Burkholderia Species Are the Most Common and Preferred Nodulating Symbionts of the Piptadenia Group (Tribe Mimoseae)

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Abstract

Burkholderia legume symbionts (also called α-rhizobia) are ancient in origin and are the main nitrogen-fixing symbionts of species belonging to the large genus Mimosa in Brazil. We investigated the extent of the affinity between Burkholderia and species in the tribe Mimoseae by studying symbionts of the genera Piptadenia (P.), Parapiptadenia (Pp.), Pseudopiptadenia (Ps.), Pitryocarpa (Py.), Anadenanthera (A.) and Microlobius (Mi.), all of which are native to Brazil and are phylogenetically close to Mimosa, and which together with Mimosa comprise the “Piptadenia group”. We characterized 196 strains sampled from 18 species from 17 locations in Brazil using two neutral markers and two symbiotic genes in order to assess their species affiliations and the evolution of their symbiosis genes. We found that Burkholderia are common and highly diversified symbionts of species in the Piptadenia group, comprising nine Burkholderia species, of which three are new ones and one was never reported as symbiotic (B. phenoliruptrix). However, α-rhizobia were also detected and were occasionally dominant on a few species. A strong sampling site effect on the rhizobial nature of symbionts was detected, with the symbiont pattern of the same legume species changing drastically from location to location, even switching from β to α-rhizobia. Coinoculation assays showed a strong affinity of all the Piptadenia group species towards Burkholderia genotypes, with the exception of Mi. foetidus. Phylogenetic analyses of neutral and symbiotic markers showed that symbiosis genes in Burkholderia from the Piptadenia group have evolved mainly through vertical transfer, but also by horizontal transfer in two species.

Introduction

Legumes have developed a symbiosis with a polyphyletic group of bacteria commonly called rhizobia. This symbiosis leads to the formation of a specialized organ, the root nodule, within which rhizobia differentiates into bacteroids. Bacteria fix atmospheric nitrogen, and feed the plant with combined nitrogen in exchange for carbon compounds derived from photosynthesis by the legume host. Over the last several decades, numerous diversity studies have focused on rhizobia, but their diversity and the number of investigated legumes hosts remain far from being complete due to the large number of legume species (>18000) [1].

Most rhizobia belong to a large diversity of alphaproteobacterial genera: Azorhizobium, Allorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium (Ensifer), Devosia, Methylobacterium, Ochrobactrum, Phyllobacterium, and more recently Aminobacter [2] and Microvirga [3], whereas Burkholderia and Cupriavidus are members of the betaproteobacteria [4], [5], [6], [7]. The terms α and β-rhizobia have thus been raised to distinguish each class of symbionts [7], [8]. Burkholderia is a highly diversified genus, including more than 70 species that have colonised a wide diversity of niches, ranging from soil and water to plants and animals [9], [10].

Diversity studies of rhizobia and legume host range have shown that the vast majority of nodulating legume species interact with α-rhizobia [7]. To date, β-rhizobia are much more restricted in terms of host range, and most species described so far interact with Mimosa species in their major area of diversification in central Brazil and other parts of the tropical World (for a review see [7]). Mimosa species symbionts include mainly Burkholderia species, such as B. tuberum [11], [12], B. mimosarum [13], [14], [15], [16], B. phymatum, [12], [16], [17], B. nodosa [18], B. sabiae [19], B. symbiotica [20], B. diazotrophica [21], and two species of Cupriavidus: C. taiwanensis [5] and C. necator [22]. Interestingly, further studies have shown that nodulation by Burkholderia could be extended to other legumes, such as some native/endemic African and Australian species in the subfamily Papilionoideae [23], [24]. For example, B. tuberum STM678 nodulates many Cyclopa species [23], and it harbors distinct nodulation genes compared to Mimosa-nodulating Burkholderia, suggesting South African and South American
burkholderias acquired their noduleation genes in distinct transfer events [9], [14], [12], [7].

The relationship between β-rhizobia and Mimosa spp. was investigated in more depth in two studies reported by [11] and [25]. These authors analyzed the diversity of symbionts from nodules of c. 70 diverse Mimosa species growing in the two major biomes of Brazil (Cerrado and Caatinga) in which the genus Mimosa has evolved and diversified into more than 200 native and endemic species [26]. They concluded on the generic character of noduleation in the genus Mimosa that the preferred symbionts of this genus in Brazil are Burkholderia. Identical tree topologies between neutral and symbiotic markers were also observed on Brazilian and French Guianan Mimosa-nodulating burkholderia [11], [12], indicating a monophyletic origin and single acquisition of symbiotic genes by a Burkholderia ascensor, followed by vertical transfer of noduleation genes during species diversification [11]. On the other hand, based on phylogenies of noduleation genes, Cipriavidus taiwanensis was found to be a more recent symbiont of Mimosa spp., which acquired noduleation genes from a Burkholderia ascensor [8], [12], [27]. Although most species of Mimosa are mainly nodulated by Burkholderia spp., and a few by Cupriavidus spp., some of them can also form effective symbioses with α-rhizobia [8], [11], [28], [29]. Later studies have underlined the existence of genetic and environmental factors that could affect the preference of legumes species for noduleation with either α- or β-rhizobia; these include soil pH or the presence of combined nitrogen [11], [12], [16], [24], [25].

The Mimosa genus is closely related to a number of genera including Piptadenia (P.), Parapiptadenia (Pp.), Pityrocarpa (P.), Anadenanthera (A.), Styphnolopodium (S.), and Mucilago (M.) [syn. Goldmania] within the tribe Mimosae, Jobson & Luckow [30] have investigated the phylogeny of these different genera and subdivided the Piptadenia genus sensu stricto (or Eupiptadenia clade) that is a sister clade to Mimosa, and includes, for example P. flava (type species), P. floribunda, P. gonoacantha, P. paniculata, the Pityrocarpa clade (P. leucocorys, P. monoliformis, P. obliqua) that is closer to the genera Styphnolopodium and Parapiptadenia than to the Eupiptadenia clade; and finally P. viridiflora that is outgrouped from the previous genera and represent a particular case deserving a new generic name [30]. Interestingly, all these genera contain woody species native to South America, particularly to Brazil [31], [30]. Some of them are currently exploited by locals owing to their economical values, such as A. peregrina [32], [33], Pp. rigida [34], [35] and P. gonoacantha [36]. Although their ability to establish associations with rhizobia is documented [37], [38], [39], [40], [41], [42], [43], information about the rhizobial diversity and symbiotic efficiency on these plant species is scarce. A recent study of symbionts of Parapiptadenia rigida in Uruguay demonstrated that this species is nodulated by rhizobial strains belonging to the genera Burkholderia, Cupriavidus and Rhizobium, among which the Burkholderia genotypes were the predominant group [44]. Symbiosis with β-rhizobia in the tribe Mimosaceae thus appears to extend outside Mimosa and be more common than previously expected.

In this study our objectives were (i) to investigate the extent of Burkholderia affinity within the tribe Mimosaceae by focusing on native species in the Piptadenia genus and in related genera in the Piptadenia Group described by Jobson & Luckow [30]; (ii) to characterize the diversity of rhizobia and their symbiotic genes in this group of legumes; (iii) to examine further symbiotic specificity within the Piptadenia Group.

