), and it is likely that the Neotropical "*B. tuberum* - *B. sprentiae*" will be allocated to a new and separate species which preferentially nodulates *Mimosa* and related mimosoids.

The *nodC* gene was sequenced for all isolates, indicating their capacity for nodulation. Probably owing to PCR amplification problems, we were unable to obtain amplicons of the *nifH* gene for some of the isolates. This does not imply that such isolates are ineffective symbionts that are not able to fix N_2, since unsuccessful PCR reactions may be attributed to different causes, such as primer mismatch or poor DNA quality (Howieson et al. 2013).

The strains that grouped with α-proteobacteria in the *nodC* and *nifH* phylogenies were closely related to *Rhizobium* sp. strain JPY479. This strain was originally isolated from nodules of *M. xanthocentra* in Mato Grosso – Brazil, and it is one of only two α-proteobacterial strains isolated from *Mimosa* spp. in the study of Bontemps et al. (2010). According to Bontemps et al. (2010) the *nifH* and *nodC* genes of these strains were related to previously described α-proteobacterial symbionts of *Mimosa* isolated elsewhere; they are close to *R. tropici* and group with *Rhizobium* strains commonly isolated from Mexican *Mimosa* species (Bontemps et al. 2016).

The authentication experiment confirmed that all isolates tested were able to nodulate with *M. pudica*. Although both *Paraburkholderia* and *Rhizobium* induced the formation of nodules in this host, the nodules formed by *Rhizobium* were small and did not appear to be effective. *Mimosa pudica* is known to be a trap plant suitable for β-rhizobia, but the same is not generally true for α-rhizobium, and this may have influenced the effectiveness of nodulation on *M. pudica* by the α-rhizobial strains (Chen

et al. 2005a; Elliott et al. 2009; Bontemps et al. 2010, 2016; Mishra et al. 2012; Klonowska et al. 2012; Gehlot et al. 2013; Melkonian et al. 2014). On the other hand, five out of the nine *Rhizobium* isolates were tested and effectively nodulated the alternative host Siratro, which is a promiscuous papilionoid species, confirming that the *Rhizobium* strains obtained here are genuine legume symbionts.

Our study revealed that the predominance of certain rhizobia in *Mimosa* nodules depends on the soil properties. The most acid and less fertile soils (Brasília and Cavalcante) favoured the association of *Mimosa* with *Paraburkholderia*, while the soil with pH close to neutral and with higher fertility (Posse) led to the association with *Rhizobium*. Only one *Paraburkholderia* strain (POS_MSP2) was isolated in Posse.

Previous studies have demonstrated that *Mimosa* species tend to associate with α-proteobacteria when growing in soils with neutral/alkaline pH, such as those from Posse. For example, mimosas in India, where soil pH ranged from 7.8 to 8.2 (Gehlot et al. 2013), and in Mexico from 6.5 to 7.8 (Bontemps et al. 2016), were found in association with α-proteobacteria (*Ensifer* and *Rhizobium*). Interestingly, reports of *Rhizobium* associated with *Mimosa* in Brazilian soils, and in the Cerrado in particular, are very rare (Bontemps et al. 2010; Reis Junior et al. 2010). In fact, most host species used in our experiment have already been reported as nodulating with *Paraburkolderia* (Bontemps et al. 2010; Reis Junior et al. 2010), but the only one that has been previously found nodulating with *Rhizobium* was *M. xanthocentra* (Bontemps et al. 2010). One possible reason for such high prevalence of *Paraburkholderia* is that previous studies have mainly sampled in sites of low fertility acidic soils, which are more typical in the Cerrado region, the main centre of *Mimosa* diversity (Simon and Proença 2000). For the very few previous records of *Rhizobium* isolation from *Mimosa* carried out in this biome, there is no specific information on soil characteristics, except for that of *R. altiplani*, which was isolated from *M. pudica* growing in an alkaline soil substrate in a disturbed area close to Brasília (Baraúna et al. 2016). It is important to consider that several patches of highly-fertile limestone soil associated with seasonally dry forests, such as those found in Posse, occur in central Brazil. Therefore, it is likely that in such localities a range of *Rhizobium* strains or even other genera from the α-proteobacteria, as well as less acid-tolerant β-proteobacteria like *Cupriavidus*, would be prevalent.

In addition to environmental factors, such as soil fertility and pH, nitrogen concentration also seems to play a role in rhizobial dominance. In a competition experiment between rhizobia strains, when *Mimosa* is growing in substrate with low

concentration of N, the nodulation by *Paraburkholderia* stands out in relation to *Rhizobium*. This situation is only reversed when there is an increase of N concentration in the environment (Elliott et al. 2009; Suárez-Moreno et al. 2012). Our results support these findings since soils from Posse, where *Rhizobium* predominates, have six-fold higher concentrations of nitrogen than in Brasília or Cavalcante (Table 2).

Although *Paraburkholderia* is not restricted to acidic soils, species of this genus have acquired mechanisms to tolerate acidity that enabled them to grow on soils where many other rhizobia groups cannot survive well, which would result in competitive advantages (Stopnisek et al. 2014). A predominance of *Paraburkholderia* was also found in some legume genera in the Cape region of South Africa, where poor and acidic soils seem to favour the association of *Paraburkholderia* with endemic legumes (Garau et al. 2009; Lemaire et al. 2015, 2016).

Contrary to our expectations we did not find pronounced host specificity, since most *Mimosa* species tested were able to nodulate with bacteria from different classes. Most species nodulated with bacteria of both the genera *Paraburkholderia* and *Rhizobium*. The exception was *M. claussenii* which appears not to associate with *Rhizobium*, as evidenced by the fact that it was the only species that failed to nodulate in the soil of Posse wherein a predominance of *Rhizobium* was found. Interestingly, even among the two endemic species, *M. kalunga* and *Mimosa* sp., which are found only in the Cavalcante region, no specificity in terms of α and β-rhizobia was observed. These results partly contradict expectations about symbiotic relationships between rhizobia and endemic *Mimosa* spp. (Bontemps et al. 2010), supporting the view that these plants are more promiscuous than previously thought (Thrall et al. 2011).

This lack of specificity between plant species and bacteria may be related to the presence of different nodulation genes in the genome of several rhizobial strains, allowing a response to different types of flavonoids secreted by the plant, resulting in bacterial infection in different legume species (Peix et al. 2015; Sprent et al. 2017). From a host perspective, even habitat specialists and narrow endemic plant species seem to maintain their ability to communicate and associate with various types of rhizobia, even with those outside their restricted habitat of occurrence. In this case, the capacity of symbiotic association with different types of rhizobia remains dormant in the host genome, even after many generations of coevolution and strong habitat/symbiont specialization. This was observed, for example, with the symbionts of native and endemic *Mimosa* species in Mexico: although their dominant symbionts were α-rhizobia, species belonging to recently diverged clades (1 – 3 my old) with close

relatives in South America had retained their ability to nodulate with *Paraburkholderia* whereas species belonging to clades which had evolved in Mexico for up to 20 my had lost that ability (Bontemps et al. 2016).

Lemaire et al. (2015, 2016) surveying symbiotic relationships in a range of South African native legumes found that the plant genera investigated showed large variation in symbiotic specificity. *Amphithalea* and *Podalyria* were exclusively nodulated by *Paraburkolderia*, whereas *Argyrolobium*, *Otholobium* and *Psoralea* were associated only with α-proteobacteria (*Mesorhizobium* and *Rhizobium*). In contrast, *Aspalathus* and *Indigofera* were associated with a wide diversity of rhizobia including both α- and β-proteobacteria. This large variation in symbiotic preference within these groups may be related to environmental conditions, such as pH, elevation, geology and biogeography (Lemaire et al 2015, 2016), as previously proposed in other studies (Mishra et al. 2012; Bournaud et al. 2013; Howieson et al. 2013; Gehlot et al. 2013).

Our results indicate that different *Mimosa* species have the ability to associate with different types of rhizobia (α- and β-proteobacteria), suggesting low specificity between host and bacterium. Even endemic plant species, when grown on soils from different localities, were able to nodulate with a range of bacteria present in non-native soil rhizospheres. Soil factors such as pH, nitrogen, organic matter and fertility seem to favour the predominance of certain types of rhizobia, irrespective of host identity, thus influencing the establishment of symbiotic relationships.

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Figure Legends

Figure 1. Plants of six *Mimosa* species (A: *M. acutistipula*, B: *M. xanthocentra*, C: *M. radula*, D: *Mimosa* sp., E: *M. kalunga*, F: *M. claussenii*) used in the trap experiment with soils from three different sites in central Brazil (P: Posse, B: Brasília, C: Cavalcante) (S: sand - negative control). Scale bar = 5 cm.

Figure 2. Maximum-likelihood tree based on concatenated 16S rRNA, *recA* and *gyrB* gene sequences (2571 bp) showing the phylogenetic relationship between *Mimosa* nodulating rhizobia isolates (this work) and reference strains (GenBank). Support values (1000 bootstrap replicates > 50%) are shown. Scale bar in number of substitutions per site. Isolates code: Sites/Soils: POS = Posse (blue); BSB = Brasília (red); CAV = Cavalcante (green). Host plant: MAC = *M. acutistipula*; MCL = *M. claussenii*; MKA = *M. kalunga*; MRA = *M. radula*; MSP = *Mimosa* sp.; MXA = *M. xanthocentra*.

Figure 3. Maximum-likelihood tree based on the nodC gene sequences (610 bp) showing the phylogenetic relationship between *Mimosa* nodulating rhizobia isolates (this work) and reference strains (GenBank). Support values (1000 bootstrap replicates > 50%) are shown. Scale bar in number of substitutions per site. Isolates code: Sites/Soils: POS = Posse (blue); BSB = Brasília (red); CAV = Cavalcante (green). Host plant: MAC = *M. acutistipula*; MCL = *M. claussenii*; MKA = *M. kalunga*; MRA = *M. radula*; MSP = *Mimosa* sp.; MXA = *M. xanthocentra*.

Figure 4. Maximum-likelihood tree based on the nifH gene sequences (300 bp) showing the phylogenetic relationship between *Mimosa* nodulating rhizobia isolates (this work) and reference strains (GenBank). Support values (1000 bootstrap replicates > 50%) are shown. Scale bar in number of substitutions per site. Isolates code: Sites/Soils: POS = Posse (blue); BSB = Brasília (red); CAV = Cavalcante (green). Host plant: MAC = *M. acutistipula*; MCL = *M. claussenii*; MKA = *M. kalunga*; MRA = *M. radula*; MSP = *Mimosa* sp.; MXA = *M. xanthocentra*.

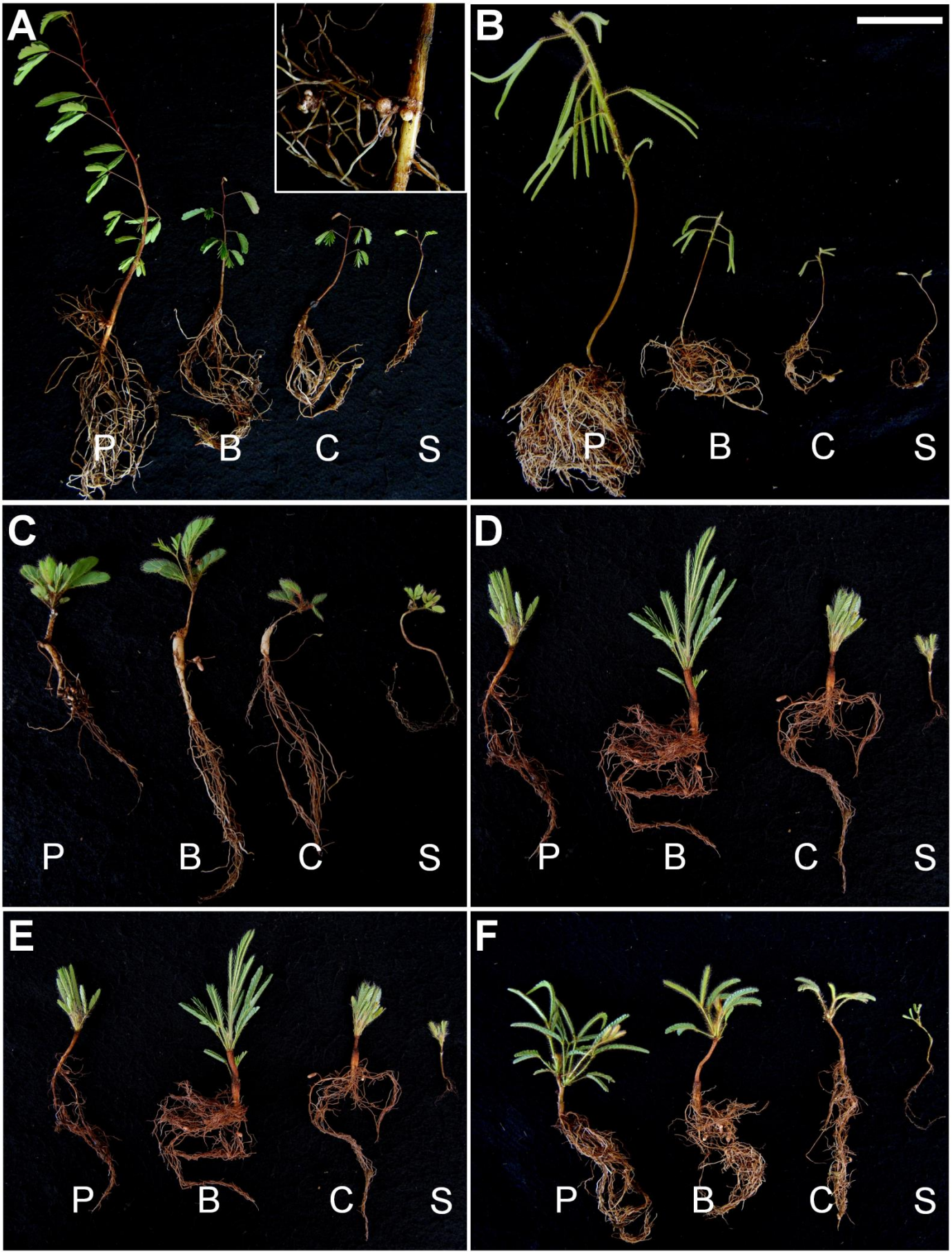

Figure 1.

