



Soil characteristics drive contrasting patterns of association between symbiotic rhizobia of endemic and widespread *Mimosa* species in Brazil

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ABSTRACT

Neotropical Beta-rhizobia have a particular affinity to the large legume (Fabaceae) genus *Mimosa* and some of its relatives in the tribe Mimosae of the Caesalpinioideae subfamily. However, little is still known about the ecology of this interaction, especially the relationship between the rhizobia of “widespread” pan-tropical *Mimosa* species like *M. pudica* and the rhizobia that nodulate endemic *Mimosa* species that are very restricted in their habitats. The objective of this study was to examine the microsymbionts of *Mimosa* spp. and other mimosoids in climates ranging from tropical to subtropical, humid to semi-arid, with varied soil characteristics and altitudes, with the aim of testing the hypothesis that widespread species have more cosmopolitan symbiont preferences than endemic ones. Nodules were sampled from >30 *Mimosa* spp. and related taxa in 13 Brazilian states covering all five national regions; many of the species were endemics or biome-restricted, but particular attention was also paid to sample nodules from the widespread species *M. pudica* at all locations. The *Mimosa* symbionts comprised 21 potential 16S rRNA and *recA* groups at the species level, with 17 belonging to the genus *Paraburkholderia*, including four lineages that may represent new species. The remaining genotypes consisted of 14 strains in two lineages of *Cupriavidus* that were mainly isolated from *M. pudica* growing at low altitudes, and a single lineage of *Rhizobium* also from *M. pudica*. In addition, a strain of *Trinickia symbiotica* was isolated from *M. misera*. It is concluded that diverse genotypes of *Paraburkholderia* dominate as symbionts of *Mimosa* in the acidic soils of its main center of radiation in Central Brazil but that *Cupriavidus* and *Rhizobium* comprise a significant minority of symbionts of widespread *Mimosa* spp., especially *M. pudica*, in lowland or disturbed areas with less acidic soils. *Mimosa* symbiont selection is thus driven either by edapho-climatic characteristics for widespread species and/or by co-evolution of the symbiotic partners for endemic species.

1. Introduction

Rhizobia are a polyphyletic group of soil-borne bacteria that form symbioses with leguminous plants (Fabaceae) and the non-legume *Parasponia* (Cannabaceae) (Sprent et al., 2017; Ardley and Sprent,

2021). They induce nodule formation on the roots (and occasionally the stems) of their hosts and supply them with nitrogen-rich compounds obtained through a process called Biological Nitrogen Fixation (BNF) in which atmospheric N₂ (dinitrogen) is converted to NH₃ (ammonia) within the nodules via the enzyme nitrogenase (Remigi et al., 2016;

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Sprent et al., 2017). Currently confirmed rhizobia belong to the *Alphaproteobacteria* and *Betaproteobacteria* bacterial classes and are commonly referred to as Alpha- and Beta-rhizobia, respectively (Gyaneshwar et al., 2011; Peix et al., 2015; Sprent et al., 2017; Ardley and Sprent, 2021). The Alpha-rhizobia consist of *Rhizobium* and *Bradyrhizobium* plus several other genera mainly in the order *Hyphomicrobiales* (formerly *Rhizobiales*). In contrast, the Beta-rhizobia consist of only three genera to date, all in the order *Burkholderiales*: *Paraburkholderia*, *Cupriavidus* and *Trinickia* (Andrews and Andrews, 2017; Estrada-de los Santos et al., 2018; Ardley and Sprent, 2021).

Among the six legume sub-families, only two of them, the Caesalpinioideae and the Papilionoideae, harbor nodulating genera. Nodulation is almost ubiquitous in the Papilionoideae (97 % of genera nodulated), but much rarer in the Caesalpinioideae (33 %), with the notable exception of the Mimosae tribe (93 % nested within it (Sprent, 2009; Sprent et al., 2017; de Faria et al., 2022)). Alpha-rhizobia, such as *Rhizobium* and *Bradyrhizobium*, nodulate several genera across both nodulating sub-families, but nodulation by the Beta-rhizobia is so far reported in only a few legume genera, most particularly with some neotropical members of the Mimosae tribe, but also with a range of endemic papilionoid legumes in the Fynbos biome at the tip of South Africa (Elliott et al., 2007b; Garau et al., 2009; Beukes et al., 2013; Lemaire et al., 2015, 2016; Mavima et al., 2021, 2022). Arguably the most widely reported legume genus with which all three known Beta-rhizobial genera can nodulate is the large mimosoid genus *Mimosa* (Chen et al. 2003a, 2003b, 2005a, 2005b; Barrett and Parker, 2005, 2006; Elliott et al., 2007a; Bontemps et al., 2010; dos Reis Junior et al., 2010; Lammel et al., 2013; Platero et al., 2016; Estrada-de los Santos et al., 2018), but they are also reported to nodulate other mimosoid genera, such as members of the “Piptadenia Group” (Taulé et al., 2012; Bournaud et al., 2013, 2017), and the large neotropical genus *Calliandra* (Silva et al., 2018; Zilli et al., 2021).