To achieve these objectives, we isolated rhizobia from diverse species in the Piptadenia Group in their native areas in Brazil, characterized their taxonomic and symbiotic diversity, and assessed their host specificity via coinoculation assays. We found a large diversity of Burkholderia species, but also α-rhizobia, and with a few exceptions an affinity of most plant species towards Burkholderia rather than to β-rhizobia. We discuss the evolutionary patterns of both taxonomic and symbiotic markers and the putative co-adaptation between Burkholderia and Piptadenia Group species.

Materials and Methods

Bacterial Isolates Sampling and Maintenance

Collection of material was authorized by IBAMA N.° 058/2006. The bacterial collection was built through several sampling campaigns performed between 1984 and 2010, from 9 states in Brazil (Rio de Janeiro, Sao Paulo, Minas Gerais, Bahia, Mato Grosso do Sul, Paraná, Pernambuco, Espirito Santo and Pará). Sample locations were concentrated on the main centers of diversification of the Piptadenia Group (Mata Atlantica) and are presented in Figure S1. Details on sampling (gps coordinates, year, season, soil type and pH) are presented in Table S1. STM strains were sampled from trees from various places of Rio de Janeiro State in April 2010; SFM, BR and CVRD strains were sampled from plants growing in plant nursery beds in various states of Brazil (Table S2). Nodules sampled from the field as described in [37] were dried on silicagel until symbiont isolation. Nodules were then rehydrated for 30 min in sterile distilled water, surface sterilized by immersion 30 seconds in 70% ethanol followed by 1 to 3 min in 3% hydrogen peroxide, and washed three times in sterile distilled water. Nodules were then individually crushed and streaked on yeast mannitol agar (YMA, [45]) plates containing bromophenol blue. All YMA plates were incubated at 28°C. Single colonies were picked and checked for purity by repeated streaking and microscope examination. All pure isolates were stored at 80°C in YM broth plus 20% (w/v) glycerol. For Styphnolopodium species, no nodules could be found on two locations, so a plant trapping approach was developed by growing seedlings on a top soil harvested under each tree, and nodules were harvested and processed as described above. As these rhizobia were trapped, they were not treated as natural symbionts, and were only included on Figure 1C to assess rhizobial patterns among the Piptadenia group. Strains from previous studies on P. flava [42], Pp. rigida [44], [46], as well as P. gonoacantha and Pp. pterosperma [46] were also included in this study. A total of 196 strains were included in the study, of which 63 “representative” strains are listed in Table 1 and were chosen as one strain per host plant per geographical origin per unique 16S rDNA ribotype (as described in molecular method section).

Plant Nodulation and Specificity Tests

Seeds of P. gonoacantha, Pp. monoliformis, Mi. foetidus and A. colubrina were obtained from Instituto Brasileiro de Florestas (Londrina, Paraná, Brasil) or Embrapa Agrobiologia (Seropédica; Brazil). M. pudica and siratro seeds were obtained from B&T World Seeds (Paguignan, France) and from University Cheikh Anta Diop (Dakar, Senegal), respectively. P. gonoacantha seeds were scarified and surface sterilized using 96% H2SO4 and 3% calcium hypochlorite (2 min and 3 min, respectively), while A. colubrina, A. peregrina, and Pp. rigida seeds were immersed for 3 min in 3% calcium hypochlorite; and Mi. foetidus seeds were sterilized with 96% H2SO4 during 30 min. Pp. monoliformis seeds were germinated by immersion in concentrated H2SO4 for 5 min, then washed with sterile dH2O and afterwards were soaked in 3% calcium hypochlorite for 3 min. All species seeds were then washed four times with sterile dH2O, before being germinated on 0.8% water
agar plates at 28°C in the dark. *M. pudica* and siratro seeds were sterilized and germinated as described in [12].

For nodulation tests, all species were grown in Gibson tubes containing Jensen medium [45] filled with dH2O, except *Piptadenia* species for which tubes were filled with sterile attapulgite (OIL DRI US Special, IIIR, Damolin) and supplemented with sterile dH2O. All plants were then grown in a chamber at 26°C (relative humidity 40%) with a 16 h light/8 h night cycle. Inoculation was performed by adding 1 ml of exponential bacterial culture grown in broth YM medium. Cross-contamination was investigated by

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**Figure 1. Comparison between Piptadenia’s group plant phylogeny and the occurrence of alpha and beta-rhizobia as nodule symbionts in the field or during coinoculation experiments.** The plant phylogeny (A) is based on a trnL-F/trnK-matK combined dataset, and was built by parsimony with TNT1.1 (default parameters, on www.phylogeny.fr) using the Jobson & Luckow [30] dataset (downloaded from Treebase, study number S1763, and amended with the *P. trisperma* from this study). The % of α and β-rhizobia per legume host (in bold) from field sampling (B) or from the coinoculation experiment (C) are represented as white (*Burkholderia*) and black (α-rhizobia) squares, with the number of strains sampled within each square. *: statistics of symbionts from [44] and [11]. The grey colored square for *Stryphnodendron* indicates that % of α-rhizobia originates from a trapping experiment on soil (see Material & Methods section).

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### Table 1. Listing of strains used in this study.