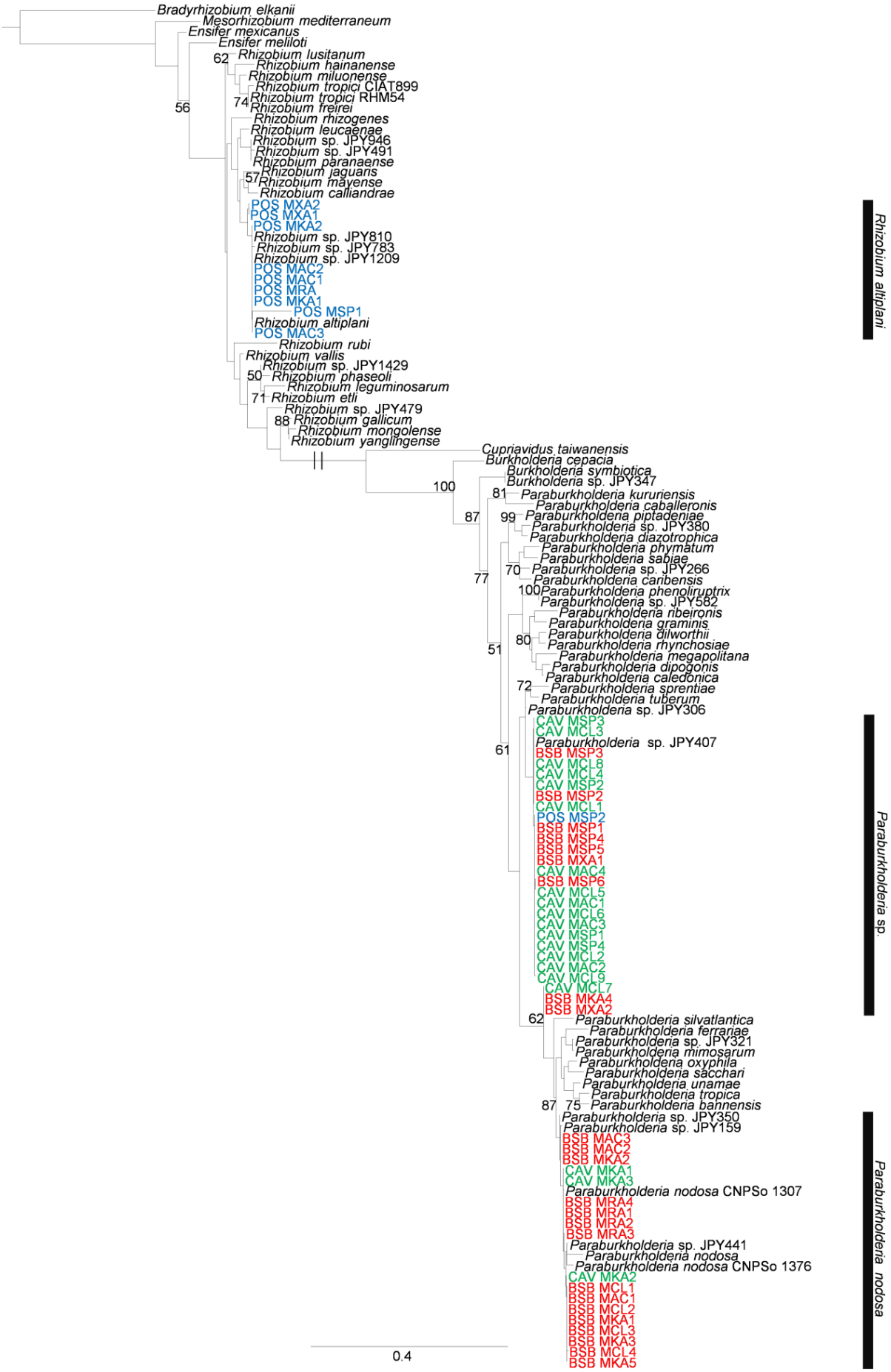
Bradyrhizobium elkanii
Mesorhizobium mediterraneum
Ensifer mexicanus
Ensifer meliloti
Rhizobium lusitanum
Rhizobium hainanense
Rhizobium miluonense
Rhizobium tropici CIAT899
Rhizobium tropici RHM54
Rhizobium freirei
Rhizobium rhizogenes
Rhizobium leucaenae
Rhizobium sp. JPY946
Rhizobium sp. JPY491
Rhizobium paranaense
Rhizobium jaguaris
Rhizobium mayense
Rhizobium calliandrae
POS MXA2
POS MXA1
POS MKA2
Rhizobium sp. JPY810
Rhizobium sp. JPY783
Rhizobium sp. JPY1209
POS MAC2
POS MAC1
POS MRA
POS MKA1
POS MSP1
Rhizobium altiplani
POS MAC3
Rhizobium rubi
Rhizobium vallis
Rhizobium sp. JPY1429
Rhizobium phaseoli
Rhizobium leguminosarum
Rhizobium etli
Rhizobium sp. JPY479
Rhizobium gallicum
Rhizobium mongolense
Rhizobium yanglingense
Cupriavidus taiwanensis
Burkholderia cepacia
Burkholderia symbiotica
Burkholderia sp. JPY347
Paraburkholderia kururiensis
Paraburkholderia caballeronis
Paraburkholderia piptadeniae
Paraburkholderia sp. JPY380
Paraburkholderia diazotrophica
Paraburkholderia phymatum
Paraburkholderia sabiae
Paraburkholderia sp. JPY266
Paraburkholderia caribensis
Paraburkholderia phenoliruptrix
Paraburkholderia sp. JPY582
Paraburkholderia ribeironis
Paraburkholderia graminis
Paraburkholderia dilworthii
Paraburkholderia rhynchosiae
Paraburkholderia megapolitana
Paraburkholderia dipogonis
Paraburkholderia caledonica
Paraburkholderia sprentiae
Paraburkholderia tuberum
Paraburkholderia sp. JPY306
CAV MSP3
CAV MCL3
Paraburkholderia sp. JPY407
BSB MSP3
CAV MCL8
CAV MCL4
CAV MSP2
BSB MSP2
CAV MCL1
POS MSP2
BSB MSP1
BSB MSP4
BSB MSP5
BSB MXA1
CAV MAC4
BSB MSP6
CAV MCL5
CAV MAC1
CAV MCL6
CAV MAC3
CAV MSP1
CAV MSP4
CAV MCL2
CAV MAC2
CAV MCL9
CAV MCL7
BSB MKA4
BSB MXA2
Paraburkholderia silvatlantica
Paraburkholderia ferrariae
Paraburkholderia sp. JPY321
Paraburkholderia mimosarum
Paraburkholderia oxyphila
Paraburkholderia sacchari
Paraburkholderia unamae
Paraburkholderia tropica
Paraburkholderia bannensis
Paraburkholderia sp. JPY350
Paraburkholderia sp. JPY159
BSB MAC3
BSB MAC2
BSB MKA2
CAV MKA1
CAV MKA3
Paraburkholderia nodosa CNPSo 1307
BSB MRA4
BSB MRA1
BSB MRA2
BSB MRA3
Paraburkholderia sp. JPY441
Paraburkholderia nodosa
Paraburkholderia nodosa CNPSo 1376
CAV MKA2
BSB MCL1
BSB MAC1
BSB MCL2
BSB MKA1
BSB MCL3
BSB MKA3
BSB MCL4
BSB MKA5
0.4
Rhizobium altiplani
Paraburkholderia sp.
Paraburkholderia nodosa

Figure 2.

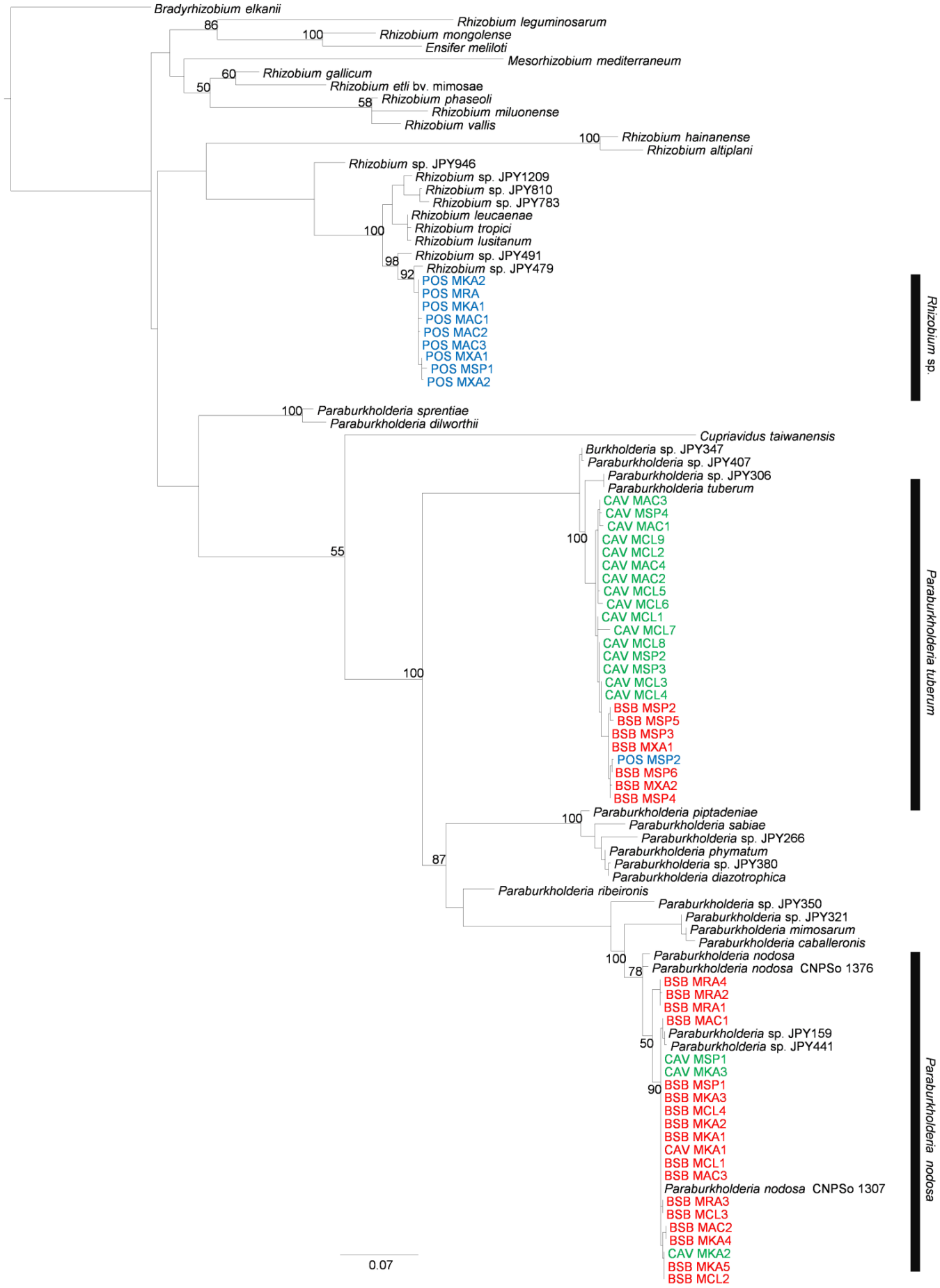
Bradyrhizobium elkanii
Rhizobium leguminosarum
Rhizobium mongolense
Ensifer meliloti
Mesorhizobium mediterraneum
Rhizobium gallicum
Rhizobium etli bv. mimosae
Rhizobium phaseoli
Rhizobium miluonense
Rhizobium vallis
Rhizobium hainanense
Rhizobium altiplani
Rhizobium sp. JPY946
Rhizobium sp. JPY1209
Rhizobium sp. JPY810
Rhizobium sp. JPY783
Rhizobium leucaenae
Rhizobium tropici
Rhizobium lusitanum
Rhizobium sp. JPY491
Rhizobium sp. JPY479
POS MKA2
POS MRA
POS MKA1
POS MAC1
POS MAC2
POS MAC3
POS MXA1
POS MSP1
POS MXA2
Paraburkholderia sprentiae
Paraburkholderia dilworthii
Cupriavidus taiwanensis
Burkholderia sp. JPY347
Paraburkholderia sp. JPY407
Paraburkholderia sp. JPY306
Paraburkholderia tuberum
CAV MAC3
CAV MSP4
CAV MAC1
CAV MCL9
CAV MCL2
CAV MAC4
CAV MAC2
CAV MCL5
CAV MCL6
CAV MCL1
CAV MCL7
CAV MCL8
CAV MSP2
CAV MSP3
CAV MCL3
CAV MCL4
BSB MSP2
BSB MSP5
BSB MSP3
BSB MXA1
POS MSP2
BSB MSP6
BSB MXA2
BSB MSP4
Paraburkholderia piptadeniae
Paraburkholderia sabiae
Paraburkholderia sp. JPY266
Paraburkholderia phymatum
Paraburkholderia sp. JPY380
Paraburkholderia diazotrophica
Paraburkholderia ribeironis
Paraburkholderia sp. JPY350
Paraburkholderia sp. JPY321
Paraburkholderia mimosarum
Paraburkholderia caballeronis
Paraburkholderia nodosa
Paraburkholderia nodosa CNPSo 1376
BSB MRA4
BSB MRA2
BSB MRA1
BSB MAC1
Paraburkholderia sp. JPY159
Paraburkholderia sp. JPY441
CAV MSP1
CAV MKA3
BSB MSP1
BSB MKA3
BSB MCL4
BSB MKA2
BSB MKA1
CAV MKA1
BSB MCL1
BSB MAC3
Paraburkholderia nodosa CNPSo 1307
BSB MRA3
BSB MCL3
BSB MAC2
BSB MKA4
CAV MKA2
BSB MKA5
BSB MCL2
0.07
Rhizobium sp.
Paraburkholderia tuberum
Paraburkholderia nodosa

Figure 3.