Current data suggest that except for the South African Fynbos biome (see earlier), Beta-rhizobia originated in Central and South America and co-evolved with the ancestors of mimosoid genera over the last 50 million years (Bontemps et al., 2010; Gyaneshwar et al., 2011); much more recently (last 500 years), some Beta-rhizobia were dispersed globally by the human-mediated transport of soil and neotropical plants from the Americas to much of the tropical world, such as South East Asia and Australia, mostly with *M. pudica*, *M. diplotricha*, and *M. pigra*, all of which are now established as highly invasive weeds in these environments (Chen et al., 2005b; Parker et al., 2007; Klonowska et al., 2012; Liu et al., 2020; Gehlot et al., 2013; Melkonian et al., 2014). Over the last two decades, most studies have identified the Beta-rhizobial genus *Paraburkholderia* as the predominant nodule-forming bacteria of *Mimosa* spp., mainly occurring in acidic tropical soils in Central and South America, indicating soil pH as a determinant factor for the selection of *Paraburkholderia* as a symbiont (Chen et al., 2005a; Bontemps et al., 2010; dos Reis Junior et al., 2010; Mishra et al., 2012; de Castro Pires et al., 2018). This is supported by studies from other types of soils, e.g., *Mimosa* spp. growing in basic soils in India and Mexico preferred Alpha-rhizobial symbionts (Gehlot et al., 2013; Bontemps et al., 2016), as did those in unusually high pH soils in central Brazil (de Castro Pires et al., 2018), whereas those in high-pH soils in Uruguay nodulated exclusively with neutral-alkaline-preferring *Cupriavidus* spp. (Platero et al., 2016; De Meyer et al., 2015a).

Mimosa in the tribe Mimosae is one of the largest genera in the subfamily Caesalpinioideae with >600 species (LPWG, 2023); almost 60 % of its species (approximately 380) are native or endemic to Brazil (Simon and Proença, 2000; Dutra et al., 2020), with a secondary center of radiation in Mexico (Simon et al., 2011). *Mimosa* species extend from northern Argentina in the south to the southern USA in the north, where they grow in diverse soils and climates, but are particularly endemic in higher altitude locations, e.g., in the Cerrado and Caatinga biomes of Brazil (Simon and Proença, 2000; Simon et al., 2009, 2011; dos Reis Junior et al., 2010). The ecology of the symbiosis between *Mimosa* and

rhizobia is relatively understudied, especially considering the environmental factors affecting the symbiosis with various rhizobial types of such a widely distributed legume genus. However, earlier work by Bontemps et al. (2010) involving a large-scale survey of symbionts from 47 *Mimosa* species native or endemic to the Brazilian Cerrado, Caatinga and Pantanal biomes suggested the co-evolution of native Brazilian *Mimosa* species with nodulating symbionts in the betaproteobacterial genus *Burkholderia*. Bontemps et al. (2010) identified seven Species Complexes (SC) based on the 16S rRNA-*recA* sequences of 143 strains; these same deep-branched SC were congruent with the lineages recovered in the phylogenies of the symbiosis-essential genes, *nodC* and *nifH*, suggesting little horizontal gene transfer (HGT) had occurred. It was thus concluded that their partnership with *Mimosa* was “ancient” rather than inherited recently from Alpha-rhizobia via HGT. Bontemps et al. (2010) also noted that one of the larger SC in their study, SC5, predominated as symbionts of mainly endemic species growing above 1000 m, e.g., in the Chapada dos Veadeiros in the state of Goiás (GO) and the Chapada Diamantina in the state of Bahia (BA), both locations wherein *Mimosa* shows high levels of diversification and endemism (Simon and Proença, 2000; Simon et al., 2011), and hence further concluded that elevation also played a role (directly or indirectly via the host) in the selection of symbionts by *Mimosa* species endemic to highland regions.