| Original Host/Strain | Ribotype | nodC clade | Bacterial species | Geographical Origin and source | Nb | Nod# |
|----------------------|----------|------------|-------------------|--------------------------------|----|------|
| **Piptadenia gonoacantha** |
| STM7321 | R1 | C12 | *Rhizobium* sp. 1 | 2 (RJ), this study | 4 | Pg, Sir |
| STM7300 | R1 | C12 | *Rhizobium* sp. 1 | 2 (RJ), this study | 15 | Pg, Sir |
| SMF1181_6 | R1 | NT | *R. tropici* | 17 (SP), this study | 2 | NT |
| STM7315 | B1 | C3 | *B. sabiae* | 2 (RJ), this study | 2 | Pg, Mp, Pm, Ap, Ac, Ppr |
| STM7319 | B1 | C3 | *B. sabiae* | 2 (RJ), this study | 1 | Pg, Mp, Sir |
| SMF1181_1 | B2 | NT | *B. nodosa* | 17 (SP), this study | 1 | NT |
| P. gonoacantha_1 | B3 | NT | *B. nodosa* | 16 (SP), this study | 2 | Pg |
| P. gonoacantha_3 | B4 | NT | *Burkholderia* sp. 3 | 16 (SP), this study | 2 | Pg |
| P. gonoacantha_8 | B5 | C2 | *Burkholderia* sp. 3 | 16 (SP), this study | 1 | Pg |
| STM7296 | B6 | C2 | *Burkholderia* sp. 3 | 4.2 (RJ), this study | 1 | Pg, Mp |
| STM7317 | B7 | C4 | *B. phenoliruptrix* | 2 (RJ), this study | 1 | Pg, Mp, Mf, Ap, Ac, Ppr, Pm |
| BR4812 | B8 | NA | *B. diazotrophica* | [46] | 1 | Pg |
| **Piptadenia trisperma** |
| STM7351 | B1 | C1 | *B. sabiae* | 5 (RJ), this study | 2 | Mp |
| STM7353 | B9 | C5 | *B. nodosa* | 5 (RJ), this study | 4 | Mp, Sir, Pg, Mf, Pm, Ap, Ac, Ppr |
| STM7348 | B2 | C5 | *B. nodosa* | 5 (RJ), this study | 2 | Mp, Sir |
| **Piptadenia paniculata** |
| STM7339 | R2 | NA | *R. tropici* | 4.1 (RJ), this study | 1 | Nod- on Mp & Sir |
| STM7342 | R3 | NA | *Rhizobium* sp. 3 | 4.1 (RJ), this study | 1 | Nod- on Mp & Sir |
| STM7330 | R1 | C12 | *Rhizobium* sp. 1 | 4.1 (RJ), this study | 1 | Pg, Sir |
| STM7333 | R4 | C9 | *Bradyrhizobium* sp. 1 | 4.1 (RJ), this study | 1 | Sir |
| STM7334 | R5 | C9 | *Bradyrhizobium* sp. 1 | 4.1 (RJ), this study | 1 | Sir |
| STM7331 | R6 | C9 | *Bradyrhizobium* sp. 3 | 4.1 (RJ), this study | 5 | Sir |
| STM7332 | R7 | C11 | *Bradyrhizobium* sp. 1 | 4.1 (RJ), this study | 6 | Sir |
| STM7329 | R8 | C9 | *Bradyrhizobium* sp. 3 | 4.1 (RJ), this study | 3 | Sir |
| STM7324 | B7 | C4 | *B. phenoliruptrix* | 4.1 (RJ), this study | 1 | Mp, Sir, Pg |
| **Piptadenia adiantoides** |
| SMF1758_4 | R9 | NT | *Rhizobium* sp. 6 | 10 (MG), this study | 2 | Pa |
| SMF1758_8 | R1 | NT | *R. tropici* | 10 (MG), this study | 1 | Pa |
| **Piptadenia monoliformis** |
| SMF774_1 | B10 | C3 | *B. phenoliruptrix* | 3 (RJ), this study | 1 | Mp |
| **Piptadenia viridiflora** |
| SMF1356_6 | R1 | C12 | *Rhizobium* sp. 1 | 12 (BA), this study | 4 | NT |
| SMF1356_7 | B1 | C3 | *B. sabiae* | 12 (BA), this study | 1 | NT |
| JPY570 (CAE9) | B11 | C3 | *Burkholderia* sp. 1 | Bahia, Gross et al.3 | 1 | Pv, Pg, Mp |
| JPY565 (CAE1) | B11 | C3 | *Burkholderia* sp. 1 | Bahia, Gross et al.3 | 1 | Pv, Pg, Mp |
| **Piptadenia stipulacea** |
| JPY584 (D84) | B12 | C3 | *Burkholderia* sp. 4 | Bahia, Gross et al.3 | 1 | Ps, Pg, Mp |
| **Piptadenia flavia** |
| UPRMB060 | R10 | C8 | *R. gallicum* | [42] | 1 | Pf |
| UPRMB061T1 | R10 | C8 | *R. gallicum* | [42] | 1 | Pf |
| **Anadenanthera peregrina** |
| STM7420 | R1 | NT | *Rhizobium* sp. 1 | 2 (RJ), this study | 1 | Nod- Mp & Sir |
| STM7426 | R1 | NT | *R. tropici* | 2 (RJ), this study | 1 | Nod- Mp & Sir |
| SMF466_6 | R1 | C12 | *R. leucaenae* | 6 (MGS), this study | 5 | NT |
| II_10R | B13 | C3 | *B. sabiae* | 16 (SP), this study | 1 | NT |
| STM7419 | B1 | C3 | *B. sabiae* | 2 (RJ), this study | 1 | Mf |
| STM7384 | B1 | C3 | *B. sabiae* | 2 (RJ), this study | 6 | Mf |
Table 1. Cont.

| Original Host/Strain Ribotype | nodC clade | Bacterial species & Geographical Origin and source | Nb | Nod# |
|------------------------------|------------|---------------------------------------------------|-----|------|
| SMF362_15                    | B2         | C5       | B. nodosa 8 (PA), this study                          | 1   | NT   |
| STM7399                      | B8         | C3       | B. diazotrophica 2 (RU), this study                   | 5   | Ap, Mp |
| SMF362_13                    | B14        | NT       | B. caribensis 8 (PA), this study                      | 3   | NT   |
| IIIA_4A                      | B10        | C3       | B. phenoliruptrix 16 (SP), this study                 | 1   | NT   |
| STM7437                      | B7         | C4       | B. phenoliruptrix 2 (RU), this study                  | 15  | Mp   |
| STM7415                      | B7         | C4       | B. phenoliruptrix 2 (RU), this study                  | 3   | Mp   |
| *Anadenanthera colubrina*    |            |          |                                                     |     |      |
| Angicol_417                  | R1         | C12      | *Rhizobium* sp. 2 7 (PE), this study                   | 2   | NT   |
| STM7444                      | B15        | C3       | B. diazotrophica 2 (RU), this study                   | 1   | Nod- on Mp,Sir |
| STM7439                      | B16        | C3       | B. diazotrophica 2 (RU), this study                   | 2   | Mp   |
| STM7445                      | B17        | C3       | B. diazotrophica 2 (RU), this study                   | 3   | Mp   |
| STM7443                      | B18        | NT       | B. diazotrophica 2 (RU), this study                   | 3   | NT   |
| STM7452                      | B19        | C3       | B. diazotrophica 2 (RU), this study                   | 1   | Mp   |
| STM7454                      | B7         | C4       | B. phenoliruptrix 2 (RU), this study                  | 5   | Mp   |
| *Parapiptadenia pterosperma* |            |          |                                                     |     |      |
| STM7365                      | B1         | NT       | B. sabiae 5 (RU), this study                          | 1   | Pg   |
| STM7373                      | B1         | C1       | B. sabiae 5 (RU), this study                          | 1   | Mp, Pg, Mf, Ap, Ac, Pm |
| CVRDI.2                      | B20        | C3       | *Bradyrhizobium* sp. 15 (ES), this study              | 1   | Pppt |
| STM7363                      | B9         | C5       | B. nodosa 5 (RU), this study                          | 5   | Mp, Sir, Pg |
| STM7358                      | B2         | C5       | B. nodosa 5 (RU), this study                          | 2   | Mp, Sir |
| BR9001                      | B2         | C5       | B. nodosa 15 (ES), [46]                               | 1   | Pppt |
| BR9002                      | B9         | C5       | B. nodosa 15 (ES), [46]                               | 2   | Pppt |
| SMF142_3                     | B2         | NT       | B. nodosa 11 (MGS), this study                        | 4   | NT   |
| BR9003                      | C5         | B. nodosa [46]                                 | 1   | Pppt |
| *Parapiptadenia rigida*      |            |          |                                                     |     |      |
| P. rigida_2                  | B1         | C1       | B. sabiae 16 (SP), this study                         | 3   | NT   |
| UYPR3.611                    | C1         | B. sabiae | Uruguayan, [44] 1                                    | 1   | Ppr  |
| UYPR3.113                    | C1         | B. caribensis | Uruguayan, [44] 1                                  | 1   | Ppr  |
| UYPR7.63                     | C13        | B. mesoamericanum | Uruguayan, [44] 1                                | Ppr |
| BR9004                      | C5         | B. nodosa [46]                                 | 1   | Ppr  |
| *Parapiptadenia blanchetti*  |            |          |                                                     |     |      |
| EG100                        | B21        | C3       | B. diazotrophica 14 (BA), Gross et al.³               | 1   | Mp   |
| *Microlobius foetidus*        |            |          |                                                     |     |      |
| STM7379                      | R1         | C12      | *R. tropici* 1 (RU), this study                       | 2   | Mf, Ap |
| STM7378                      | R6         | NA       | *Bradyrhizobium* sp. 4 1 (RU), this study             | 4   | Mf, Sir |
| STM7375                      | R8         | C10      | *Bradyrhizobium* sp.2 1 (RU), this study              | 1   | Sir |
| *Pseudopiptadenia contorta*  |            |          |                                                     |     |      |
| CVRDI.5                      | B3         | C5       | B. nodosa 15 (ES), this study                         | 2   | Psc  |
| CVRDI.7                      | B2         | C5       | B. nodosa 15 (ES), this study                         | 2   | Psc  |
| *Pseudopiptadenia psilostachya* |       |          |                                                     |     |      |
| SMF613_4                     | R1         | NT       | *R. tropici* 9 (Para), this study                     | 3   | NT   |
| *Pseudopiptadenia bahiana*   |            |          |                                                     |     |      |
| EG118                        | B1         | C1       | B. sabiae 13 (BA), Gross et al.³                      | 1   | Mp   |
| *Stryphnodendron* sp. (trapping)* |       |          |                                                     |     |      |
| STM9027                      | R11        | NT       | *Bradyrhizobium* sp. 18 (RU), this study              | 1   | Str  |
| STM9026                      | R12        | NT       | *Bradyrhizobium* sp. 18 (RU), this study              | 4   | Str  |
| STM9018                      | R13        | NT       | *Bradyrhizobium* sp. 18 (RU), this study              | 15  | Str  |