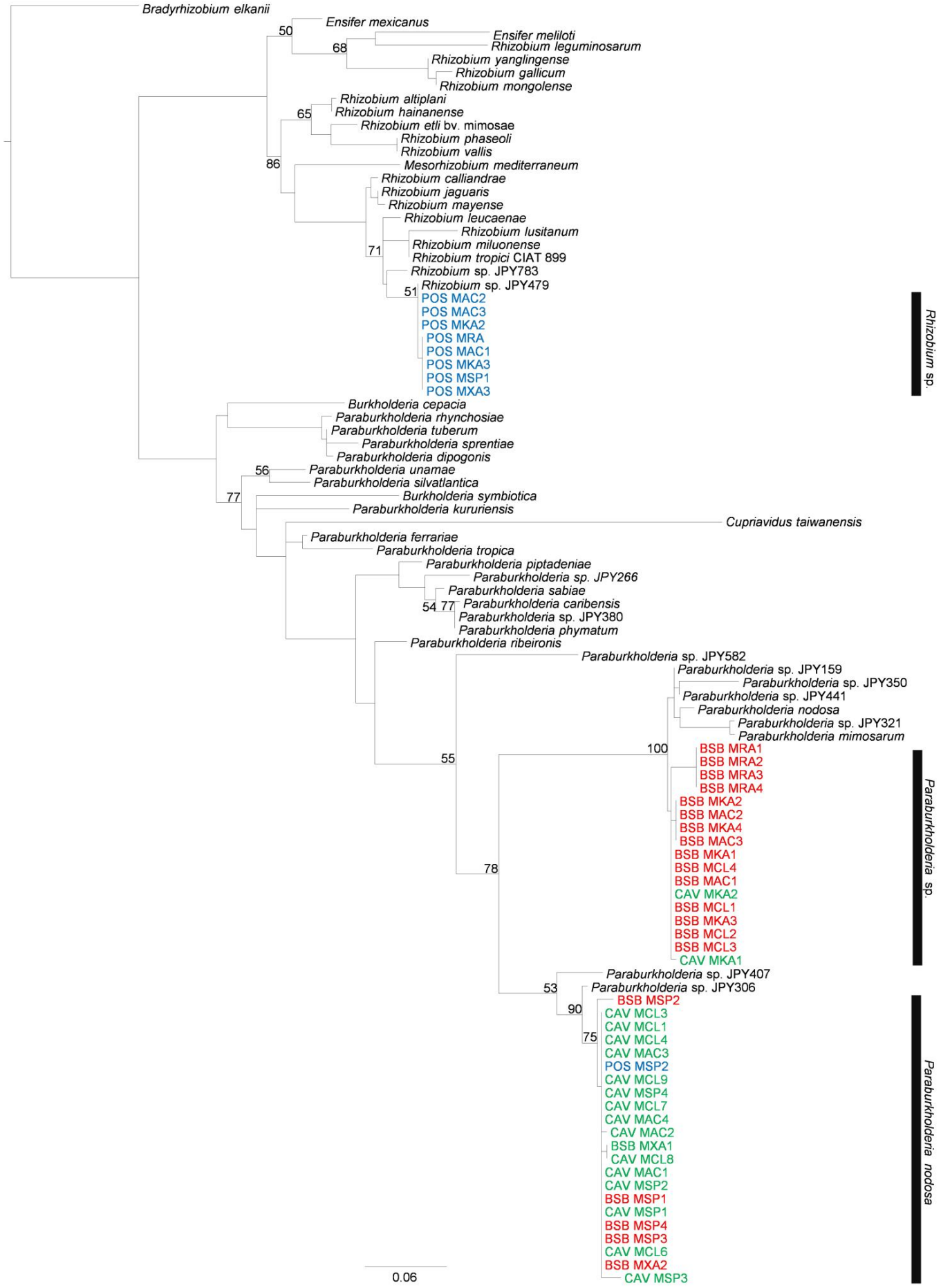
Bradyrhizobium elkanii
Ensifer mexicanus
Ensifer meliloti
Rhizobium leguminosarum
Rhizobium yanglingense
Rhizobium gallicum
Rhizobium mongolense
Rhizobium altiplani
Rhizobium hainanense
Rhizobium etli bv. mimosae
Rhizobium phaseoli
Rhizobium vallis
Mesorhizobium mediterraneum
Rhizobium calliandrae
Rhizobium jaguaris
Rhizobium mayense
Rhizobium leucaenae
Rhizobium lusitanum
Rhizobium miluonense
Rhizobium tropici CIAT 899
Rhizobium sp. JPY783
Rhizobium sp. JPY479
POS MAC2
POS MAC3
POS MKA2
POS MRA
POS MAC1
POS MKA3
POS MSP1
POS MXA3
Rhizobium sp.
Burkholderia cepacia
Paraburkholderia rhynchosiae
Paraburkholderia tuberum
Paraburkholderia sprentiae
Paraburkholderia dipogonis
Paraburkholderia unamae
Paraburkholderia silvatlantica
Burkholderia symbiotica
Paraburkholderia kururiensis
Cupriavidus taiwanensis
Paraburkholderia ferrariae
Paraburkholderia tropica
Paraburkholderia piptadeniae
Paraburkholderia sp. JPY266
Paraburkholderia sabiae
Paraburkholderia caribensis
Paraburkholderia sp. JPY380
Paraburkholderia phymatum
Paraburkholderia ribeironis
Paraburkholderia sp. JPY582
Paraburkholderia sp. JPY159
Paraburkholderia sp. JPY350
Paraburkholderia sp. JPY441
Paraburkholderia nodosa
Paraburkholderia sp. JPY321
Paraburkholderia mimosarum
BSB MRA1
BSB MRA2
BSB MRA3
BSB MRA4
BSB MKA2
BSB MAC2
BSB MKA4
BSB MAC3
BSB MKA1
BSB MCL4
BSB MAC1
CAV MKA2
BSB MCL1
BSB MKA3
BSB MCL2
BSB MCL3
CAV MKA1
Paraburkholderia sp.
Paraburkholderia sp. JPY407
Paraburkholderia sp. JPY306
BSB MSP2
CAV MCL3
CAV MCL1
CAV MCL4
CAV MAC3
POS MSP2
CAV MCL9
CAV MSP4
CAV MCL7
CAV MAC4
CAV MAC2
BSB MXA1
CAV MCL8
CAV MAC1
CAV MSP2
BSB MSP1
CAV MSP1
BSB MSP4
BSB MSP3
CAV MCL6
BSB MXA2
CAV MSP3
Paraburkholderia nodosa
50
68
65
86
71
51
56
77
54
77
100
55
78
53
90
75
0.06

Figure 4.

Table 1. Description of the three sites in central Brazil (Posse, Brasília and Cavalcante) where seeds from *Mimosa* species and soil samples were collected.

City/State	Site name	Coordinates	Soil classification*	Vegetation	Species collected
Posse/GO	Sabonete farm	14°04'00"S; 46°49"17'W; 630m	Oxisol (Nitisol) with limestone rock outcrop, high fertility and high pH	Seasonal Deciduous Forest (Mata Seca Decidual)	*Mimosa acutistipula* and *Mimosa xanthocentra*
Brasília/DF	Brasília National Park	15°43'39"S; 47°56'55" W; 1038m	Oxisol (Ferralsol), deep, low fertility and low pH	Woody Savanna (Cerrado)	*Mimosa claussenii* and *Mimosa radula*
Cavalcante/GO	RPPN Serra do Tombador	13°40'48"S; 47°49'21"W; 794m	Inceptsol (Cambisol) with rocky material derived from sandstone, low fertility and low pH	Shrubby Savannah (Cerrado Rupestre)	*Mimosa kalunga* and *Mimosa* sp.

* Soil classification: American System; between parenthesis World Reference Base.

Table 2. Chemical and granulometric characteristics of the soil samples from the three collection sites (Posse, Brasília and Cavalcante), in the depth of 0 to 20 cm. CEC: Cation exchange capacity; OM: Organic matter

Collection sites	Ca cmolc/dm3	Mg cmolc/dm3	Al cmolc/dm3	H+AL cmolc/dm3	K mg/dm3	CEC	P mg/dm3	OM %	$pHCaCl_2$	N %	Clay %	Silt %	Sand %
Posse	15.0	1.6	0.0	1.9	291	19.2	8.1	11.0	6.3	0.80	30	8	62
Brasília	0.6	0.2	0.6	7.5	48	8.4	0.5	4.5	4.2	0.14	48	12	40
Cavalcante	0.2	0.1	0.8	2.9	40	3.3	0.5	1.7	4.3	0.13	9	4	87

Table 3. Total biomass and number of nodules (mean ± standard deviation) of the six *Mimosa* species in the different soil types from central Brazil (Posse, Brasília and Cavalcante). Original site of each species in grey.

Species	**Posse soil**		**Brasília soil**		**Cavalcante soil**		**Control (Sand)**	
	Biomass* (g)	Nº nodules	Biomass* (g)	Nº nodules	Biomass* (g)	Nº nodules	Biomass (g)	Nº nodules
M. acutistipula	0.52a ±0.21	14.3a ±3.0	0.11b ±0.02	8.0b ±1.7	0.16b ±0.06	8.0b ±4.6	0.02 ±0.01	0 ±0
M. claussenii	0.70a ±0.39	0.0b ±0.0	0.53a ±0.13	7.3a ±2.5	0.24a ±0.03	7.0a ±2.3	0.05 ±0.02	0 ±0
M. kalunga	0.41a ±0.05	3.0a ±1.7	0.19b ±0.00	3.5a ±1.3	0.24b ±0.02	0.8a ±0.7	0.03 ±0.01	0 ±0
M. radula	0.20a ±0.20	1.8a ±2.4	0.11a ±0.03	2.0a ±1.8	0.06a ±0.02	0.6a ±0.5	0.02 ±0.01	0 ±0
***Mimosa* sp.**	0.49a ±0.14	1.6a ±2.0	0.31a ±0.09	3.8a ±0.8	0.28a ±0.01	3.5a ±2.6	0.04 ±0.02	0 ±0
M. xanthocentra	2.31a ±0.72	120.6a ±16.7	0.14b ±0.00	8.2b ±7.1	0.04b ±0.01	0.0b ±0.0	0.02 ±0.01	0 ±0

*Values followed by the same letter, in the lines, are not different according to Duncan's test ($p < 0.05$).

Table 4. Bacterial (rhizobia) isolates obtained from a trap experiment using six species of *Mimosa* growing in soils from three different sites in central Brazil. Identification of the isolates at the genus level was based on the sequencing of five genes. The nodulation column indicates the result of an authentication experiment using *M. pudica* as plant host (+++ = effective nodulation; + = ineffective nodulation). For some *Rhizobium* isolates, an authentication experiment was also performed in the alternative host *Macroptilium atropurpureum* (nodulation effectiveness in parentheses).

Isolate	Identification (genus)	Host species	Site	Nodulation
POS_MAC1	*Rhizobium*	*M. acutistipula*	Posse	+ (+++)
POS_MAC2	*Rhizobium*	*M. acutistipula*	Posse	+
POS_MAC3	*Rhizobium*	*M. acutistipula*	Posse	+ (+++)
POS_MKA1	*Rhizobium*	*M. kalunga*	Posse	+
POS_MKA2	*Rhizobium*	*M. kalunga*	Posse	+ (+++)
POS_MRA	*Rhizobium*	*M. radula*	Posse	+ (+++)
POS_MSP2	*Paraburkholderia*	*Mimosa* sp.	Posse	+++
POS_MSP1	*Rhizobium*	*Mimosa* sp.	Posse	+ (+++)
POS_MXA2	*Rhizobium*	*M. xanthocentra*	Posse	+
POS_MXA1	*Rhizobium*	*M. xanthocentra*	Posse	+
BSB_MAC1	*Paraburkholderia*	*M. acutistipula*	Brasília	+++
BSB_MAC2	*Paraburkholderia*	*M. acutistipula*	Brasília	+++
BSB_MAC3	*Paraburkholderia*	*M. acutistipula*	Brasília	+++
BSB_MCL4	*Paraburkholderia*	*M. claussenii*	Brasília	+++
BSB_MCL3	*Paraburkholderia*	*M. claussenii*	Brasília	+++
BSB_MCL2	*Paraburkholderia*	*M. claussenii*	Brasília	+++
BSB_MCL1	*Paraburkholderia*	*M. claussenii*	Brasília	+++
BSB_MKA3	*Paraburkholderia*	*M. kalunga*	Brasília	+++
BSB_MKA1	*Paraburkholderia*	*M. kalunga*	Brasília	+++
BSB_MKA5	*Paraburkholderia*	*M. kalunga*	Brasília	+++
BSB_MKA2	*Paraburkholderia*	*M. kalunga*	Brasília	+++
BSB_MKA4	*Paraburkholderia*	*M. kalunga*	Brasília	+++
BSB_MRA3	*Paraburkholderia*	*M. radula*	Brasília	+++
BSB_MRA1	*Paraburkholderia*	*M. radula*	Brasília	+++
BSB_MRA4	*Paraburkholderia*	*M. radula*	Brasília	+++
BSB_MRA2	*Paraburkholderia*	*M. radula*	Brasília	+++
BSB_MSP6	*Paraburkholderia*	*Mimosa* sp.	Brasília	+++
BSB_MSP1	*Paraburkholderia*	*Mimosa* sp.	Brasília	+++
BSB_MSP4	*Paraburkholderia*	*Mimosa* sp.	Brasília	+++
BSB_MSP2	*Paraburkholderia*	*Mimosa* sp.	Brasília	+++
BSB_MSP5	*Paraburkholderia*	*Mimosa* sp.	Brasília	+++

BSB_MSP3	*Paraburkholderia*	*Mimosa* sp.	Brasília	+++
BSB_MXA1	*Paraburkholderia*	*M. xanthocentra*	Brasília	+++
CAV_MAC4	*Paraburkholderia*	*M. acutistipula*	Cavalcante	+++
CAV_MAC2	*Paraburkholderia*	*M. acutistipula*	Cavalcante	+++
CAV_MAC3	*Paraburkholderia*	*M. acutistipula*	Cavalcante	+++
CAV_MAC1	*Paraburkholderia*	*M. acutistipula*	Cavalcante	+++
CAV_MCL9	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL7	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL6	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL5	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL4	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL1	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL2	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL3	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MCL8	*Paraburkholderia*	*M. claussenii*	Cavalcante	+++
CAV_MKA2	*Paraburkholderia*	*M. kalunga*	Cavalcante	+++
CAV_MKA3	*Paraburkholderia*	*M. kalunga*	Cavalcante	+++
CAV_MKA1	*Paraburkholderia*	*M. kalunga*	Cavalcante	+++
CAV_MSP4	*Paraburkholderia*	*Mimosa* sp.	Cavalcante	+++
CAV_MSP3	*Paraburkholderia*	*Mimosa* sp.	Cavalcante	+++
CAV_MSP1	*Paraburkholderia*	*Mimosa* sp.	Cavalcante	+++
CAV_MSP2	*Paraburkholderia*	*Mimosa* sp.	Cavalcante	+++

Supporting Information

Table S1. *Mimosa* species used in this work, vouchers (deposited in Embrapa Cenargen herbarium), growth habit and geographic distribution according to Barneby (1991) and Simon et al. (2010).