Since it was published, almost all the symbionts in the study of Bontemps et al. (2010) have been largely moved to a new genus *Paraburkholderia* (Sawana et al., 2014), with a small number of strains being incorporated into another new genus, *Trinickia* (Estrada-de los Santos et al., 2018). In terms of the current taxonomy of *Burkholderia* sensu lato, the *Burkholderia* SCs identified by Bontemps et al. (2010) contain the following species: SC1 (*Trinickia symbiotica*), SC2 (*P. sabiae*, *P. caribensis*, *P. phymatum*), SC3 (*P. diazotrophica*, *P. franconis*), SC4 (*P. mimosarum*), SC5 (*P. nodosa* and *P. guartelaensis*), SC6 (*P. tuberum* sv. *mimosae*, now moved to *P. atlantica* and *P. youngii*), and SC7 (*P. phenoliruptrix*). It should be noted, however, that all the SCs also contained several strains that are not yet allocated to a formally described species.

The overall objective of this study was to test the hypothesis that widespread *Mimosa* species have more cosmopolitan symbiont preferences than endemic ones in the main center of this legume genus, Brazil. Towards this aim, the occurrence of nodulating *Mimosa* spp. was surveyed across their native range in several states of Brazil, encompassing various biomes. The range of rhizobial genotypes is almost certainly at least partly related to soil characteristics (Elliott et al., 2009; Lammel et al., 2013; Bontemps et al., 2016; de Castro Pires et al., 2018; Soares Neto et al., 2022), given the solid biogeographical relationships between particular *Mimosa*-nodulating symbionts and their native environments. However, it is difficult to establish which factors other than co-evolution are involved in the selection of symbionts by many *Mimosa* spp. because the plants are either highly endemic or biome-restricted (Simon and Proença, 2000; Simon et al., 2011). Therefore, in order to mitigate the effects of co-evolution, the present study had a particular emphasis on the symbionts of widespread/invasive species that colonize many types of soils and which are “promiscuous” in terms of their rhizobial preferences, e.g., *M. pudica* (see aforementioned references). The symbionts from these were compared to type strains, but also to reference strains from previous studies of the symbionts of *Mimosa* (and other mimosoids, such as *Calliandra* and members of the “Piptadenia group”) in South America and the wider neotropics (Barrett and Parker, 2005, 2006; Bontemps et al., 2010; Taulé et al., 2012; Bournaud et al., 2013, 2017; Silva et al., 2018), as well as with strains isolated from *Mimosa* growing in pantropical invasive environments.

2. Material and methods

2.1. Sampling of plants, nodules and soil

Specimens of *Mimosa* species, including aerial parts (for identification), root nodules, and soil samples were collected in various sites and

altitudes in the states representing all five regions of Brazil: Amazonas (AM), Roraima (RR) (Northern region), Bahia (BA), Ceará (CE), Maranhão (MA), (Northeast region), Distrito Federal (DF), Goiás (GO), Mato Grosso (MT) (Central-West region), Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ), Sao Paulo (SP) (Southeast region) and Santa Catarina (SC) (Southern region) from 2009 to 2018 (Table S1). For each survey, branches with leaves were collected (including reproductive structures, when available), and a voucher for each specimen was deposited and then later identified at the herbarium of Embrapa-CENARGEN. In parallel, plants were excavated to access their roots, and nodules (when present) were sampled from the roots visibly connected to the parent plant. The nodules were transferred to sterilized plastic tubes (2 ml) containing silica gel. Rhizosphere soil samples were also collected from around the nodulated roots down to a depth of 20 cm, placed in plastic bags, and then dried at room temperature until analysis; some soils were also used for “trapping” rhizobia from *M. pudica* seedlings (see below for details about seed germination).

2.2. Isolation and screening of bacteria from the nodules

Nodules were washed with sterile deionized water, then surface disinfected by immersion in 95 % ethanol for 30 s, followed by immersion in 3 % sodium hypochlorite for 5 min, and four washes in sterile distilled water. Nodules were then crushed and streaked onto Medium 79 (Fred and Waksman, 1928), otherwise known as yeast mannitol agar or YMA (Vincent, 1970), and incubated at 28 °C from 2 to 7 days. Single colonies were transferred to new plates and were then grown in liquid YM media for 2 to 5 days (until visual growth of the bacteria was observed). From each isolate, a 1 ml aliquot was mixed with 1 ml sterile glycerol and frozen at –80 °C, while another aliquot was used to extract DNA, according to Bontemps et al. (2010) with some modifications.