Symbols: % ribotype number as defined in Mat&Methods.
Species affiliation based on the 16S-recA phylogeny from Figure 2A.
using uninoculated negative controls randomly placed between treatments. Plants were observed for nodulation over a period of 30–60 dai, depending on plant species. Nitrogen fixation was estimated by visual observation of plant vigour and foliage color. For plant-rhizobium coinoculation experiments, plants were grown in Gibson tubes filled with attapulgite, moistened with microculture counting. Plants were incubated in the same conditions than as described above, watered with dH_{2}O and harvested 4 weeks post inoculation. Eight nodules were harvested per nodulated plant (12 replicates), surface sterilized 3 min with 3% HCl at 98°C, followed by 7 cycles of 10 seconds at 96°C then 4°C, with a final cycle of 2 min at 4°C, and 1 μl was used to amplify a recA gene fragment as described above. PCR templates were sequenced as previously described.

**Results**

**Table 1. Cont.**

| Number of isolates from the same host, location and 16 S haplotype per representative strain listed in the first column. |
|---|

| Geographic region | Number of isolates | Nodulation status | Nodulation test obtained from the representative strain |
|---|---|---|---|
| Brazil | 12 | 11 | Burkholderia |
| Indonesia | 5 | 4 | Rhizobium |
| India | 3 | 3 | Bradyrhizobium |

| Plant phylogenetic markers trnL-F and trnK-matK of *Styphnadenia* sp. and *P. trisperma* were PCR-amplified and sequenced as described by [30], from leaves collected from the field. |

**Phylogenetic and Sequence Analyses**

Nucleotide sequences from 16 S rDNA, recA, nodC and nifH genes were corrected with CHROMAS PRO v1.33 (Technelyx Pty Ltd), aligned using Muscle3.6 [52], and alignments were manually curated with GENEDOC [53]. Screening and classification of each 16 S rDNA haplotype (unique sequence in our dataset) was performed using MOTHUR using the unique.seqs command [54]. Phylogenetic trees were constructed by neighbor joining and likelihood methods using MEGA5 [55] and PAUP4 [56], or by Bayesian analyses using Mr Bayes [57] using priors from a GTR+I+G model (with parameters previously estimated by ML under PAUP4). Parsimony analyses on trnL-F+trnK-matK plant markers were performed on TNT1.1 (default parameters, www.phylogeny.fr) using the Jobson & Luckow [30] dataset (downloaded from Treebase, www.treebase.org, study number S1763), amended with new sequence from this study (*P. trisperma*). Bootstrapping analyses were conducted on MEGA5.

**Nucleotide Sequence Accession Numbers**

The sequences have been deposited in EMBL database under accession numbers HE983632 to HE983825 and HF536727 to HF536767, and are listed by gene for each strain in Table S3. Sequence alignments are available upon request.

**Building a Collection of Rhizobia from Piptadenia Group Species**

We sampled at least one representative species of the different clades and genera in the Piptadenia Group (see species in bold in Figure 1A): *Piptadenia sensu stricto* (the “Eupiptadenia” clade e.g. *P. gonoacantha*, *P. paniculata*, *P. adiantoides*), the Pityrocarpa clade (*P. verruciflora* clade, and *Anadenanthera*, *Para-piptadenia*, *Styphnadenia* and *Pseudopiptadenia* clades. The species assignment of each plant host was confirmed by the Botanical garden of Rio de Janeiro (R. Ribeiro). For five species (*Styphnadenia adstringens*, *P. trisperma*, *P. paniculata*, *P. gonoacantha* and *A. peregosa*), the taxonomic position was confirmed by sequencing the trnL-F marker, and comparing it to the Jobson & Luckow dataset [30]. Among all sampled legume species, we confirmed that 17 of them are nodulated, and one, *Piptadenia trisperma* is a new report for nodulation (according to nodulation data in GRIN database, http://www.ars-grin.gov/sbmljw/cgi-bin/nodulation.pl and [58]). The rhizobial collection was composed of 196 isolates and details are presented in Table 1, where they are classified according to original host legume,
representative strain and site of isolation. Representative strains for each location and legume host were chosen according to their 16 S rDNA sequence haplotype (named ribotype in Table 1); one strain per legume host and per sampling location with a unique 16 S rDNA sequence based on a 800 bp alignment was kept for further analyses, and designated as representative strains (for a total of 63 strains per host). Each location and legume host were chosen according to their 16 S rDNA sequence based on a 800 bp alignment was kept for further analyses, and designated as representative strains (for a total of 63 strains per host location). These strains were kept as the original host could not be tested. Details about nodule size, shape and functionality for each *Bradyrhizobium* species are given in Table S4 and Figure S2, together with pictures of nodules on *M. pudica* and *P. gonoacantha*. All nodulating strains appeared to fix nitrogen with *M. pudica* (based on red color within nodules usually linked to presence of leghaemoglobin, and plant development, see Table S4), except for 15 strains which were not tested. No nodulation was observed on *M. pudica* (carrying nodC1 variant) and *P. gonoacantha* (based on 16 S rDNA, and these grouped closed to *Bradyrhizobium* strains isolated from various hosts from the Piptadenia Group, except the BSP3 clade which was sampled only from *P. gonoacantha*. Some clades (BSa, BP, BC, BD, BN) contained strains isolated both from the genus *Mimosa* and the Piptadenia Group, while others were only found associated to the Piptadenia Group (BPL, BSP1, BSP3, BSP4) or to *Mimosa* species (BM, BSY, BSP2, BT). In the case of the *α*-rhizobia (Figure S3), strains were clustered into 8 new clades of *Rhizobium* and 4 of *Bradyrhizobium*. *Bradyrhizobium* sp. 1 (BSP1), *R. tropici* and BSP3 were the most represented clades with 25, 10 and 8 strains, respectively. *R. tropici* strains were found on the most important number of legume host (6 species). Strains of *R. leucanum* and *R. gallicum* were also found on *P. peregrina* and *P. flava* [42], respectively. *Rhizobium* isolates from *P. rigida* from the Taulé et al. study [44] were grouped within the *R. mesoamericanum* species (UPR7.63 & STM3625; Figure S4), a frequently sampled symbiotic species from *Mimosa pudica* [12], [60], [61]. In *Bradyrhizobium*, four clades could be distinguished and these were named *Bradyrhizobium* sp1 (BrSP1) to BrSP4, with no close relationship to any described species (except BrSP4 that was closely related to *Br. elkanii*). No obvious pattern could be deduced between host plants and α-rhizobial clades. Strains trapped from two soils of *Styphnoedron* sp. were only characterized on the basis of their 16 S rDNA, and these grouped closed to *Bradyrhizobium* sp. 1 (Figure S4).