Species	Voucher number	Growth habit	Geographic distribution
Mimosa acutistipula (Mart.) Benth. var. *acutistipula*	M. F. Simon 2455	Tree	Northeast, midwest and southeast (Brazil and eastern Bolivia), mostly in seasonally dry forests on fertile soils
Mimosa claussenii Benth. var. *claussenii*	E. B. A. Dias 439	Shrub	Widespread across Cerrado region in central Brazil
Mimosa kalunga M.F. Simon & C.E. Hughes	L. M. Borges 1069	Subshrub	Endemic to Cavalcante, Goiás (Brazil)
Mimosa radula Benth. var. *imbricata* (Benth.) Barneby	J. R. Santos 742	Subshrub	Widespread across Cerrado region in central Brazil
Mimosa sp.	L. M. Borges 1061	Shrub	Endemic to Cavalcante, Goiás (Brazil)
Mimosa xanthocentra Mart. var. *xanthocentra*	M. F. Simon 2454	Subshrub	Widespread in South America, ruderal

Table S2. Sequences of primers used in this study.

Region (gene)	Primer	Primer sequence (5'- 3')	Reference
16S rRNA	16S f27	AGAGTTTGATCCTGGCTCAG	Lane (1991)
	16S r1492	ACGGYTACCTTGTTACGACTT	Heuer et al. (1997)
recA	recA41F	TTC GGCAAGGGMTCGRTSATG	Vinuesa et al. (2005)
	recA640R	ACATSACRCCGATCTTCATGC	
gyrB	gyrB343F	TTCGACCAGAAYTCCTAYAAGG	Martens et al. (2008)
	gyrB1043R	AGCTTGTCCTTSGTCTGCG	
nodC	nodC540F	TGATYGAYATGGARTAYTGGYT	Sarita et al. (2005)
	nod1160R	CGYGACAGCCANTCKCTATTG	
nifH	nifH-F	AAAGGYGGWATCGGYAARTCCACCAC	Loiret et al. (2004)
	nifH-R	TTGTTSGCSGCRTACATSGCCATCAT	

Table S3. Genbank accession numbers of the strains used in this study according to each gene.

Strain	**16S**	**recA**	**gyrB**	**nifH**	**nodC**
Bradyrhizobium elkanii	NR 117947	KF532941	HE576484	HQ231535	D28965
Burkholderia cepacia	U96927	AF143786	HQ849191	HQ699896	-
Burkholderia sp. JPY347	FN543709	FN543852	-	-	FN543572
Burkholderia symbiotica	FN543707	FN543850	HF952726	FN543990	-
Cupriavidus taiwanensis	FN908230	FN908248	-	NC 010529	FR853112
Ensifer meliloti	D14509	CP004140	HQ438227	KC848573	AY664618
Ensifer mexicanus	DQ411930	DQ411951	-	DQ411936	-
Mesorhizobium mediterraneum	KX226348	AJ294369	KP251585	EU267716	DQ407293
Paraburkholderia bannensis	AB561874	-	-	-	-
Paraburkholderia caballeronis	EF139186	-	-	-	KF484909
Paraburkholderia caledonica	AF215704	HQ849134	HQ849189	-	-
Paraburkholderia caribensis	Y17009	HQ398576	HQ849190	AJ505317	-
Paraburkholderia diazotrophica	FN543713	FN543856	KM655754	FN543995	FN543576
Paraburkholderia eburnea	JQ692176	-	-	-	-
Paraburkholderia ferrariae	DQ514537	HQ398577	HQ849193	EF158799	-
Paraburkholderia graminis	U96939	AY619653	HQ849199	-	-
Paraburkholderia kururiensis	AB024310	AY619654	HQ849201	AY098590	-
Paraburkholderia megapolitana	AM489502	HQ398583	-	-	-
Paraburkholderia mimosarum	AY752958	HQ398584	HQ849202	AY883420	FR853108
Paraburkholderia nodosa	AY773189	HQ398586	HQ849204	AY533866	HE983823
Paraburkholderia nodosa CNPSo 1307	KU097038	KU097186	KU097096	-	KU097141
Paraburkholderia nodosa CNPSo 1376	KU097054	KU097202	KU097112	-	KU097157
Paraburkholderia phymatum	AJ302312	AY619667	HQ849208	AJ505319	DQ888213
Paraburkholderia piptadeniae	LN875219	LN875227	LN875234	LN875250	LN875243
Paraburkholderia ribeironis	LN875221	LN875230	LN875236	HF536754	LN875244
Paraburkholderia rynchosiae	EU219865	HE994064	HE994045	EU219869	-
Paraburkholderia sabiae	AY773186	EU294397	LT632431	AY533867	KT390825
Paraburkholderia sacchari	AF263278	HQ849156	HQ849212	-	-
Paraburkholderia sp. BSB MAC1					
Paraburkholderia sp. BSB MAC2					
Paraburkholderia sp. BSB MAC3					
Paraburkholderia sp. BSB MCL1					
Paraburkholderia sp. BSB MCL2					
Paraburkholderia sp. BSB MCL3					
Paraburkholderia sp. BSB MCL4					
Paraburkholderia sp. BSB MKA1					
Paraburkholderia sp. BSB MKA2					
Paraburkholderia sp. BSB MKA3					
Paraburkholderia sp. BSB MKA4					
Paraburkholderia sp. BSB MKA5					
Paraburkholderia sp. BSB MRA1					
Paraburkholderia sp. BSB MRA2					
Paraburkholderia sp. BSB MRA3					

Paraburkholderia sp. BSB MRA4					
Paraburkholderia sp. BSB MSP1					
Paraburkholderia sp. BSB MSP2					
Paraburkholderia sp. BSB MSP3					
Paraburkholderia sp. BSB MSP4					
Paraburkholderia sp. BSB MSP5					
Paraburkholderia sp. BSB MSP6					
Paraburkholderia sp. BSB MXA1					
Paraburkholderia sp. BSB MXA2					
Paraburkholderia sp. CAV MAC1					
Paraburkholderia sp. CAV MAC2					
Paraburkholderia sp. CAV MAC3					
Paraburkholderia sp. CAV MAC4					
Paraburkholderia sp. CAV MCL1					
Paraburkholderia sp. CAV MCL2					
Paraburkholderia sp. CAV MCL3					
Paraburkholderia sp. CAV MCL4					
Paraburkholderia sp. CAV MCL5					
Paraburkholderia sp. CAV MCL6					
Paraburkholderia sp. CAV MCL7					
Paraburkholderia sp. CAV MCL8					
Paraburkholderia sp. CAV MCL9					
Paraburkholderia sp. CAV MKA1					
Paraburkholderia sp. CAV MKA2					
Paraburkholderia sp. CAV MKA3					
Paraburkholderia sp. CAV MSP1					
Paraburkholderia sp. CAV MSP2					
Paraburkholderia sp. CAV MSP3					
Paraburkholderia sp. CAV MSP4					
Paraburkholderia sp. POS MSP2					
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Paraburkholderia sp. JPY266	FN543671	FN543814	-	FN543954	FN543533
Paraburkholderia sp. JPY306	FN543694	FN543837	-	FN543977	FN543557
Paraburkholderia sp. JPY321	FN543702	FN543845	-	FN543985	FN543566
Paraburkholderia sp. JPY350	FN543711	FN543854	-	FN543993	FN543574
Paraburkholderia sp. JPY380	FN543717	FN543860	-	FN543998	FN543578
Paraburkholderia sp. JPY407	FN543731	FN543874	-	FN544011	FN543592
Paraburkholderia sp. JPY441	FN543749	FN543892	-	FN544029	FN543610
Paraburkholderia sp. JPY582	FN543775	FN543918	-	FN544053	-
Paraburkholderia tropica	AJ420332	HQ849161	HQ849216	AY098592	-
Paraburkholderia tuberum	AJ302311	HQ849162	HQ849217	AJ302315	FR853110
Paraburkholderia unamae	AY221956	DQ514539	-	EF158804	-
Paraburkholderia dilworthii	HQ698907	HG422553	HE994041	-	HG934336
Paraburkholderia dipogonis	JX009147	JX009158	-	JX009151	-
Paraburkholderia oxyphila	AB488693	KU180357	KU180358	-	-
Paraburkholderia phenoliruptrix	AY435213	HQ398589	HQ849207	-	-

Paraburkholderia silvatlantica	AY965240	HQ849157	HQ849213	EF158808	-
Paraburkholderia sprentiae	HQ698903	HE99406	HE994059	HG422564	HG425193
Rizhobium altiplani	KX022634	KX022644	-	KX022684	KX022674
Rizhobium calliandrae	JX855164	JX855191	KF761514	JX855219	-
Rizhobium etli	DQ648575	CP005950	JN129340	EU386144	EU386149
Rizhobium freirei	EU488742	EU488827	KJ603458	-	-
Rizhobium gallicum	U86343	AY907357	HQ438235	EU418410	AF217270
Rizhobium hainanense	U71078	HQ394252	HQ438236	AY934876	DQ010039
Rizhobium jaguaris	JX855169	JX855192	KF761516	JX855222	-
Rizhobium leguminosarum	U29386	AM236080	JQ795182	DQ450935	AY665788
Rizhobium leucaenae	X67234	AJ294372	JN129330	JN580767	JN580662
Rizhobium lusitanum	AY738130	KF206875	KC293525	AY943644	HM852098
Rizhobium mayense	JX855172	JX855195	KF761515	JX855225	-
Rizhobium miluonense	EF061096	HM047131	KF963015	JN580786	JN580680
Rizhobium mongolense	U89817	AY907358	-	AY929540	GQ507367
Rizhobium paranaense	EU488753	EU488826	KF738135	-	-
Rizhobium phaseoli	EF141340	EF113136	KC293518	JN580758	HM441255
Rizhobium rhizogenes	D14501	AM182126	HQ438225	-	-
Rizhobium rubi	AY626395	AM182122	HQ438207	-	EU281314
Rizhobium sp. POS MAC1					
Rizhobium sp. POS MAC2					
Rizhobium sp. POS MAC3					
Rizhobium sp. POS MKA1					
Rizhobium sp. POS MKA2					
Rizhobium sp. POS MRA					
Rizhobium sp. POS MSP1					
Rizhobium sp. POS MXA1					
Rizhobium sp. POS MXA2					
Rhizobium sp. JPY1209	KP760722	KP760808	-	-	KP760578
Rhizobium sp. JPY1429	KP760748	KP760832	-	-	-
Rhizobium sp. JPY479	FN543788	-	-	FN544065	FN543645
Rhizobium sp. JPY491	FN543789	-	-	-	FN543646
Rhizobium sp. JPY783	KP760673	KP760762	-	KP760497	KP760534
Rhizobium sp. JPY946	KP760693	KP760781	-	KP760510	KP760550
Rhizobium sp. JPY810	KP760681	KP760769	-	-	KP760540
Rizhobium tropici CIAT 899	U89832	EU488815	JN129327	JX863573	-
Rizhobium tropici RHM54	JQ085251	JQ085274	-	-	JQ085263
Rizhobium vallis	FJ839677	GU211770	-	GU211767	GU211769
Rizhobium yangligense	AF003375	KF206887	-	AY929541	-

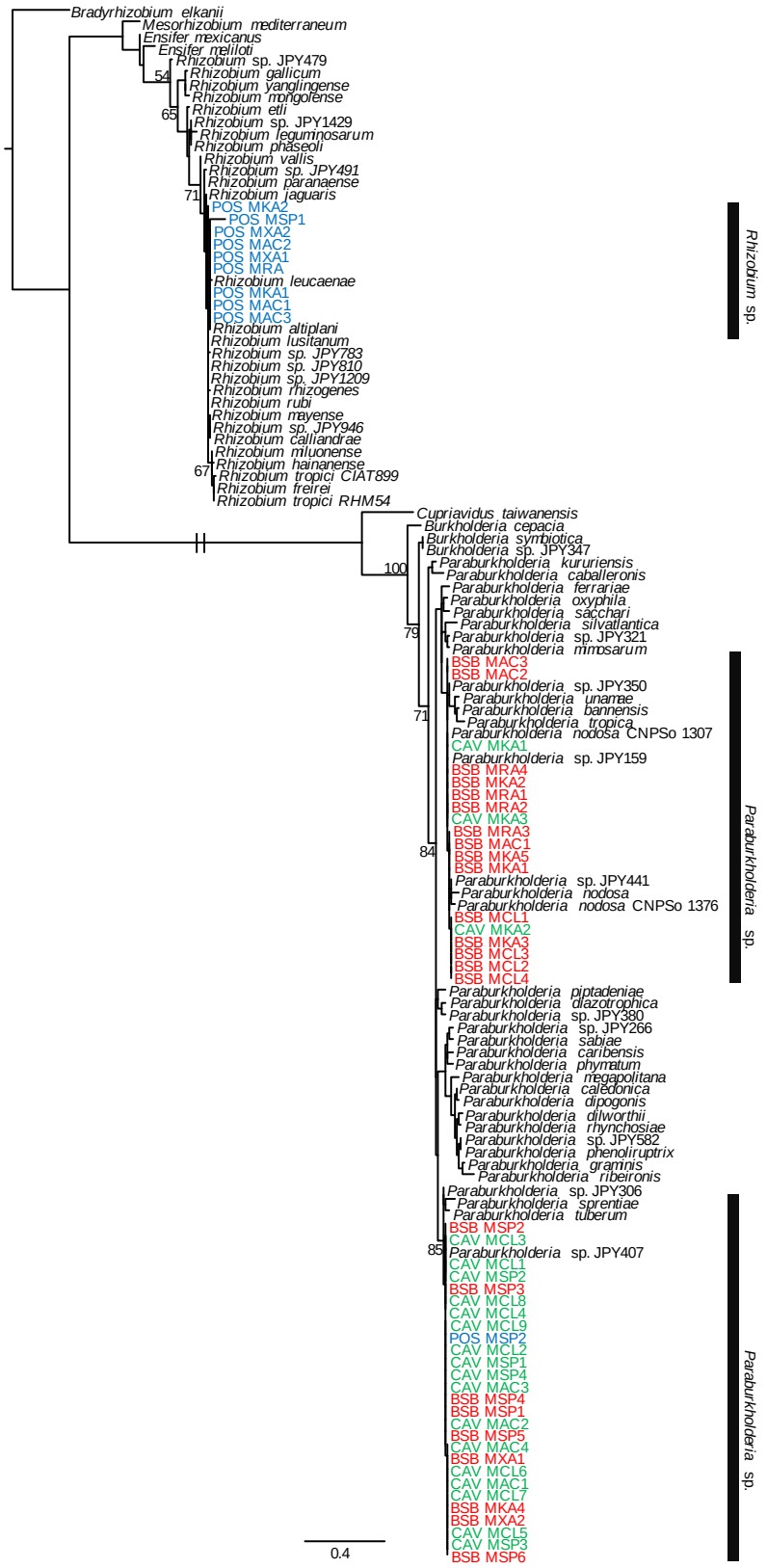

Figure S1. Maximum-likelihood tree based on the 16S rRNA gene sequences (1340 bp) showing the phylogenetic relationship between *Mimosa* nodulating rhizobia isolates (this work) and reference strains (GenBank). Support values (1000 bootstrap replicates > 50%) are shown. Scale bar in number of substitutions per site. Isolates code: Sites/Soils: POS = Posse (blue); BSB = Brasília (red); CAV = Cavalcante (green). Host plant: MAC = *M. acutistipula*; MCL = *M. claussenii*; MKA = *M. kalunga*; MRA = *M. radula*; MSP = *Mimosa* sp.; MXA = *M. xanthocentra*.