2.3. Sequencing of the 16S rRNA, recA, nodC and nifH genes

Novel bacterial strains isolated during the present study (BR- and EG-strains, Table S1) were studied by Sanger sequencing of the 16S rRNA gene as well as the housekeeping gene *recA* (recombinase A) and the *nifH* (nitrogenase reductase) and *nodC* (N-acetyl glucosaminyl transferase) symbiosis-essential genes. DNA was extracted from pelleted bacterial cells grown in liquid YM medium using the Wizard Genomic DNA Purification System (Promega). Genes of interest were amplified from template DNA by PCR using GoTaq polymerase (Promega) according to the recommendations of the supplier. The 16S rRNA region was amplified using primers 27F and 1492R (Weisburg et al., 1991), with the specific cycling conditions as specified in previous studies (Bontemps et al., 2010, 2016). To amplify the *recA* gene, different conditions were used for each bacterial genus, as previously described, using the primers Burk *recA* F and Burk *recA* R for *Paraburkholderia* described in Mishra et al. (2012), Cupri *recA* F and Cupri *recA* R for *Cupriavidus* (Andrus et al., 2012) and 41F and 640R for *Rhizobium* (Vinuesa et al., 2005). The *nodC* gene was amplified using previously described conditions and primers: nodCBurk2 F and nodCBurk2 R for *Paraburkholderia* (Bontemps et al., 2010) and nodCCtai468 F and nodCCtai1231 R for *Cupriavidus* and 540 F and 1160 R for *Rhizobium* (Mishra et al., 2012). Concerning the *nifH* gene, primers used were Burk *nifH* F and Burk *nifH* R for *Paraburkholderia* (Chen et al., 2006), nhcf3 and nhcr4 for *Cupriavidus* (Andam et al., 2007) and *nifH* F and *nifH* R for *Rhizobium* (Chen et al., 2005a, 2005b); for DNA of challenging strains from any of the three genera, the *nifH* primers PolF and PolR (Poly et al., 2001) were also successfully used.

Amplicons were visually inspected by gel electrophoresis and prepared for sequencing by treatment with alkaline phosphatase FastAP and exonuclease ExoI (Thermo Scientific) for 15 min at 37 °C followed by deactivation for 20 min at 85 °C. Sequencing reactions were performed bi-directionally using the primers also used for PCR and the Big Dye kit (Applied Biosystem). Purified reaction products were analyzed

using a ABI3500 capillary sequencer (Applied Biosystem). Sequence files were quality-processed and assembled into contigs using BioNumerics version 7.1 (Applied Mathematics, Belgium). Sequences were deposited and received GenBank accession numbers (Table S1).

2.4. Phylogenetic analysis using the 16S rRNA, recA, nodC and nifH genes

Alpha- (*Rhizobium*) and Beta- (*Paraburkholderia* and *Cupriavidus*) *proteobacteria* sequences were analyzed separately to prevent long branches and loss of phylogenetic resolution. Multiple sequence alignments of datasets consisting of previously published sequences, the sequences of type strains representative of described species and the sequences obtained in this study were generated in MEGA 7 (Kumar et al., 2016) using MUSCLE (Edgar, 2004). Type strain information was obtained from the List of Prokaryotic names with Standing in Nomenclature (LPSN; <https://lpsn.dsmz.de>; Parte et al., 2020). The 16S rRNA and *recA* gene datasets were concatenated after aligning and trimming the individual alignments in MEGA 7. Visually inspected multiple alignments were exported in fasta format, and maximum-likelihood phylogenetic trees were constructed using IQ-tree Webserver (Trifinopoulos et al., 2016) with ultrafast bootstrap analysis (Minh et al., 2013) and 1000 iterations. For each alignment, the most appropriate substitution model was selected using the IQ-tree Webserver model selection tool (ModelFinder; Kalyaanamoorthy et al., 2017). Final drawings of phylogenetic trees were generated using iTOL (Interactive Tree of Life, Letunic and Bork, 2021).

2.5. Genome sequencing and average nucleotide identity (ANI) analysis

The genomes for reference strains/species were obtained from the National Center for Biotechnology Information (NCBI; Benson et al., 2017, Table S1). The genomes of 46 strains were sequenced during this study (Table S1). The sequencing was performed by MicrobesNG (University of Birmingham, UK) as per Estrada-de los Santos et al. (2018), after which genomes were assembled using SPAdes v3.7 (Bankevich et al., 2012; Nurk et al., 2013). These whole genome assemblies have been deposited in the NCBI database under the accessions listed in Table S1.

These 46 newly-genome-sequenced strains were selected to represent most of the distinct lineages observed within the phylogenies. The genomes of phylogenetically closely-related type strains of *Paraburkholderia*, *Cupriavidus* and *Trinickia* were also selected for comparison. For all pairs of genome sequences, average nucleotide identity (ANI) values were determined using FastANI with default settings (k-mer size = 16; fragment size = 3000) (Jain et al., 2018).