### Distribution of Rhizobial Species per Host Plant and Sampling Locations

A distribution of rhizobial species according to their plant host and site of sampling is presented in Figure 3. We observed a strong site sampling effect since the diversity pattern of encountered species was completely divergent for the same host sampled on different sites. This pattern heterogeneity of symbionts was even found between *α* and β-rhizobia, since *P. gonoacantha* and *A. peregrina* nodules hosted mainly *Rhizobium* strains in some sites but *Bradyrhizobium* species on other sites, indicating no strict specificity of these species for *α* or β-rhizobia. *Burkholderia nodosa* was particularly frequent on the three different sampling sites of *P. phasmatum*, and was also found in *P. trisperma*, *A. peregrina* and *P. gonoacantha*. *Burkholderia nodosa* was also a promiscuous species in the Piptadenia Group (found in *A. peregrina*, *P. viridiflora*, *P. pterosperma*, *P. gonoacantha*). On the other hand, BSP1 and BSP3 species were only found in *P. gonoacantha* nodules. On the plant side, some species exhibited the same pattern of symbionts diversity in the same geographical area (for example *P. trisperma* and *P. phasmatum* in Cabo Frio, RJ; Figure 3). However, the opposite could also be found, as *P. gonoacantha* and *P. paniculata* in Cabo Frio also exhibited divergent

### Taxonomic Characterization of the Piptadenia Symbionts Collection

To further characterize the rhizobial collection at the taxonomic level and assign a putative species to each strain, a phylogenetic analysis of all representative strains based on a partition of two neutral markers (643 bp of the variable part of the 16 S rRNA gene, and a 644 bp fragment of the recA gene), was built following a Bayesian analysis (see Methods). The recA gene is a more highly resolved phylogenetic marker than the 16 S rDNA gene, and has proven to be a valuable tool for discriminating species within the *Burkholderia* genus [11], [12], [59]. We also included type strains of the most closely related species in the genera *Burkholderia, Rhizobium* and *Bradyrhizobium*. A phylogenetic tree of the 16 S-recA dataset is presented in Figure 2A (only for the genus *Burkholderia*, as α-rhizobia are presented in Figure S3); and individual gene marker phylogenies are available as Figure S4. The bacterial collection was clustered into 9 highly supported clades (posterior probabilities >0.9, bootstrap values >75%) in the genus *Burkholderia* (107 strains), 8 clades in *Rhizobium* (48 strains) and 4 in *Bradyrhizobium* (41 strains). In *Burkholderia*, most strains belonged to clades associated with the species *B. nodosa* (BN, 31 strains), *B. phasmatiopsis* (BPL, 27 strains), *B. sabina* (BSa, 21 strains), and *B. diazotrophica* (BD, 18 strains). Other strains belonged to *B. phymatum* (BP, one strain), *B. caribensis* (BC, two strains), and three potentially undescribed species: *Burkholderia* sp. 1, 3 and 5 (BSP1, two strains, BSP3, four strains, BSP4, one strain). *Burkholderia* strains were identified among all legume species in this study, except for *Mi. fistulosus, Ps. polistachya* and *P. ativendoides*. For these three species, few rhizobial isolates were obtained (3–7), all belonging to *Rhizobium* and/or *Bradyrhizobium* genera. Each clade includes strains isolated from various hosts from the Piptadenia Group, except the BSP3 clade which was sampled only from *P. gonoacantha*. Some clades (BSa, BP, BC, BD, BN) contained strains isolated both from the genus *Mimosa* and the Piptadenia Group, while others were only found associated to the Piptadenia Group (BPL, BSP1, BSP3, BSP4) or to *Mimosa* species (BM, BSY, BSP2, BT). In the case of the α-rhizobia (Figure S3), strains were clustered into 8 new clades of *Rhizobium* and 4 of *Bradyrhizobium*. *Bradyrhizobium* sp. 1 (BSP1), *R. tropici* and BSP3 were the most represented clades with 25, 10 and 8 strains, respectively. *R. tropici* strains were found on the most important number of legume host (6 species). Strains of *R. leucanum* and *R. gallicum* were also found on *P. peregrina* and *P. flava* [42], respectively. *Rhizobium* isolates from *P. rigida* from the Taulé et al. study [44] were grouped within the *R. mesoamericanum* species (UPR7.63 & STM3625; Figure S4), a frequently sampled symbiotic species from *Mimosa pudica* [12], [60], [61]. In *Bradyrhizobium*, four clades could be distinguished and these were named *Bradyrhizobium* sp1 (BrSP1) to BrSP4, with no close relationship to any described species (except BrSP4 that was closely related to *Br. elkanii*). No obvious pattern could be deduced between host plants and α-rhizobial clades. Strains trapped from two soils of *Styphnoedron* sp. were only characterized on the basis of their 16 S rDNA, and these grouped closed to *Bradyrhizobium* sp. 1 (Figure S4).

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Piptadenia’s Affinity for *Burkholderia* Symbionts
patterns of symbiotic association, one being nodulated by β- and the other by α-rhizobial species.