Figure S2. Maximum-likelihood tree based on the recA gene sequences (540 bp) showing the phylogenetic relationship between *Mimosa* nodulating rhizobia isolates (this work) and reference strains (GenBank). Support values (1000 bootstrap replicates > 50%) are shown. Scale bar in number of substitutions per site. Isolates code: Sites/Soils: POS = Posse (blue); BSB = Brasília (red); CAV = Cavalcante (green). Host plant: MAC = *M. acutistipula*; MCL = *M. claussenii*; MKA = *M. kalunga*; MRA = *M. radula*; MSP = *Mimosa* sp.; MXA = *M. xanthocentra*.

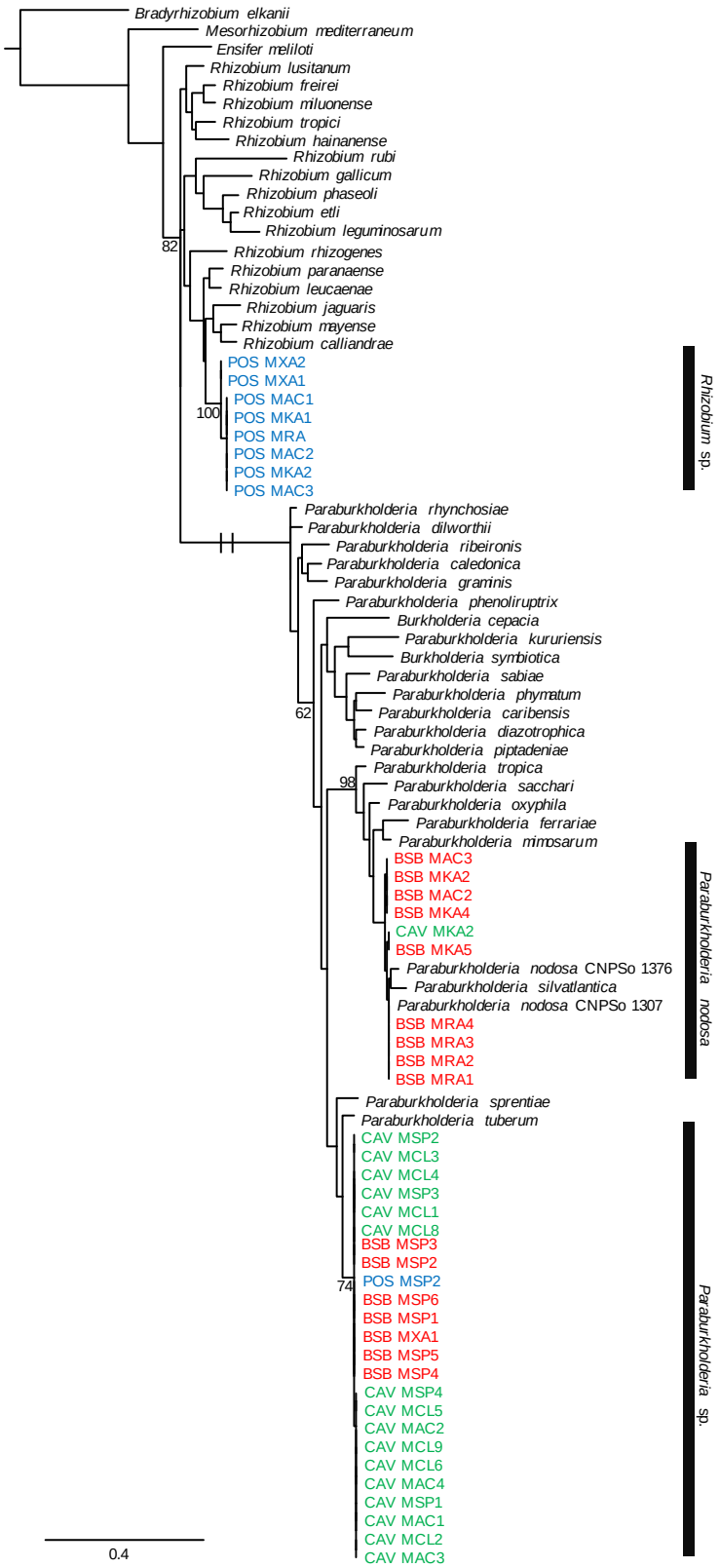

Figure S3. Maximum-likelihood tree based on the gyrB gene sequences (540 bp) showing the phylogenetic relationship between *Mimosa* nodulating rhizobia isolates (this study) and reference strains (GenBank). Support values (1000 bootstrap replicates > 50%) are shown. Scale bar in number of substitutions per site. Isolate codes: Sites/Soils: POS = Posse (blue); BSB = Brasília (red); CAV = Cavalcante (green). Host plant: MAC = *M. acutistipula*; MCL = *M. claussenii*; MKA = *M. kalunga*; MRA = *M. radula*; MSP = *Mimosa* sp.; MXA = *M. xanthocentra*.

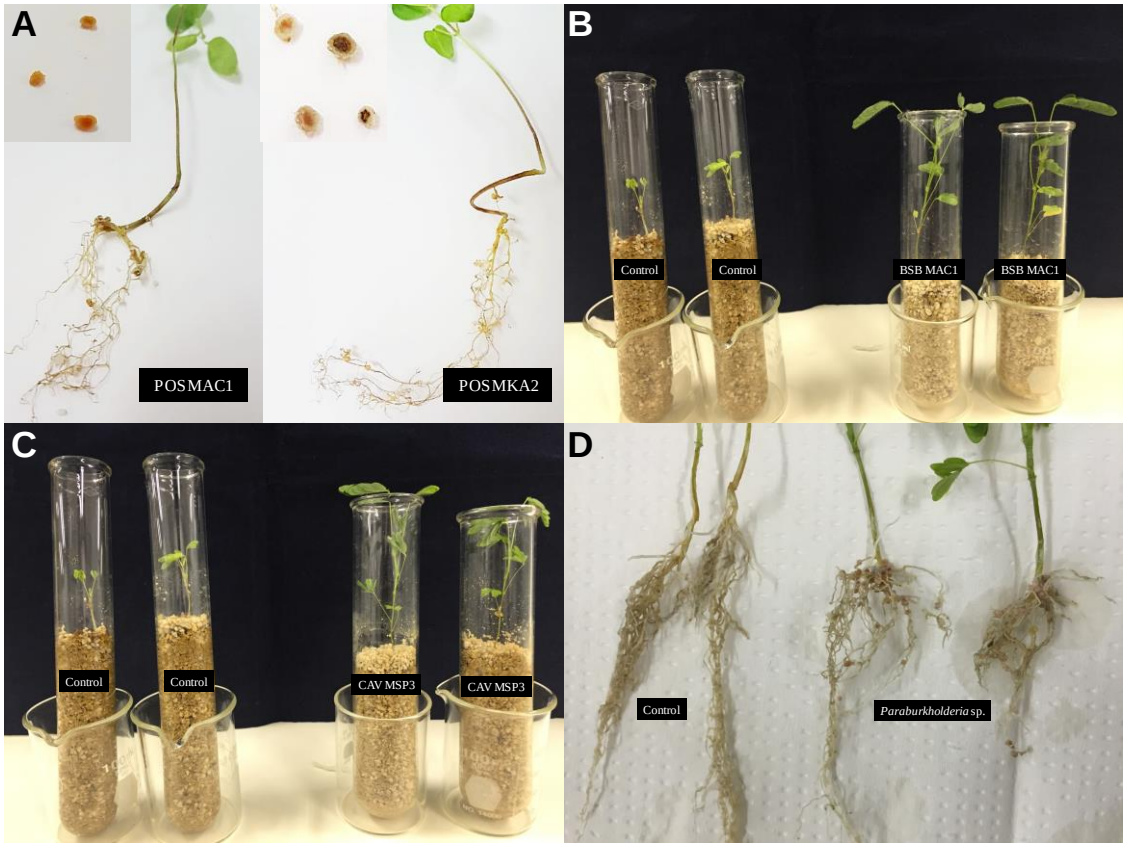

Figure S4. Authentication experiment using seedlings of *Macroptilium atropurpureum* (A) and *Mimosa pudica* (B-D) inoculated with different rhizobia obtained in trap experiment (results representing the three rhizobia lineages found are shown). Inoculated plants look healthier and taller than control (non-inoculated) plants (B-C), providing evidence for the symbiotic capacity of rhizobia tested. A. *Rhizobium* isolates POS_MAC1 and POS_MKA2 isolated from *M. acutistipula* and *M. kalunga*, respectively, growing on Posse soil; B. *Paraburkholderia* isolate BSB_MAC1 isolated from *M. acutistipula* growing on Brasília soil; C. *Paraburkholderia* isolate CAV_MSP3C1 isolated from *Mimosa* sp. growing on Cavalcante soil; D. root nodules of plants inoculated with *Paraburkholderia* isolate (right) and absence of nodules in non-inoculated plants (left).

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CAPÍTULO II

Revelando as associações simbióticas entre espécies de *Stryphnodendron* (Leguminosae) e bactérias fixadoras de nitrogênio

Introdução

Stryphnodendron é um gênero neotropical de leguminosas com aproximadamente 30 espécies, que ocorre desde a América Central até o Sudeste do Brasil (Scalon 2007). As espécies de *Stryphnodendron* crescem em uma grande variedade de ambientes, com destaque para florestas úmidas (Amazônia e Mata Atlântica) e savanas (Cerrado). O gênero é representado por espécies subarbustivas anãs, por arbustos e até árvores com 40 metros de altura, sendo mais comuns aquelas entre dois a oito metros (Occhioni 1990). Filogeneticamente, o gênero pertence ao grupo informal Piptadenia (Simon et al. 2016) no clado mimosoida (LPWG 2017).

Esse gênero possui uma considerável importância econômica. Uma das espécies mais utilizadas, *S. adstringens*, possui uma das maiores fontes naturais de tanino em sua casca, e é utilizada na medicina popular como cicatrizante anti-inflamatório e analgésico (Rodrigues 1893; Panizza et al. 1988). Existe também uma pomada feita do extrato da casca e é empregada em feridas de pele (Minatel et al. 2010). Porém, por causa do seu valor medicinal, as populações naturais de *S. adstringens* estão diminuindo significativamente devido ao extrativismo predatório (Borges Filho e Felfili 2003).

Assim como boa parte das leguminosas, as espécies de *Stryphnodendron* provavelmente contribuem com a fixação de nitrogênio atmosférico nos ecossistemas onde ocorrem. Todavia, os estudos sobre nodulação com esse gênero ainda são escassos. Apenas alguns trabalhos sobre a ocorrência de nodulação em leguminosas da região amazônica mostraram que três espécies de *Stryphnodendron* apresentavam nódulos, *S. guianense*, *S. pulcherrimum* e *S. racemiferum*, sendo que, a primeira espécie apresentou nódulos em viveiro e as duas últimas em ambiente natural e viveiro (Moreira et al. 1992; Faria e Lima 1998). Compilações de espécies noduladoras de leguminosas reportam apenas quatro espécies de *Stryphnodendron* arbóreas como noduladoras (Sprent 2005).

Ainda mais escassos são os estudos envolvendo a identificação de simbiontes associados a *Stryphnodendron*. Existe na literatura, até o presente momento, apenas um trabalho onde foi registrada a presença de bactérias do gênero *Bradyrhizobium* em nódulos de *Stryphnodendron* sp. originária do Rio de Janeiro. Além disso, há registros não publicados na Guiana Francesa da espécie *Stryphnodendron pulcherrimum* nodulando com *Rizobium tropici* e *Paraburkholderia nodosa* (Bournaud et al. 2013), sugerindo que este gênero, assim como outros membros pertencentes ao grupo Piptadenia, como *Parapiptadenia*, *Piptadenia* e *Mimosa* (Simon et al. 2016) também formam nódulos em simbiose com β-proteobacteria do gênero *Paraburkholderia*

(Moulin et al. 2015). Apesar de *Bradyrhizobium* ser um simbionte frequentemente associado a espécies de leguminosas (Fonseca et al. 2012; Silva et al. 2014; Lemaire et al. 2015; Beukes et al. 2016), existe apenas um trabalho com três registros de associação simbiótica de *Bradyrhizobium* com *Stryphnodendron* (Bournaud et al. 2013). Uma eventual associação entre *Bradyrhizobium* e espécies de *Stryphnodendron* comprovaria a elevada diversidade de simbiontes associados a gêneros pertencentes a esse grupo. De qualquer forma, estudos adicionais serão necessários para confirmar esse resultado.