2.6. Nodulation tests

All the BR, EG and JHI isolates were tested for their nodulation capability by inoculation of liquid cultures onto *M. pudica* seedlings under sterile conditions. The JPY strains were all confirmed to nodulate their cognate hosts and/or *M. pudica* by Bontemps et al. (2010). For the BR, EG and JHI strains, germinated seedlings were obtained by breaking seed dormancy (scarification) with concentrated sulfuric acid for 10 min, followed by surface disinfection (sequential immersion in 97 % ethanol for 30 s, 5 min in a 30 % hydrogen peroxide solution and finally five washes in sterile water). The seeds were then germinated in Petri dishes with 1 % water agar at 28 °C for 48 h in the dark. Germinated seeds were transferred to sterilized glass tubes (200 × 30 mm) containing vermiculite as substrate moistened with a nitrogen-free nutrient solution with pH 6.8 (Norris and t Mannetje, 1964). Each plant was then inoculated with 1 ml of YM broth containing the bacterium of interest in the late logarithmic growth phase; *Cupriavidus taiwanensis* LMG19424^T was used as a positive control (Elliott et al., 2007a) and sterile culture medium as a negative control. Plants ($n = 3$ per treatment) were grown

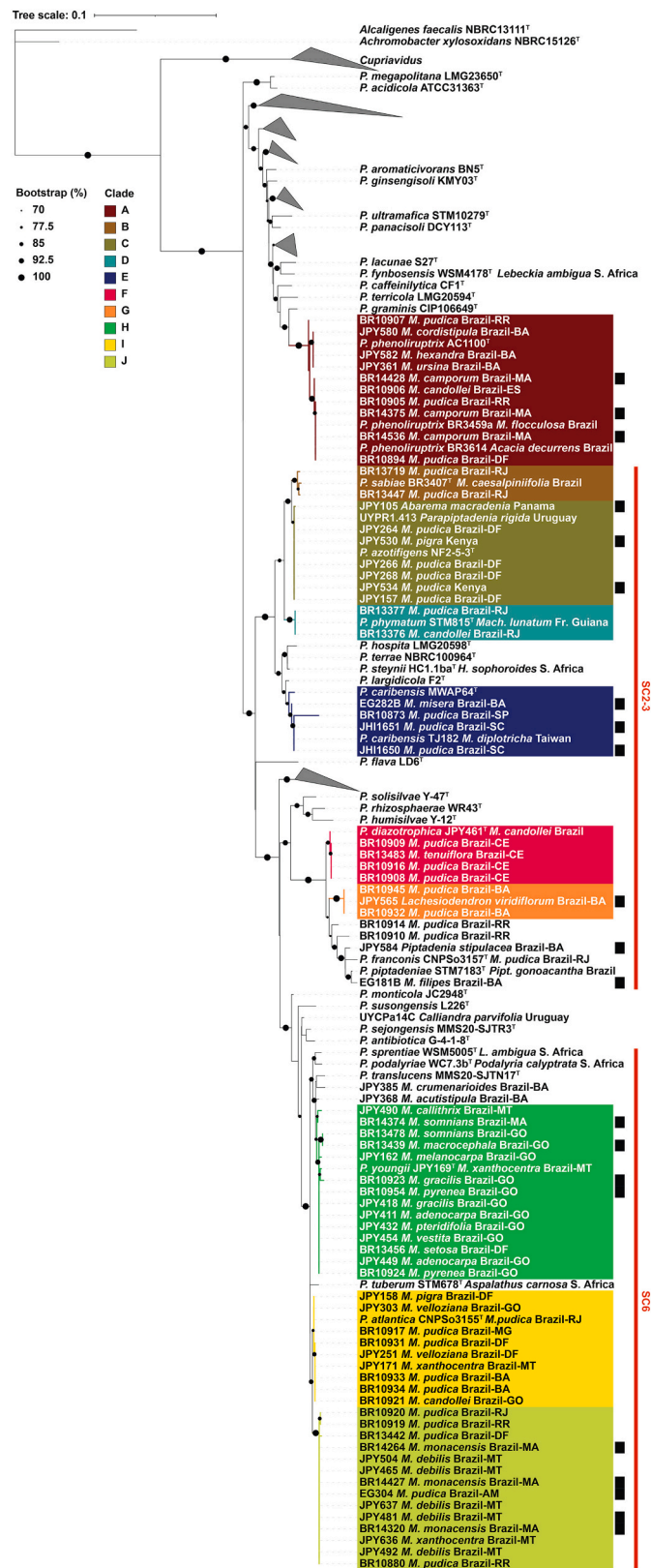


Fig. 1. 16S rRNA-*recA* *Paraburkholderia*. Phylogenetic trees of strains from the genus *Paraburkholderia* and emphasizing clades A–J (a) and K–Q (b). IQ-TREE was used to reconstruct the phylogeny of 260 concatenated and aligned *Paraburkholderia* and *Cupriavidus* 16S rRNA and *recA* sequences. There was a total of 1189 nucleotide positions and 252 parsimony-informative sites and ultrafast bootstrap analysis (1000 iterations) and the ‘TIM2 + F + I + G4’ best-fit model according to Bayesian Information Criterion were applied. The phylogenetic relationships of the genus *Paraburkholderia* are shown and the strains pertaining to the genus *Cupriavidus* were collapsed (gray triangle). Some clades of non-symbiotic *Paraburkholderia* strains were also collapsed to reduce the size of the tree. Phylogenetic clades A to Q are color-coded. Plant host species and geographic origin of relevant strains are indicated. For Brazilian strains and when available, the state of origin is also indicated using two-letter abbreviations (e.g., Brazil-DF). *Alcaligenes faecalis* NBRC13111^T and *Achromobacter xylosoxidans* NBRC15126^T were used as outgroup.

Paraburkholderia species clusters (SC), as described in Bontemps et al. (2010), are indicated by red vertical lines to the right of the phylogram. Black squares indicate strains from the present study with complete genome sequences. Abbreviations: S. Africa = South Africa, Fr. Guiana = French Guiana, N. Caledonia = New Caledonia, *Pipt. gonoacantha* = *Piptadenia gonoacantha*, *Mach. Lunatum* = *Machaerium lunatum*, *H. sophoroides* = *Hypocalyptus sophoroides*, *L. ambigua* = *Lebeckia ambigua*. Details in Table S1.