Phylogeny of Symbiosis-related Genes of Piptadenia’s Rhizobia

Phylogenetic analyses were performed on fragments of the nodC (involved in the synthesis of the Nod factor core) and nifH (involved in nitrogen fixation) genes to evaluate the origin and evolution of symbiosis in Piptadenia Group microsymbionts. The nodC and nifH data sets used for phylogenies included representative strains listed in Table 1, as well as reference strains from each clade of the Bontemps et al. [11] and Mishra et al. [12] diversity studies on Mimosa species. A nodC ML phylogeny is shown in Figure 2B, while the nifH ML tree is presented as Figure S5. All nodC sequences from Burkholderia symbiotic species of the Mimoseae tribe were monophyletic compared to α-rhizobia. Burkholderia strains from the Piptadenia Group were clustered into five nodC clades (nodC1 to nodC5 on Figure 2B). Some nodC clades corresponded to already described bacterial species, such as B. nodosa (nodC3), B. phymatum (nodC1), B. diazotrophica (nodC2), while other species had strains carrying different alleles of nodC (e.g. B. sabiae with nodC1 or nodC3 alleles; B. phenoliruptrix with nodC3 and nodC4 alleles). In the case of B. phenoliruptrix, strains carrying either nodC3 or nodC4 were both efficient symbionts of M. pudica, while B. sabiae nodC variants (nodC1, nodC3) exhibited different nodulation phenotypes on M. pudica (inefficient/efficient, Table S4). Some clades carried strains sampled from Mimosa and Piptadenia Group species (nodC3, nodC5), while others were specific to either the Piptadenia Group (nodC1, nodC2, nodC4) or to Mimosa (nodC6, nodC7). The large nodC3 clade hosted strains from every studied legume species, except for P. rigida that was restricted to the nodC1 clade. Overall, no clear pattern was identified concerning nodC cladogenesis and legume hosts (except the nodC2 clade that was specific to P. gonoacantha). A better correspondence was found between the nodC and 16 S-recA trees. However, although several 16 S-recA clades corresponded to nodC clades, horizontal gene transfer was also identified in species which carried different nodC alleles (e.g. B. sabiae with BSa-1 and 2, and B. phenoliruptrix with BPL-1 & 2, Figure 2B). The nifH gene ML tree (Figure S5) reflected the same cladogenesis as the nodC tree, but with a few exceptions e.g. some strains in the nodC1 clade (UYPR1.313, UYPR3.611 and Prigida2) were clustered in a different clade in the nifH tree (nifH3).

In the case of α-rhizobia from the Piptadenia Group (Figure S2B), nodC sequences from Rhizobium strains (R. tropici, RSP1, RSP2, R. leguminosarum) were clustered in a clade (nodC12) together with R. tropici strains from diverse Mimosa species isolated from Papua New Guinea (NGR181) and Puerto Rico (UPRM8021) [28], [42]. There was no sequence variation on nodC fragment.

Figure 3. Distribution of rhizobial species according to host plant and sampling regions. The % of each species per legume host was calculated according to sampled isolates listed in Table 1. Sampling region is listed below each histogram while on top are indicated the number of isolates. Abbreviations: SER, RJ: Seropédica, Rio de Janeiro; MGS: Mato Grosso do Sul; PAR: Paraná; SP: São Paulo; CB, RJ: Cabo Frio, Rio de Janeiro; BU, RJ: Búzios, Rio de Janeiro; ES: Espírito Santo; MG: Minas Gerais; PHF: SP: Paraíbuna horto florestal, São Paulo; BA: Xique-Xique, Bahia; IFSP: SP, IFSP, São Paulo; Ac: Anadenanthera colubrina; Ap: A. peregrina; Mf: Microlobius foetidus; Pg: Piptadenia gonoacantha; Ppan: P. paniculata; Pt: P. trisperma; Ppv: P. viridiflora; Pppt: Parapiptadenia pterosperma; Ppr: Parapiptadenia rigida.

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Figure 2. Comparison of phylogenies between neutral and symbiotic markers in beta-rhizobia. The neutral marker phylogeny (A) is based on a partition of 600 bp of 16 S DNA and 600 bp of recA genes, and was built by a Bayesian analysis with priors estimated by ML (see Experimental procedure section). Numbers at ach nodes indicates posterior probabilities (upper number) and bootstraps values (lower number) from a ML analyses built in parallel (with a GTR+I+G model, 1000 bootstrap replicates). Bootstraps are only indicated when >50%. Node values in bold indicates supported nodes (both by posterior probabilities and bootstrap) retained for clades delineation. The nodC phylogeny is based on 437 bp alignments, and was built by ML using a GTR+I+G model, with 1000 bootstraps replicates (% indicated at node trees if >50%). Abbreviations used: B: Burkholderia, C: Cupriavidus, BPL: Burkholderia phenoliruptrix, BT: B. tuberum, BSa: B. sabiae, BD: B. diazotrophica, BSy: B. symbiotta, BM: B. mimosarum, BN: B. nodosa, BSP1,2,3,4: Burkholderia sp. 1 to 4. doi:10.1371/journal.pone.0063478.g002
between UPRM8021 and the bean symbiont. The only exceptions were \( R. \text{gallicum} \) between UPRM8021 and the bean symbiont.

In order to assess plant specificity in the Piptadenia Group, we carried out a coinoculation experiment with 12 rhizobial genotypes, representative of species and \( \text{nodC} \) clades on 6 Piptadenia Group species (\( \text{Mf}, \text{Pm}, \text{Ppr}, \text{Pg}, \text{Ap}, \text{Ac} \)) for which seeds were available. The final range of nodules analyzed were 12 to 60 on 5 to 9 plant replicates. The nodule occupants were then identified as described in the Material and Methods section. Table 2 shows the percentage of nodule occupancy per bacterial genotype for each plant host. The percentage for \( \gamma \)-rhizobia was also included in Figure 1C for comparison with wild nodule sampling % and plant phylogeny. Overall, all plant species showed a preference for \( \text{Burkholderia} \) genotypes, except for \( \text{Mi. foetidus} \) which was nodulated preferentially by \( \text{bradyrhizobia} \). One \( \text{Burkholderia} \) genotype, STM7353 (BN, \( \text{nodC5} \)) was the most prevalent (in 38 to 85% of nodules) over all other genotypes on all plant hosts (except for \( \text{Mf} \), 9% of nodules), followed by STM7317 (BPL, \( \text{nodC4} \)) that occupied 30 to 33% of plant hosts (except on \( \text{Mf} \), 9%, and \( \text{Pg} \), 3%). Interestingly, the two \( \text{Br. sabiae} \) genotypes (each carrying either the \( \text{nodC1} \) or \( \text{nodC3} \) allele) did not show the same nodulation pattern: STM7373 was present at around 20% on \( \text{Mf} \) and \( \text{Ac} \) while STM7315 (\( \text{nodC3} \)) was absent, and conversely STM7315 was present at 20% on \( \text{Pm} \) while STM7373 was absent. Such results indicate potential host plant specificity towards several \( \text{nodC} \) alleles, although competitiveness for nodulation also interferes with these results. Genotypes from the \( \text{Rhizobium} \) and \( \text{Bradyrhizobium} \) genera were poorly competitive, and only STM7379 (RT, \( \text{nodC2} \)) and STM7378 (BSP4) were identified in one host, \( \text{Mi. foetidus} \) (these two strains originating from nodules of this species). It is interesting to note that if \( \text{Mi. foetidus} \) favors \( \gamma \)-rhizobia symbionts, this plant species selected its native genotypes rather than \( \gamma \)-rhizobia from \( \text{P. paniculata} \), thus underlining specific affinities between this host and its symbionts. Another case of potential host plant specificity is that of STM7296 (BSP3, \( \text{nodC2} \)), as this poorly competitive strain was only detected on its original plant host, \( \text{P. gonoacantha} \).