Diante do exposto fez-se necessária uma investigação mais aprofundada envolvendo a nodulação em espécies de *Stryphnodendron*, como também sobre a associação simbiótica entre espécies do gênero e os rizóbios. Neste trabalho foram realizados testes de nodulação com espécies representativas do gênero, a fim de incrementar o conhecimento sobre as bactérias que nodulam com *Stryphnodendron.* Bactérias isoladas dos nódulos foram identificadas molecularmente com base no gene 16S rRNA. As perguntas abordadas neste estudo foram: as espécies testadas são capazes de formar nódulos? Qual o principal grupo de rizóbios que nodula com as espécies de *Stryphnodendron* testadas? Os rizóbios associados à *Stryphnodendron* são predominantemente β-proteobacterias, assim como os simbiontes de gêneros próximos de leguminosas? Há diferenças nos rizóbios encontrados em espécies de *Stryphnodendron* provenientes de diferentes ambientes e tipos de solo?

Material e Métodos

Experimento com plantas-armadilha

Devido à dificuldade em se obter nódulos de raízes de plantas adultas em campo, foi realizado um experimento em casa de vegetação com planta-armadilha (Mishra et al. 2012; Bontemps et al. 2016) a fim de verificar a nodulação em plântulas de espécies de *Stryphnodendron*. Sementes de todas as espécies coletadas foram utilizadas no experimento de plantas-armadilha cultivadas em seu solo de origem, para verificar se as plantas tinham capacidade de nodular.

Para realização de testes de nodulação com planta-armadilha, foram utilizadas sementes de dez espécies de *Stryphnodendron* provenientes de diferentes localidades nos biomas Amazônia e Cerrado representando variados tipos de solo e vegetação (Tabela 1; Figura 1). As espécies amostradas abrangem as três principais linhagens de *Stryphnodendron* (Simon et al. 2016) e formas de vida que variam de subarbustos, arbustos a árvores, que crescem em ambientes tanto florestais quanto savânicos. Amostras de solo contendo a rizosfera associada aos locais de ocorrência das espécies de plantas foram coletadas. As características físico-químicas dos solos de cada local, na profundidade de 0 a 20 cm (Tabela 2), foram analisadas de acordo com as metodologias propostas por Embrapa (1997). As coletas de amostras de sementes e solo foram realizadas pela autora e também pela equipe da Embrapa Cenargen e colaboradores.

Tabela 1. Espécies de *Stryphnodendron* utilizadas em experimento de nodulação com planta-armadilha. Formas de vida conforme Scalon (2007) e vouchers depositados no herbário CEN, exceto o SM Faria 1070 (Herbário RB).

Espécie	Forma de vida	Voucher	Procedência	Fisionomia	Distribuição Geográfica
S. adstringens (Mart.) Coville	Árvore	AB Giroldo 28	Brasília – DF	Cerrado s.s.	Brasil Central
S. confertum Heringer & Rizzini	Subarbusto	MF Simon 2599	Brasília – DF	Campo sujo	Distrito Federal e Goiás
S. cristalinae Heringer	Subarbusto	RC Pires 100	Cristalina – GO	Campo sujo	Endêmica de Goiás
S. duckeanum Occhioni	Árvore	AA Santos 3478	Porto Velho – RO	Floresta ombrófila	Predominante no Norte do Brasil
S. fissuratum E.M.O.Martins*	Árvore	RF Haidar 1733	Aruanã – GO	Cerrado s.s.	Mato Grosso e Goiás
S. guianense (Aubl.) Benth.*	Árvore	SM Faria 1070	Porto Trombetas – PA	Floresta ombrófila	Norte e Nordeste do Brasil
S. heringeri Occhioni	Subarbusto	RC Pires 99	Água Fria – GO	Cerrado s.s.	Endêmica de Goiás
S. pulcherrimum (Willd.) Hochr.	Árvore	AA Santos 3417	Porto Velho – RO	Floresta ombrófila	Predominante no Norte e Nordeste do Brasil
S. pumilum Glaz.	Subarbusto	MF Simon 2656	Brasília – DF	Cerrado s.s.	Distrito Federal e Goiás
S. rotundifolium Mart.*	Árvore	G Pereira-Silva 2334	Ipameri – GO	Cerrado s.s.	Ampla distribuição no Brasil

* Para essas espécies foi utilizado, no experimento de planta-armadilha, o solo de outra localidade de ocorrência da espécie (*S. fissuratum*, Nova Xavantina-MT; *S. rotundifolium*, Pirenópolis-GO) ou mistura de todos os solos (*S. fissuratum*, *S. guianense*).

Tabela 2. Localização e características químicas e granulométricas das amostras de solo (profundidade de 0 a 20 cm) utilizadas no experimento de planta-armadilha com espécies de *Stryphnodendron*.

Solos	Água Fria/GO	Brasília/DF	Cristalina/GO	Nova Xavantina/MT	Pirenópolis/GO	Porto Velho/RO SD*	Porto Velho/RO SP*
Latitude	14°54'39"S	15°41'26"S	16°48'19"S	14°41'46"S	15°48'48"S	09°36'45"S	09°26'26"S
Longitude	47°48'41"O	47°55'24"O	47°39'19"O	52°21'10"O	48°55'05"O	65°28'04"O	64°47'06"O
Altitude	1000m	1060m	1170m	300m	950m	143m	105m
Ca (cmolc/dm3)	0,90	0,90	0,20	3,20	0,10	4,05	0,20
H+Al (cmolc/dm3)	3,60	6,30	3,00	2,80	3,77	-	-
Mg (cmolc/dm3)	0,30	0,80	0,20	0,20	0,06	0,10	0,10
Al (cmolc/dm3)	1,00	1,50	0,50	0,20	0,95	2,90	4,00
K (cmolc/dm3)	0,09	0,36	0,04	0,32	-	0,10	0,07
CTC	4,89	8,36	3,44	6,52	3,93	12,10	16,24
P (cmolc/dm3)	3,40	1,80	2,10	3,00	-	1,20	1,50
N (%)	0,15	0,25	0,10	0,27	-	-	-
MO (%)	2,8	5,4	2,2	5,4	1,2	3,1	2,8
pHCaCl2	4,20	4,20	4,30	4,90	4,06	3,70	3,90
Argila (%)	50	59	10	35	-	40	23
Silte (%)	11	12	4	9	-	10	7
Areia (%)	39	29	86	56	-	50	70

Ca, cálcio; H+AL, hidrogênio + alumínio; Mg, magnésio ; Al, alumínio; K, potássio; CTC, capacidade de troca de cátions; P, fósforo; N, nitrogênio; MO, matéria orgânica; pH(CaCl2), pH em solução de cloreto de cálcio.
*SD, solo de origem de *S. duckeanum*; SP, solo de origem de *S. pulcherrimum*.

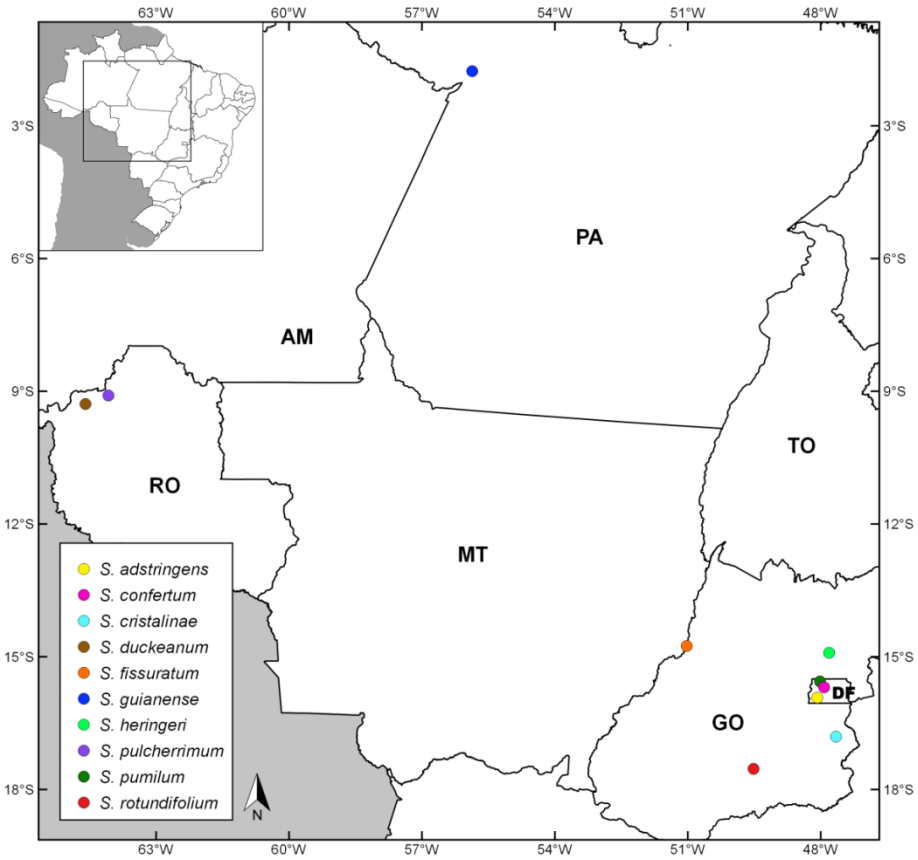

Figura 1. Local de coleta das sementes das dez espécies de *Stryphnodendron* utilizadas no estudo de nodulação.

As sementes foram imersas em álcool 70% por 30 segundos e em solução de hipoclorito de sódio (2,5% de cloro ativo) por cinco minutos para esterilização da superfície. Posteriormente, as sementes foram colocadas em peneira de malha de 23 mm para serem lavadas cinco vezes com água destilada estéril. A dormência foi quebrada lixando a testa das sementes, depois foram colocadas em placas de Petri com papel filtro umedecido. Após dez dias, as plântulas foram transferidas para vasos de 300 ml preenchidos com solo.

Cada espécie foi plantada em uma mistura de 50% solo de origem com 50% areia esterilizada, em quatro repetições, que foram cultivadas em casa de vegetação por quatro meses. Em alguns casos, as espécies foram cultivadas em solo proveniente de localidade diferente de onde a semente foi coletada, mas ainda assim, em local de ocorrência da espécie. *S. guianense* foi cultivada em uma mistura de todos os solos, pois o solo de origem não estava disponível para essa espécie. *S. fissuratum* foi cultivada em uma mistura de todos os solos, pois, a princípio, também não havia disponibilidade de solo de ocorrência da espécie. No entanto, em seguida, conseguiu-se obter esse solo e *S. fissuratum* também foi semeada no solo de origem da espécie (Tabela 1).

Isolamento e preservação dos rizóbios

Os nódulos selecionados obtidos no experimento com planta-armadilha foram reidratados em água destilada estéril por 2,5 horas. Em seguida, foi feita a esterilização superficial dos nódulos em álcool (95%) por 30 segundos, seguido por solução de hipoclorito de sódio 2,5% por cinco minutos e lavagem em água estéril por cinco vezes. Após esse procedimento, foram adicionados 500 µl de solução salina estéril e os nódulos foram esmagados com uma pinça esterilizada. A solução resultante foi diluída serialmente até 10^{-3} em solução salina estéril. Depois disso, foram plaqueados 100 µl das duas últimas diluições (10^{-2} e 10^{-3}) em meio 79 com vermelho Congo (Vincent 1970), utilizando-se duas repetições para cada diluição. As placas foram incubadas por dois a 12 dias a 30ºC. Em seguida, colônias individuais foram selecionadas para obtenção de culturas de bactérias puras. Duas repetições de cada isolado foram estocadas em meio 79 sólido, em frascos âmbar de 10 ml a 4ºC e quatro repetições de cada isolado foram armazenadas em longo prazo em meio 79 líquido com 30% glicerol a -80ºC.

Extração de DNA, amplificação e sequenciamento de genes para identificação molecular dos isolados

As bactérias isoladas a partir do experimento com as plantas-armadilha foram cultivadas em meio 79 com vermelho Congo sólido por 72 horas, a 28ºC. Em seguida, uma colônia purificada foi inserida em meio 79 líquido por 24 horas, também a 28°C. Posteriormente, a extração de DNA dos isolados foi realizada com a utilização do Pure Link Genomic DNA Kits (Invitrogen), seguindo as instruções do fabricante.

O DNA extraído de cada isolado foi utilizado como molde para as reações de PCR e sequenciamento do gene 16S rRNA, o qual é amplamente utilizado em estudos filogenéticos com bactérias e identificação molecular de rizóbios (Peix et al. 2015). Os produtos de PCR foram gerados e sequenciados nas duas direções no Centro de Recursos Biológicos Johanna Döbreiner – Embrapa Agrobiologia. As sequências obtidas foram comparadas a sequências depositadas do banco de dados GenBank (Tabela S1).

Análises filogenéticas

Para caracterização dos isolados ao nível taxonômico, sequências representativas de linhagens de bactérias foram obtidas do GenBank e alinhadas com as sequências

geradas neste estudo utilizando o algoritmo ClustalW implementado no programa BioEdit 4.8.4 (Hall 1999) e manualmente corrigidas. A análise filogenética baseada no gene 16S rRNA foi feita seguindo a análise de máxima verossimilhança (ML), implementado pelo programa RAxML-HPC v.8, usando o modelo de substituição nucleotídica GTR-CAT (Stamatakis 2006) a partir do portal CIPRES (Miller et al. 2010). Para obter valores de suporte, o conjunto de dados foi reanalisado mil vezes usando método de bootstrap (Felsenstein 1985). A árvore filogenética foi construída com as sequências alinhadas para a região anteriormente citada, inferindo-se distância genética entre as amostras. Estirpes de referência pertencentes aos gêneros *Bradyrhizobium*, *Bacillus*, *Ensifer*, *Mesorhizobium*, *Paenibacillus Paraburkholderia* e *Rhizobium*, foram incluídas na árvore filogenética do gene 16S rRNA.

Experimento de autenticação

A capacidade de nodulação das bactérias isoladas no experimento com planta-isca foi confirmada por meio de experimento de autenticação (Lemaire et al. 2015). Como na literatura ainda não há uma espécie-modelo para estudos de nodulação em *Stryphnodendron*, a espécie *S. fissuratum* foi escolhida como "hospedeiro-modelo", por ser uma espécie que atingiu crescimento rápido em relação às outras espécies e obteve maior quantidade de nódulos no experimento com planta-isca. Inicialmente, a dormência das sementes foi quebrada com imersão em ácido sulfúrico concentrado por cinco minutos, que depois foram lavadas com água estéril por, pelo menos, dez vezes. As sementes foram pré-geminadas em uma bandeja com algodão, embebidas com dH_2O estéril e incubadas por 48h a 27ºC.