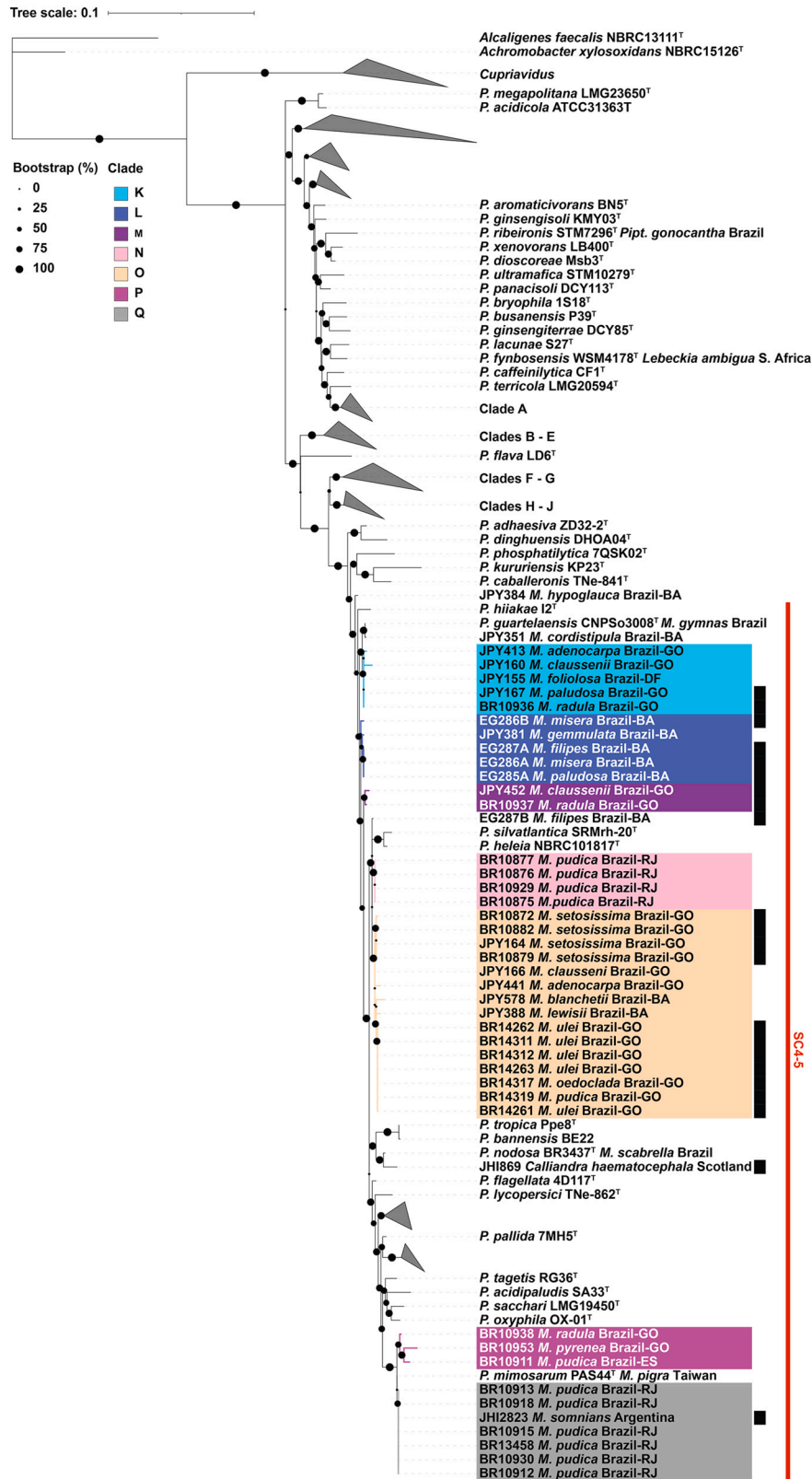


Fig. 1. (continued).

in a growth room for 45 days at 28 °C and with a 16 h light photoperiod. Bacteria were re-isolated from any nodules formed, and their identity confirmed by genomic profile comparison to the inoculated strains by BOX-PCR (Koeuth et al., 1995).

2.7. Soil analysis

Soil analysis was conducted in the Agricultural Chemistry Laboratory of Embrapa Agrobiologia using procedures described in Silva (2009). Briefly, the soil was first air dried and sieved to 2 mm, removing plant material. The pH of the sampled soil in water was then measured using a pH-electrode, while Ca²⁺, Mg²⁺, and Al³⁺ were extracted using 1 M KCl and analyzed by atomic absorption spectroscopy. Phosphorus (P) and potassium (K) were extracted using the Mehlich I solution (Silva, 2009), and P was analyzed by UV-spectrophotometry, and K by atomic absorption.

2.8. Statistical analyses

Analyses were performed for those rhizobial isolates from widespread *Mimosa* species for which soil samples were also taken (mainly *M. pudica*; Table S1), in order to identify correlations between biological (occurrence of bacterial genera *Cupriavidus*, *Paraburkholderia* and *Rhizobium*) and soil chemistry attributes (pH, Al³⁺, Ca, Mg, P and K), a multivariate redundancy analysis (RDA) was performed in R (R Core Team, 2021). The Monte Carlo permutation test assessed the significance of relationships between biological and chemical data matrices, considering 5 % as the probability of significance. The soil chemistry variables were centralized and standardized. A heatmap was constructed to demonstrate the relationship between 16S rRNA-*recA* groups and host-plant species, using the pheatmap package (version 1.0.12) in R and rows (plant species) were clustered using hclust based on Euclidean distance.

3. Results