### Discussion

**Burkholderia Species are Common and Highly Diverse Symbionts of the Piptadenia Group**

\( \text{Burkholderia} \) symbiont diversity has been intensively studied on collections isolated from nodules of different species of \( \text{Mimosa} \), most of them originating from Brazil [11], [14]. In the present study we extended the nodulation by \( \text{Burkholderia} \) symbionts to other species in the Piptadenia Group, including six genera that

| Strain | Species | \( \text{nodC}^b \) | \( \text{Mf} \) | \( \text{Pm} \) | \( \text{Ppr} \) | \( \text{Pg} \) | \( \text{Ap} \) | \( \text{Ac} \) |
|--------|---------|----------------|--------|--------|--------|--------|--------|--------|
| \( \text{Burkholderia} \) | STM7317 (Pg) | BPL | C4 | 9 | 32 | 33 | 3 | 32 | 31 |
| STM7296 (Pg) | BSP3 | C2 | 7 |  |  |  |  |  |  |
| STM7399 (Ap) | BD | C3.2 | 2 |  |  |  |  |  |  |
| STM7373 (Pppt) | BSa | C1 | 22 | 5 | 3 | 3 | 27 |  |
| STM7315 (Pg) | BSa | C3.1 | 21 | 8 | 2 | 8 | 4 |  |
| STM7333 (Pt) | BN | C5 | 9 | 42 | 59 | 85 | 53 | 38 |
| \( \gamma \)-rhizobia | STM7300 (Pg) | BSP1 | C12 |  |  |  |  |  |  |
| STM7379 (Mf) | RT | C12 | 17 | 2 |  |  |  |  |
| STM7342 (Ppan) | BSP3 | NT |  |  |  |  |  |  |
| STM7332 (Ppan) | BrSP1 | C11 |  |  |  |  |  |  |
| STM7329 (Ppan) | BrSP3 | C9 |  |  |  |  |  |  |
| STM7378 (Mf) | BrSP4 | NT | 43 |  |  |  |  |  |
| Total Nb nodules |  |  | 23 | 19 | 12 | 60 | 60 | 26 |

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*Original host of each strain is indicated between parenthesis;*  
*nodC numbering corresponds to \( \text{nodC} \) clades defined in Figure 2B and figure S3B;*  
*Number of nodule samples analyzed per coinoculation assay. Abbreviations: BPL: \( \text{Burkholderia} \) phenoliruptrix; BD: \( \text{Diazotrophicus} \); BSa: \( \text{Bradyrhizobium} \) subsp. \( \text{ampelophagus} \); BSP3: \( \text{Burkholderia} \) sp. 3; BN: \( \text{B. nodosa} \); BSP1: \( \text{Rhizobium} \) sp. 1; 3; RT: \( \text{Rhizobium} \) tropici; BrSP1 to 4: \( \text{Bradyrhizobium} \) 1 to 4; Ac: \( \text{Anadenanthera} \) colubrina, Ap: \( \text{A. peregrina} \); Mf: \( \text{Micrololobus} \) foetidus; Pg: \( \text{Piptadenia} \) gonoacantha; Ppr: \( \text{Parapiptadenia} \) rigida; Pm: \( \text{Pityrocarpa} \) monoliformis.  
doi:10.1371/journal.pone.0063478.t002
are sister clades to *Mimosa* in the tribe Mimoseae. The Piptadenia Group contains about 70 species (if excluding the genus *Mimosa*, that is also part of this group), which are common in tropical and subtropical America, but are mainly native to Brazil [30]. The capacity of nodulation among these clades has been investigated in previous works (see Introduction), but one of them, *P. trichophylla*, is a new report of nodulation from this study. A large diversity of *Burkholderia* was found, with nine species, five being already described as symbionts of *Mimosa* species (*B. sahiae*, *B. phymatum*, *B. carinensis*, *B. diazotrophica*, *B. nodosa*) [8], [18], [19], [17], [21]), one was not previously reported as containing symbiotic strains (*B. phenoliruptrix*, [10], [62]), and BSP1, BSP3 and BSP4 are three putative new species, whose taxonomic status is currently being investigated (Bournaud et al., unpublished). This diversity of symbiotic *Burkholderia* species mirrored the one discovered on 47 species of *Mimosa* by Bontemps et al [11] in Brazil, which also had seven clades, of which only three overlap with this study and are symbionts of both *Mimosa* and *Piptadenia* (*B. nodosa*, *B. diazotrophica* and *B. sahiae*). Overall, diversity studies on symbionts of mimosoid legumes reveal that four rhizobial species were found only associated to the Piptadenia Group (BPL, BSP1, BSP3, BSP4) while four different ones are found only on *Mimosa* species (BSP2, *B. symbiotica*, *B. mimosarum*, *B. tuberum*). *B. tuberum* is a frequent species in *Mimosa* spp. nodules in Brazil and French Guiana [11], [12] but was not detected in Piptadenia group nodules. However, these patterns are only based on our current knowledge, and given the high sampling site effect observed on the diversity of symbionts in this group, it is likely that each bacterial species could also be found in both groups of legume hosts.

The legume ability to form a symbiosis with *Burkholderia* is not antagonistic with the presence of α-rhizobia, since the two kinds of symbionts were found on six plant species (Table 1, Figure 1B), and some species had only α-rhizobia strains sampled from them. No clear conclusion about affinities of plant species towards α or β-rhizobia could be drawn for several legumes for which the nodule sampling was limited (i.e. *Py. monoliformis*, *P. flava*, *P. stipulacea*, *Pseudopiptadenia* spp.), or for which the site sampling effect was too strong (Fig. 3, *P. gonoacantha*, *A. peregrina*). In order to investigate this affinity, we conducted co-occlusion tests with 12 genotypes of α and β-rhizobia, and could conclude that *Py. monoliformis*, *Pp. rigida*, *P. gonoacantha*, *A. peregrina* and *A. colubrina* had a strong preference towards *Burkholderia* genotypes, while *Mi. foetidus* had an affinity for two genotypes of *Rhizobium* and *Bradyrhizobium*, as was also found in the wild sampling study. Another species, *P. paniculata*, remains ambiguous in terms of symbiont preference. Unfortunately we could not investigate this topic due to the unavailability of seeds. In the case of *Styphnodendron* species, trapping assays on soils recovered from the rhizosphere of two trees (identified as *S. adstringens*) trapped only *Bradyrhizobium* species. The species *S. pulcherrimum* species is nodulated by strains close to *R. tropici* but also by *B. nodosa* in French Guiana (Christine Le Roux, unpublished data), indicating that this genus is not only nodulated by α-rhizobia. The *Styphnodendron* genus thus warrants a more focused study in order to characterize its symbionts.