As plântulas foram testadas em substrato perlita de acordo com Elliot et al. (2009). Tubos de vidro (50 ml de volume) foram preenchidos até a metade com perlita. Em seguida, os tubos foram autoclavados (121º e 1 atm por 30 minutos). Solução nutritiva de Hoagland (Hoagland e Arnon 1938), sem N, foi aplicada até o ponto de saturação da perlita. Posteriormente, as plântulas foram inoculadas com 1 ml de cultura dos isolados crescidos em meio 79 por 48h. A possibilidade de contaminação cruzada foi investigada utilizando controle negativo não inoculado, inserido aleatoriamente entre os tratamentos. Todas as plantas permaneceram em câmara de crescimento com temperatura controlada (26ºC) e ciclo de 16h luz/ 8h escuro. Após dois meses, as plantas foram coletadas e a presença/ausência de nódulos foi registrada. A fixação de

nitrogênio foi estimada por observação visual do vigor da planta e a cor da folhagem, e verificação da coloração interna dos nódulos.

Resultados

Espécies que apresentaram nodulação

Das dez espécies de *Stryphnodendron* estudadas, provenientes de diferentes localidades, oito apresentaram nodulação (Figura 2). As duas espécies que não nodularam foram *S. duckeanum* e *S. pulcherrimum*, sendo ambas as amostras provenientes de Rondônia. Um novo experimento foi realizado para essas duas espécies e novamente não foi observada nodulação.

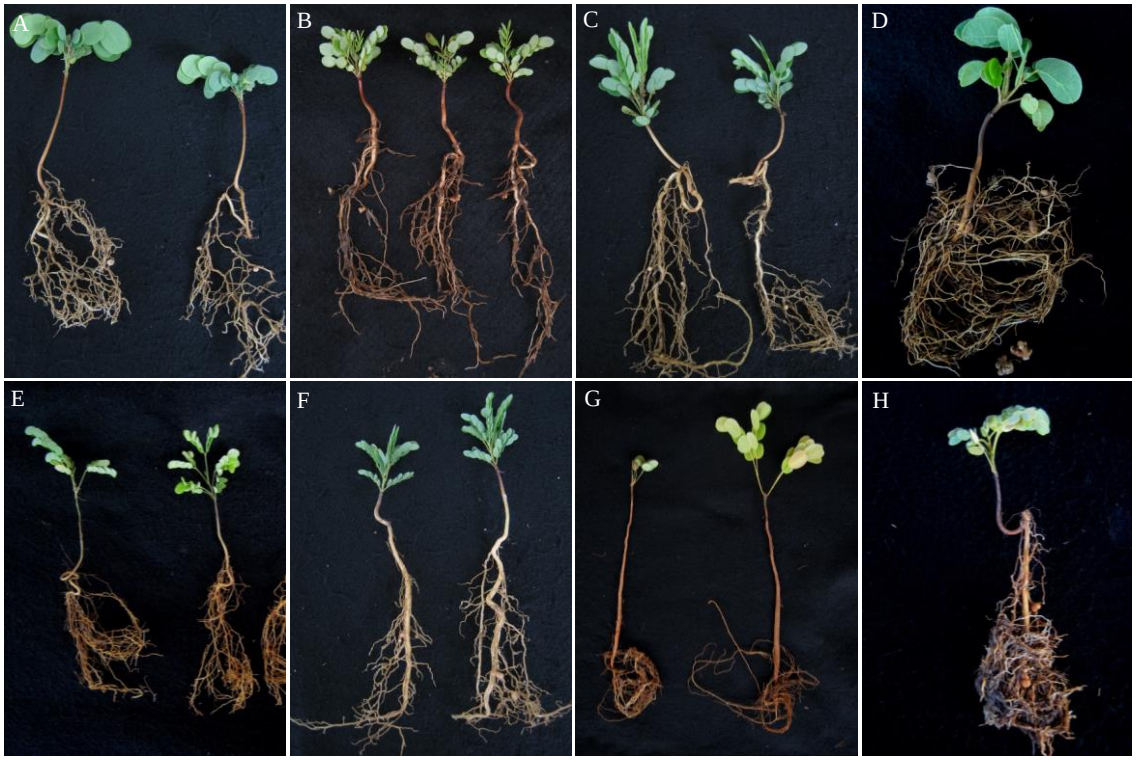

Figura 2. Espécies de *Stryphnodendron* que apresentaram formação de nódulos em experimento com planta-isca: **A** - *S. adstringens*, **B** - *S. cristalinae*, **C** - *S. confertum*, **D** - *S. fissuratum*, **E** - *S. guianense*, **F** - *S. heringueri*, **G** - *S. pumilum* e **H** - *S. rotundifolium*.

Identificação dos rizóbios usando sequências do gene 16S rRNA

Uma classificação preliminar, ao nível de gênero dos 47 isolados obtidos no experimento com planta-isca, foi realizada comparando-se as sequências do gene 16S rRNA com as sequências do banco de dados do GenBank utilizando o algoritmo BLASTN (Altschul et al. 1990). Os resultados do BLASTN classificaram os isolados em seis gêneros diferentes (*Bacillus*, *Bradyrhizobium*, *Cohnella*, *Paenibacillus*,

Paraburkholderia e *Rhizobium*) e revelaram que os isolados compartilhavam uma alta similaridade (99 - 100%) com espécies de bactérias já conhecidas. Isolados pertencentes à classe Bacilli (*Bacillus*, *Cohnella* e *Paenibacillus*), apesar de, provavelmente, não se constituírem em bactérias simbióticas, também foram obtidos de nódulos e foram tratados nesse trabalho.

Na árvore filogenética (Figura 3), construída com base no gene 16S rRNA, observa-se a discriminação de quatro grandes grupos contendo as bactérias isoladas neste trabalho, sendo dois pertencentes à classe α-proteobacteria, um à classe β-proteobacteria, e outro à classe Bacilli. As α-bactérias isoladas foram agrupadas junto aos gêneros *Rhizobium* e *Bradyrhizobium*. Dentro de *Rhizobium*, os isolados ficaram no mesmo clado de *R. miluonense*. Já em *Bradyrhizobium*, as bactérias agruparam-se em quatro clados distintos (Figura 3).

Os isolados identificados como β-proteobacteria foram agrupados ao gênero *Paraburkholderia*, formando dois clados distintos, um próximo a *P. sabiae* e o outro ao clado formado por *P. nodosa*, *P. unamae* e *P. bannensis*. Já os isolados pertencentes à classe Bacilli foram agrupados em diferentes clados compreendendo os gêneros *Bacillus* (ST 08, ST 21), *Cohnella* (ST 26) e *Paenibacillus* (14 isolados).

Figura 3. Árvore filogenética de isolados de rizóbios das espécies de *Stryphnodendron* (em negrito) combinados com sequências referência do GenBank, baseada no gene 16S rRNA (1420 bp). A árvore de consenso de maioria foi construída utilizando-se o método da máxima verossimilhança. Valores de suporte (bootstrap >50%) são mostrados. Barra de escala em número de substituições por sítio. As espécies-hospedeiras dos isolados estão representadas por diferentes cores.

Experimento de autenticação dos isolados

A habilidade de nodulação dos rizóbios obtidos no experimento com planta-isca foi testada em *Stryphnodendron fissuratum*. Dos 47 isolados obtidos, não foi possível realizar o experimento de autenticação em 17 isolados, pelo rizóbio isolado não ter crescido em laboratório ou pelo hospedeiro ter morrido antes da finalização do experimento. Apenas nove isolados induziram a formação de nódulos, sendo que cinco foram efetivos, ou seja, os nódulos apresentaram coloração róseo-avermelhada em seu interior, o que indica a presença de leg-hemoglobina e atividade de fixação de nitrogênio (Tabela 3). Os isolados que obtiveram nodulação efetiva pertencem ao gênero *Bradyrhizobium* com hospedeiros originais *S. fissuratum* e *S. pumilum*, e *Rhizobium* com hospedeiro original *S. rotundifolium*. As plantas inoculadas tinham folhas saudáveis e esverdeadas, enquanto que, as plantas controle não apresentaram nódulos nas raízes, e continham folhas amareladas, indicando deficiência de N.

Tabela 3. Resultados do experimento de autenticação de nodulação a partir de bactérias obtidas em experimento com planta-isca e testadas no hospedeiro *S. fissuratum* (nodulação efetiva= +++; nodulação não efetiva= +; não nodulou= -; não foi feito o teste= /).

Espécie	Estirpe	Solo*	Identificação	Nodulação
S. adstringens	ST 36	Brasília	*Bradyrhizobium* sp.	-
S. confertum	ST 52	Brasília	*Paenibacillus* sp.	-
S. confertum	ST 56	Brasília	*Bradyrhizobium* sp.	-
S. cristalinae	ST 60	Cristalina	*Paraburkholderia* sp.	-
S. cristalinae	ST 61	Cristalina	*Paraburkholderia* sp.	-
S. cristalinae	ST 62	Cristalina	*Paraburkholderia* sp.	+
S. fissuratum	ST 06	Mistura dos solos	*Rhizobium* sp.	-
S. fissuratum	ST 07	Mistura dos solos	*Paenibacillus* sp.	/
S. fissuratum	ST 08	Mistura dos solos	*Bacillus aryabhattai*	/
S. fissuratum	ST 11	Mistura dos solos	*Paenibacillus* sp.	/
S. fissuratum	ST 16	Mistura dos solos	*Paenibacillus* sp.	/
S. fissuratum	ST 21	Mistura dos solos	*Bacillus nealsonii*	-
S. fissuratum	ST 26	Mistura dos solos	*Cohnella xylanilytica*	-
S. fissuratum	ST 27	Mistura dos solos	*Paenibacillus* sp.	/
S. fissuratum	ST 37	Mistura dos solos	*Bradyrhizobium* sp.	/
S. fissuratum	ST 38	Mistura dos solos	*Bradyrhizobium* sp.	/
S. fissuratum	ST 39	Mistura dos solos	*Bradyrhizobium* sp.	/
S. fissuratum	ST 40	Mistura dos solos	*Bradyrhizobium* sp.	/
S. fissuratum	ST 42	Mistura dos solos	*Bradyrhizobium* sp.	/
S. fissuratum	ST 43	Mistura dos solos	*Bradyrhizobium* sp.	/
S. fissuratum	ST 48	Nova Xavantina	*Bradyrhizobium* sp.	+++

Espécie	Estirpe	Solo*	Identificação	Nodulação
S. fissuratum	ST 49	Nova Xavantina	*Bradyrhizobium* sp.	-
S. fissuratum	ST 50	Nova Xavantina	*Bradyrhizobium* sp.	+++
S. fissuratum	ST 51	Nova Xavantina	*Bradyrhizobium* sp.	+++
S. guianense	ST 30	Mistura dos solos	*Paenibacillus* sp.	/
S. heringeri	ST 53	Água Fria	*Paenibacillus* sp.	-
S. heringeri	ST 57	Água Fria	*Paenibacillus* sp.	/
S. heringeri	ST 58	Água Fria	*Paenibacillus* sp.	-
S. pumilum	ST 13	Brasília	*Bradyrhizobium* sp.	+++
S. pumilum	ST 15	Brasília	*Bradyrhizobium* sp.	+
S. pumilum	ST 31	Brasília	*Paenibacillus* sp.	-
S. pumilum	ST 44	Brasília	*Bradyrhizobium* sp.	-
S. pumilum	ST 45	Brasília	*Bradyrhizobium* sp.	-
S. rotundifolium	ST 01	Pirenópolis	*Paenibacillus* sp.	/
S. rotundifolium	ST 02	Pirenópolis	*Paenibacillus* sp.	/
S. rotundifolium	ST 03	Pirenópolis	*Paenibacillus* sp.	-
S. rotundifolium	ST 04	Pirenópolis	*Paenibacillus* sp.	/
S. rotundifolium	ST 17	Pirenópolis	*Paraburkholderia* sp.	-
S. rotundifolium	ST 18	Pirenópolis	*Rhizobium sp.*	+++
S. rotundifolium	ST 19	Pirenópolis	*Rhizobium sp.*	-
S. rotundifolium	ST 20	Pirenópolis	*Paraburkholderia* sp.	-
S. rotundifolium	ST 22	Pirenópolis	*Rhizobium* sp.	-
S. rotundifolium	ST 23	Pirenópolis	*Paraburkholderia* sp.	-
S. rotundifolium	ST 24	Pirenópolis	*Paraburkholderia* sp.	-
S. rotundifolium	ST 25	Pirenópolis	*Rhizobium* sp.	+
S. rotundifolium	ST 28	Pirenópolis	*Rhizobium* sp.	/
S. rotundifolium	ST 29	Pirenópolis	*Rhizobium* sp.	+

* Solo onde o isolado foi obtido.

Discussão

A maioria das espécies testadas neste trabalho foi capaz de formar nódulos. Dessa forma, dentre as dez espécies testadas, *S. guianense* confirmou resultados de estudos anteriores (Moreira et al. 1992; Faria e Lima 1998); sete representam novos registros de nodulação: *S. adstringens*, *S. confertum*, *S. cristalinae*, *S. fissuratum*, *S. heringeri*, *S. pumilum* e *S. rotundifolium*; e outras duas espécies não apresentaram nodulação: *S. duckeanum* e *S. pulcherrimum*. Ressalta-se que *S. pulcherrimum*, apesar de não ter nodulado no presente estudo, já foi reportada como espécie noduladora (Bournaud et al. 2013). A ampla amostragem realizada neste estudo confirma *Stryphnodendron* como um gênero capaz de nodular.

Foi encontrada uma grande variedade de simbiontes associados a espécies de *Stryphnodendron*, diferentemente do que acontecem com outros gêneros filogeneticamente próximos (grupo Piptadenia), que se associam predominantemente com β-rizóbios como *Mimosa*, *Parapiptadenia*, *Piptadenia*, *Pseudopiptadenia* e *Pityrocarpa* (Gyaneshwar et al. 2011; Bontemps et al. 2010; Bournaud et al. 2013; Moulin et al. 2015). Bactérias, pertencentes a diferentes classes e gêneros, foram encontradas nos nódulos das espécies de *Stryphnodendron* de diferentes localidades, mostrando uma relação simbiótica mais complexa do que se pensava para um gênero relativamente pequeno.

As relações filogenéticas apresentadas na Figura 3 baseadas no gene 16S rRNA puderam agrupar os isolados encontrados nas principais linhagens contidas em seis gêneros de bactérias. Porém, seriam necessárias sequências de mais genes para permitir uma melhor identificação em nível de espécie, tendo em vista que a resolução baseada apenas no gene 16S rRNA é considerada baixa para discernir espécies (Willens et al. 2001; Fosenca et al. 2012) A partir disso, o foco da discussão será nos grupos de bactérias encontradas de maneira geral, como, gêneros e classes.