3.1. Nodulation of *Mimosa* species in thirteen states of Brazil and the identification of their symbiotic bacteria

Nodules from 30 *Mimosa* and related taxa were sampled in 13 Brazilian states covering all five national regions, with *M. pudica* sampled from all states except for MA (Table S1, Fig. S1). Of these, *M. macrocephala* and *M. oedoclada* from the Cerrado biome in GO were new reports of nodulation, as was *M. carolina* from MA. These, together with data from the recent studies of Paulitsch et al. (2019a, 2019b) and Klepa et al. (2021) raises the total of known nodulated *Mimosa* species to 138 when they are added to those published in earlier studies (Sprenst, 2009; Lammel et al., 2013; Lammel et al., 2015; Bontemps et al., 2016; Platero et al., 2016; de Castro Pires et al., 2018).

We obtained 112 potentially symbiotic isolates from the nodules of these *Mimosa* spp., including 65 isolates from *M. pudica* nodules (Table S1). Phylogenies of these new isolates (all prefixed with “BR” and “EG”) were then constructed using their 16S rRNA sequences, to which were added sequences from 34 strains (all prefixed with “JPY”) described in a previous study of *Mimosa* symbionts from central Brazil (Bontemps et al., 2010) together with reference/type strains plus 15 mostly unpublished strains from the authors' strain collections (all prefixed with “JHI” or “JPY”) (Table S1). Based on 16S rRNA gene partial sequences most isolates were Beta-rhizobia, with the majority belonging

to *Paraburkholderia*, plus a few belonging to *Cupriavidus* (and some Alpha-rhizobia i.e. *Rhizobium*), as well as a single isolate of *Trinickia* (Fig. S1; Table S1). However, all 34 JPY strains were *Paraburkholderia*, confirming their former affiliation to the genus *Burkholderia*, the generic name under which they were originally published (Bontemps et al., 2010).