**Symbiosis Genes in Burkholderia from Mimosoid Legumes are Monophyletic and have been Transferred both Vertically and Horizontally Among Bacterial Species**

The *Burkholderia* strains from *Mimosa* and from the other Piptadenia Group species are monophyletic in terms of their nodC and nifH phylogenies, indicating a common ancestor at the origin of their interaction with members of the tribe Mimosaceae. The comparison of symbiotic and neutral markers revealed that vertical gene transfer is the main process for the dissemination of symbiotic genes within *Burkholderia* species. This pattern has been already observed for *Mimosa* symbionts [11], [12], and has allowed for the description of the symbiotic nature of *Mimosa*-nodulating *Burkholderia* as being “ancient”, as it has been tentatively dated at c. 50 MY ago [11]. However, we found also that two species of *Burkholderia* (*B. phenoliruptrix* and *B. sahiae*) harbor strains with different nodC alleles, which were either inherited by vertical transfer (nodC3/BSa-2, nodC4/BPL-2), or were acquired by horizontal gene transfer from another *Burkholderia* clade (nodC1/BSa-1, nodC3/BPL-1). Horizontal gene transfer thus also played a role in the co-adaptation between *Burkholderia* species and their legume hosts.

**Host Specificity between Burkholderia Genotypes and Piptadenia Group Species is not Strong**

Although gene phylogenies have informed us about the ancient character of the symbiosis between *Burkholderia* and *Piptadenia*, host specificity between the different partners is not obvious. Host plants are dispersed all over the *Burkholderia* phylogenies (Figure 2A,B), with no specificity pattern, except for that between BSP3 and *P. gonoacantha*. We tried to assess the specificity of the interactions between legume and rhizobial partners using co-inoculation experiments, and detected i) a high affinity of most tested plants towards *Burkholderia* genotypes, ii) promiscuous strains with high competitiveness for nodulation (*B. nodosa*, *B. phenoliruptrix*), as well as some trends towards specificity (e.g. *P. gonoacantha*/BSP3, *Mi. foetidus*/Bradyrhizobium). Given the strong site sampling effect detected in the sampling analyses (Figure 3), and the absence of strict host specificity, it is realistic to hypothesize that most Piptadenia Group and *Burkholderia* species interact with a relaxed host specificity, and that plant selection targets nodulation genes from the *Burkholderia* nodC monophyletic clade or from diverse α-rhizobia when no symbiotic *Burkholderia* are present, and there are few exclusions in order to maximize their chances of finding a compatible partner. The environmental (especially soil) conditions would thus play a crucial role in the survival and biogeography of the symbionts, and could be the origin of the different diversity patterns observed in our results. Several parameters have already been found to affect *Burkholderia* symbionts diversification and/or competition, such as soil pH [12], [24], [16], altitude [11], or nitrogen sources [28].

**Conclusion**

In this study we extended the symbiotic interaction with *Burkholderia* from *Mimosa* to the wider Piptadenia Group in the tribe Mimosaceae. Given the ancient and diverse character of this interaction, symbiosis with *Burkholderia* species might be present in other genera of this tribe, and possibly even in other mimosoid tribes, such as the Ingeae, as Barrett and Parker [63] have isolated *Burkholderia* strains from a member of this tribe (*Abronia macradenia*). The fact that *B. phymatum* STM815 is able to nodulate several legumes in the subfamily Mimosoideae (including species in *Leucaena*, *Prosopis*, and *Acacia*) gives clues on the possible extent of the host range of these symbionts [7]. Symbiosis with *Burkholderia* could thus be much more common and ecologically significant than anticipated, encompassing several tribes in the Mimosoideae. Given the relaxed specificity between Piptadenia Group species and their symbionts, and the diversification of *Burkholderia* into many species, larger samplings of each species in relation to soils and environmental parameters are required in order to get a clearer picture of the diversity and phylogeography of β-rhizobia, and how they have co-evolved with species in the Mimosoideae.
Supporting Information

Figure S1 Sampling sites of nodules, soil and plant material. (PPT)

Figure S2 Section of *M. pudica* and *P. gonoacantha* nodules induced by different *Burkholderia* species. Legend: sections (40 micrometer-deep) of *M. pudica* nodules at 21 days post-inoculation (A to G) induced by *B. sabiae* STM7373 (A), *B. phenoliruptrix* STM7317 (B), or *Burkholderia* sp. 3 STM7296 (C). Sections of *P. gonoacantha* nodules at 60 dpi (D to E) induced by *Burkholderia* sp. 3 STM7296 (D) or *Burkholderia* sp. 1 JPY565 (E). Scale bar: 500 micrometer on all but B (1000 μm). (PPT)

Figure S3 Phylogenies of neutral and symbiotic markers in alpha-rhizobia from the *Piptadenia* group. The phylogeny of neutral markers (A) is based on a 16 S rDNA partition and is built by Neighbor Joining from a distance matrix corrected by the Kimura 2 method, and with 1000 bootstraps replicates. See Figure 2 legend for abbreviations and Table S3 for accession numbers. (PPT)

Figure S4 Comparison of phylogenies of neutral markers in rhizobia from the *Piptadenia* group. Phylogenies of 16 S rDNA (A) and recA (B) were built by Neighbor Joining from a distance matrix corrected by the Kimura 2 method, and with 1000 bootstraps replicates. See Figure 2 legend for abbreviations and Table S3 for accession numbers. (PPT)

Figure S5 Phylogeny of the *nifH* gene in alpha and betarhizobia from the *Piptadenia* group of legumes. The phylogeny was built by ML using a GTR model with 1000 bootstraps replicates. See Figure 2 legend for abbreviations and Table S3 for accession numbers. (PPT)

Table S1 Sampling sites characteristics and date of nodule collection. Legend: \( pH \) range indicated for the region when soil was not harvested for pH determination. ND: Not Determined. (DOC)

Table S2 Primers used for PCR amplification and sequencing. (DOC)

Table S3 Accession numbers of strains from this study and reference strains. Abbreviations: B.: *Burkholderia*, Br.: *Bradyrhizobium*, R.: *Rhizobium*, C.: *Capriavidus*, Pse.: *Pseudomonas*, %: T at the end of the strain name indicates the type strain of a species. (DOC)

Table S4 Nodulation data of representative strains inoculated on *Mimosa pudica* (at 21 days post-inoculation-dpi) and *Piptadenia gonoacantha* (60 dpi). Legend: \( \phi \): Efficiency of symbiosis was estimated as effective or ineffective according to plant development and presence of leghaemoglobin in nodules. %: Letters refer to Figure S2 pictures showing nodules sections. NT: Not Tested, ND: Not Determined. Scale bar on all nodules pictures is 1000 mm. BPL: *Burkholderia phenoliruptrix*, BSA: *B. sabiae*, Bsp1-3: *Burkholderia* sp. 1 to 3, BN: *B. nodosa*, BD: *B. diazotrophica*. (XLSX)

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Author Contributions

Conceived and designed the experiments: CB SMF EG EKJ YP LM. Performed the experiments: CB SMF JMFdS PT MS CC YP LM. Analyzed the data: CB SMF JMFdS EKJ YP LM. Contributed reagents/materials/analysis tools: SMF JMFdS EG PT MS EKJ YP LM. Wrote the paper: CB SMF EKJ YP LM.

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