Os isolados identificados como *Rhizobium* foram obtidos de nódulos de *S. fissuratum* (um isolado) e *S. rotundifolium* (seis isolados), espécies ocorrentes no Cerrado. Dentre as espécies do grupo Piptadenia, *Rhizobium* é geralmente relatado em ambientes com pH tendendo para o neutro-alcalino e em solos mais férteis (Gehlot et al. 2013; Bontemps et al. 2016; capítulo 1 dessa dissertação). O fato de *Rhizobium* ter sido registrado no solo com baixo teor de matéria orgânica e ácido de Pirenópolis-GO é, de

certa forma, notável, tendo em vista a predominância de β-rizóbios nessas condições edáficas (Bontemps et al. 2010).

No clado *Bradyrhizobium*, os isolados de *S. adstringens*, *S. confertum*, *S. fissuratum* e *S. pumilum* formaram quatro clados diferentes, próximos a diversas espécies de *Bradyrhizobium* fixadoras de nitrogênio. As espécies de *Bradyrhizobium* têm uma ampla gama de hospedeiros dentre as leguminosas e elevada diversidade em áreas tropicais e subtropicais (Moreira et al. 1998; Menna e Hungria et al. 2011; Silva et al. 2014; Andrews e Andrews 2017). *Bradyrhizobim* parece ser a bactéria simbiótica dominante na família leguminosa como um todo. Esse gênero interage com várias espécies e, até mesmo, diferentes subfamílias de leguminosas já foram reportadas nodulando com *Bradyrhizobim*, não só no Brasil, como também no mundo, (Fonseca et al. 2012; Koppell e Parker 2012; Parker e Rousteau 2014; Santos et al. 2017; Sprent et al. 2017). Ao que tudo indica, é provável que esse gênero possa ser um dos primeiros simbiontes nos nódulos de leguminosas (Grupta e Mok 2007; Martinez - Romero 2009; Parker 2015). Além disso, as espécies desse gênero têm uma importância econômica, visto que, são utilizadas como inoculante agrícola na soja (*Glycine max*), um dos casos mais afortunados de FBN (Hungria et al. 2005).

Os isolados de *S. cristalinae* (três isolados), e *S. rotundifolium* (quatro isolados), ambos obtidos em solos arenosos, pH ácido, com baixo teor de nutrientes e capacidade de troca de cátions (CTC), em locais de altitude elevada no Cerrado (Cristalina e Pirenópolis; Tabela 3), se agruparam com *Paraburkholderia*. As espécies de *Paraburkholderia* são conhecidas por serem geralmente encontradas em solos mais ácidos e menos férteis e são comumente relatadas no Cerrado associadas a *Mimosa* (Bontemps et al. 2010; dos Reis Junior et al. 2010; Mishra et al. 2012; Capítulo 1 dessa dissertação). Esse rizóbio também foi o principal grupo de bactéria encontrado em nódulos de *Phaseolus vulgaris*, quando utilizadas como plantas-armadilha, em solo nativo típico do Cerrado, mostrando sua capacidade de associação com outras leguminosas (Dall'Agnol et al. 2016).

Na classe Bacilli, isolados de *S. confertum*, *S. fissuratum*, *S. guianense*, *S. heringeri*, *S. pumilum* e *S. rotundifolium* foram agrupados em diversos clados pertencentes aos gêneros *Bacillus*, *Cohnella* e *Paenibacillus*. Essas bactérias podem ser facilmente encontradas na rizosfera de várias plantas e no solo e, de modo geral, não constituem em bactérias simbióticas (Seldin et al. 1998), apesar de relato de atividade da enzima nitogenase em *Bacillus megaterium* (Ding et al. 2005). A capacidade de

nodulação dessas bactérias gram-negativas é controversa, mas é bastante plausível que elas desempenhem outro papel dentro do nódulo, podendo interagir com as bactérias simbióticas (Peix et al. 2015; Grady et al. 2016). Em estudos feitos com *Vigna unguiculata* em simbiose com *Bradyrhizobium*, observou-se uma maior fixação de nitrogênio na presença de *Baciilus* e *Paenibacillus* (Silva et al. 2007).

A maioria dos isolados obtidos não foi capaz de nodular com o hospedeiro escolhido para o experimento de autenticação, *S. fissuratum*. Apenas cinco isolados, correspondentes a α-proteobacterias, obtiveram nodulação efetiva. Alguns isolados identificados como *Paraburkholderia* e, até mesmo *Bradyrhizobium* e *Rhizobium*, nodularam *S. fissuratum*, porém os nódulos obtidos apresentaram tamanho reduzido e não se mostraram efetivos. É importante ressaltar que os resultados obtidos no experimento de autenticação são ainda preliminares. Existe a possibilidade de isolados que não formaram nódulos efetivos em *S. fissuratum* possam nodular plantas hospedeiras promíscuas como *Macroptilum atropurpureum* (Siratro) (Sessé & Moc. ex DC.) Urb. e *Mimosa pudica* L. (Mishra et al. 2012; Bournaud et al. 2013; Lemaire et al. 2015) ou ainda seus próprios hospedeiros originais pertencentes ao gênero *Stryphnodendron*. Nenhum isolado identificado como *Paenibacillus* foi capaz de nodular.

Aparentemente, algumas espécies de *Stryhpnodendron* apresentaram especificidade hospedeira, como por exemplo, a espécie endêmica *S. cristalinae*, da qual apenas bactérias do gênero *Paraburkholderia* foram isoladas dos nódulos. A espécie *S. cristalinae* é oriunda do solo mais arenoso e com um dos menores teores de matéria orgânica, o que pode ter influenciado sua associação por *Paraburkholderia*, uma vez que espécies dessa bactéria são encontradas predominantes em ambientes similares (Garau et al. 2009; Bontemps et al. 2010; dos Reis Jr et al. 2010). As espécies coletadas na região de Brasília (*S. adstringens*, *S. confertum* e *S. pumilum*), cultivadas em Latossolo Vermelho, se associaram simbioticamente com *Bradyrhizobium*, sendo comum encontrar esse rizóbio associado a diferentes espécies de leguminosas no Cerrado (Fonseca et al. 2012; Silva et al. 2014; Santos et al. 2017).

O presente trabalho sobre simbiontes em leguminosas é um dos poucos que relata Bacilli presente em nódulos de plantas. É provável que espécies desses gêneros (bactérias ambientais) estejam presentes em nódulos de diversas espécies de plantas, porém, não há indícios de que foram isolados, ou podem ter sido descartados por não se tratarem bactérias fixadoras de nitrogênio (Seldin et al. 1998). De qualquer forma, esse

grupo de bactérias, que parece diverso nas amostras isoladas, deve ser estudado com maior profundidade. Desse modo, os resultados deste trabalho geraram mais dúvidas do que respostas, como: Quão comuns são essas bactérias em nódulos de leguminosas? Qual função dessas bactérias nos nódulos? São capazes de fixar nitrogênio ou contribuir para o crescimento da planta? Possuem genes de nodulação? São capazes de induzir nodulação, mesmo na ausência de rizóbios genuínos, como aparentemente aconteceu com as espécies *S. guianense* e *S. heringeri*?

Estudos realizados com espécies dos gêneros do grupo Piptadenia têm revelado uma preferência dos hospedeiros por *Paraburkholderia*, apesar de terem sido relatados também tanto *Bradyrhizobium* quanto *Rhizobium* se associando com diferentes espécies desse grupo, as espécies de *Paraburkholderia* são as mais comuns (Bournaud et al. 2013; Moulin et al. 2015). Este é o primeiro estudo dedicado ao gênero *Stryphnodendron* e contou com uma ampla amostragem taxonômica e geográfica, abrangendo diferentes ambientes e espécies.

Conclusões

Os resultados desta pesquisa revelaram novos registros de nodulação em espécies de *Stryphnodendron* e uma interessante diversidade de simbiontes, considerando o pequeno número de espécies dentro do gênero. Essa variação de simbiontes, acrescida dos relatos para outros gêneros do grupo Pipatadenia, revelam certa amplitude em termos de preferência simbiótica, aumentando ainda mais a diversidade de simbiontes associados a gêneros/espécies pertencentes a esse grupo.

Apesar dos avanços, algumas lacunas ficaram em aberto neste trabalho, como a identificação molecular dos isolados, a qual se sugere o aprimoramento, com sequenciamento, de mais genes, a fim de se confirmar a identificação ao nível específico. Ainda é possível que haja novas espécies de bactérias simbióticas dentre os isolados obtidos. Em relação ao experimento de autenticação, seria interessante a repetição do experimento utilizando-se plantas pertencentes ao hospedeiro original, a fim de se confirmar quais dos isolados obtidos são genuínos simbiontes fixadores de N.

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Informação de Suporte

Tabela S1. Número de acesso do Genbank das estirpes utilizadas no estudo filogenético.

Estirpe	16S rRNA
Bacillus aryabhattai	KX783597
Bacillus megaterium	HM371417
Bacillus nealsonii	NR_044546
Bradyrhizobium arachidis	HM107167
Bradyrhizobium canariense	AY577427
Bradyrhizobium centrosemae	KC247115
Bradyrhizobium cytisi	EU561065
Bradyrhizobium daqingense	HQ231274
Bradyrhizobium elkanii	AF208518
Bradyrhizobium ferriligni	KJ818096
Bradyrhizobium ganzhouense	JQ796661
Bradyrhizobium huanghuaihaiense	NR_117945
Bradyrhizobium ingae	KF927043
Bradyrhizobium iriomotense	AB300992
Bradyrhizobium japonicum	NR_112552
Bradyrhizobium jicamae	AY624134
Bradyrhizobium kavangense	KP899562
Bradyrhizobium liaoningense	AF208513
Bradyrhizobium pachyrhizi	AY624135
Bradyrhizobium rifense	EU561074
Bradyrhizobium yuanmingense	AF193818
Burkholderia cepacia	U96927
Burkholderia symbiotica	FN543707
Cohnella xylanilytica	FJ001841
Cupriavidus taiwanensis	FN908230
Ensifer meliloti	D14509
Mesorhizobium mediterraneum	KX226348
Paenibacillus aestuarii	NR_116365
Paenibacillus castaneae	NR_044403
Paenibacillus chinjuensis	NR_025024
Paenibacillus chondroitinus	AB073206
Paenibacillus elgii	NR_115140
Paenibacillus frigoriresistens	NR_109546
Paenibacillus medicaginis	NR_145626
Paenibacillus ourofinensis	EU257517
Paenibacillus polymyxa	EU124564
Paenibacillus puldeungensis	NR_117451
Paenibacillus soli	DQ309073
Paenibacillus vulneris	NR_117618

Estirpe	16S rRNA
Paenibacillus xylanisolvens	NR_112906
Paenibacillus yunnanensis	NR_145625
Paraburkholderia bannensis	AB561874
Paraburkholderia diazotrophica	FN543713
Paraburkholderia ferrariae	DQ514537
Paraburkholderia hiiakae	NR_146372
Paraburkholderia mimosarum	AY752958
Paraburkholderia nodosa	AY773189
Paraburkholderia phymatum	AJ302312
Paraburkholderia piptadeniae	LN875219
Paraburkholderia ribeironis	LN875221
Paraburkholderia rynchosiae	EU219865
Paraburkholderia sabiae	AY773186
Paraburkholderia sp. JPY266	FN543671
Paraburkholderia sp. JPY306	FN543694
Paraburkholderia sp. JPY407	FN543731
Paraburkholderia tropica	AJ420332
Paraburkholderia tuberum	AJ302311
Paraburkholderia unamae	AY221956
Paraburkholderia oxyphila	AB488693
Paraburkholderia sprentiae	HQ698903
Rhizobium multihospitium	EF490014
Rhizobium sp. JPY1209	KP760722
Rhizobium sp. JPY479	FN543788
Rhizobium sp. JPY810	KP760681
Rizhobium altiplani	KX022634
Rizhobium calliandrae	JX855164
Rizhobium hainanense	U71078
Rizhobium leucaenae	X67234
Rizhobium lusitanum	AY738130
Rizhobium miluonense	EF061096
Rizhobium tropici	U89832

CONCLUSÃO GERAL

As leguminosas, em associação com bactérias fixadores de nitrogênio, são as maiores contribuintes de N biologicamente fixado para o meio ambiente. Neste trabalho, dois gêneros de leguminosas foram tratados. O gênero *Mimosa* tem um grande número de espécies, para as quais as relações simbióticas estão de certa forma amplamente documentadas na literatura. Neste trabalho aspirou-se entender as questões mais específicas entre as relações planta-hospedeira e simbionte, mediante manipulação experimental envolvendo diversas espécies e tipos de solo. Já para *Stryphnodendron*, este trabalho foi pioneiro e revelador, uma vez que estudos sobre as relações simbióticas desse gênero eram limitados.

No primeiro capítulo foi confirmada a presença tanto de α- quanto de β-proteobacterias associadas a espécies de *Mimosa*, por meio de experimentos com plantas-armadilha. Fatores ambientais como pH e fertilidade do solo parecem favorecer a predominância entre essas duas classes de rizóbios. No solo mais fértil e com pH neutro-alcalino, houve uma predominância de *Rhizobium*, enquanto que os solos que têm um pH ácido e uma fertilidade baixa favoreceram associação com *Paraburkholderia*. O presente trabalho revelou uma baixa especificidade entre as plantas hospedeiras, pois até mesmo espécies endêmicas se associaram indiscriminadamente com rizóbios pertencentes às duas classes de Proteobacteria.

No segundo capítulo foram feitos novos relatos de nodulação em espécies de *Stryphnodendron*. Uma elevada diversidade microbiana foi encontrada em associação com espécies desse gênero, contrastando com o encontrado na literatura sobre grupo Piptadenia, aos quais os integrantes são frequentemente associados com β-bactérias. Este trabalho amplia a visão da relação simbiótica no grupo Piptadenia.

Tendo em vista o benefício que essas relações simbióticas trazem para as plantas, para o meio ambiente e para a agricultura (fixação de nitrogênio, recuperação de áreas degradadas, entre outros), esse tema deve ser mais explorado. Considerando a diversidade das leguminosas que ocorrem no Brasil, ainda há muito que ser estudado quando se trata da simbiose entre plantas e bactérias.