The *recA* sequences of 100 of the BR isolates were also obtained (Fig. S2); these were combined with those of the JPY, EG and JHI strains (Table S1) to construct a 16S rRNA-*recA* phylogeny (Fig. 1), which allows for greater resolution in terms of which Beta-rhizobial species the BR, EG, JHI and JPY isolates belong to. Although many of the JPY strains from the study of Bontemps et al. (2010) have already been allocated to various species, such as *P. mimosarum* (Chen et al., 2006), *P. nodosa* (Chen et al., 2007), *P. sabiae* (Chen et al., 2008), *Trinickia* (syn. *Burkholderia*) *symbiotica* (Sheu et al., 2012), *P. diazotrophica* (Sheu et al., 2013), *P. atlantica* and *P. youngii* (Mavima et al., 2021), the 34 JPY strains re-examined here were originally placed in six of the seven 16S rRNA-*recA* SC but with no apparent affiliation to any described species at the time that they were first published (Bontemps et al., 2010). Here, we more firmly establish the identity of these so far unclassified JPY *Paraburkholderia* strains and determine the identities of the new BR, EG and JHI strains *vis-à-vis* current revisions in the taxonomy of Beta-rhizobia. A concatenated 16S rRNA-*recA* phylogeny reveals that the combined BR-EG-JHI-JPY isolates belong to 21 genotypes of rhizobia comprising 17 *Paraburkholderia* (Fig. 1, Table S1), two *Cupriavidus* genotypes (Fig. 2, Table S1), a single genotype of *Rhizobium*, and a single strain of *Trinickia*.

Nine of the rhizobial genotypes clustered with type strains from validly described species of *Paraburkholderia*, such as: *P. phenoliruptrix* (Group A *Mimosa*-associated strains from BA, DF, ES, MA and RR plus the *Acacia decurrens* symbiont, BR3614); *P. sabiae* (Group B *Mimosa* symbionts from RJ); *P. azotifigens* (Group C *Mimosa*-associated strains from DF and Kenya, plus *Parapiptadenia* and *Jupunba/Abarema* symbionts from Uruguay and Panama, respectively); *P. phymatum* (Group D *Mimosa* symbionts from RJ); *P. caribensis* (Group E *Mimosa*-associated strains from BA, SC and SP); *P. diazotrophica* (Group F *Mimosa*-associated strains from CE); *P. atlantica* and *P. youngii* (Groups H and I, respectively); *Mimosa*-associated strains mainly from north east and central Brazil: BA, DF, GO, MA, MT, but also from MG and RJ), and *P. mimosarum* (Group Q *M. pudica* symbionts from RJ and Argentina).

The remaining groups in the *Paraburkholderia* 16S rRNA-*recA* phylogeny did not contain any type strains. These were: Group G (closest to *P. diazotrophica*) comprising *Mimosa* symbionts from BA and JPY565 from the “Piptadenia group” species *Lachesiodendron viridiflorum* (syn. *Piptadenia viridiflora*) in BA; Group J (closest to *P. atlantica*/*P. youngii*) comprising *Mimosa* symbionts from AM, DF, MA, MT, RJ and RR; the related Groups K, L and M (closest to *P. guartelaensis*) comprising *Mimosa*-associated strains from BA, DF and GO; the related Groups N and O (closest to the non-symbionts *P. silvatlantica* and *P. heleia*) comprising *Mimosa* symbionts from BA, GO and RJ; and Group P (closest to *P. mimosarum*) comprising *Mimosa*-associated strains from GO and ES. There were also eleven single strain lineages based upon the approach of recognizing Groups on the basis of the smallest recovered, supported monophyletic lineage: JPY351 and JPY384 from endemic *Mimosa* spp. in BA that were all close to *P. guartelaensis* and Groups K, L and M; EG287B isolated from nodules on *M. filipes* in BA was closest to Groups N and O; JHI869 isolated from nodules on *Calliandra haematocephala* at the Royal Botanical Gardens Edinburgh, UK (this study), which was close to *P. nodosa*; UYCPa14C isolated from *Calliandra*

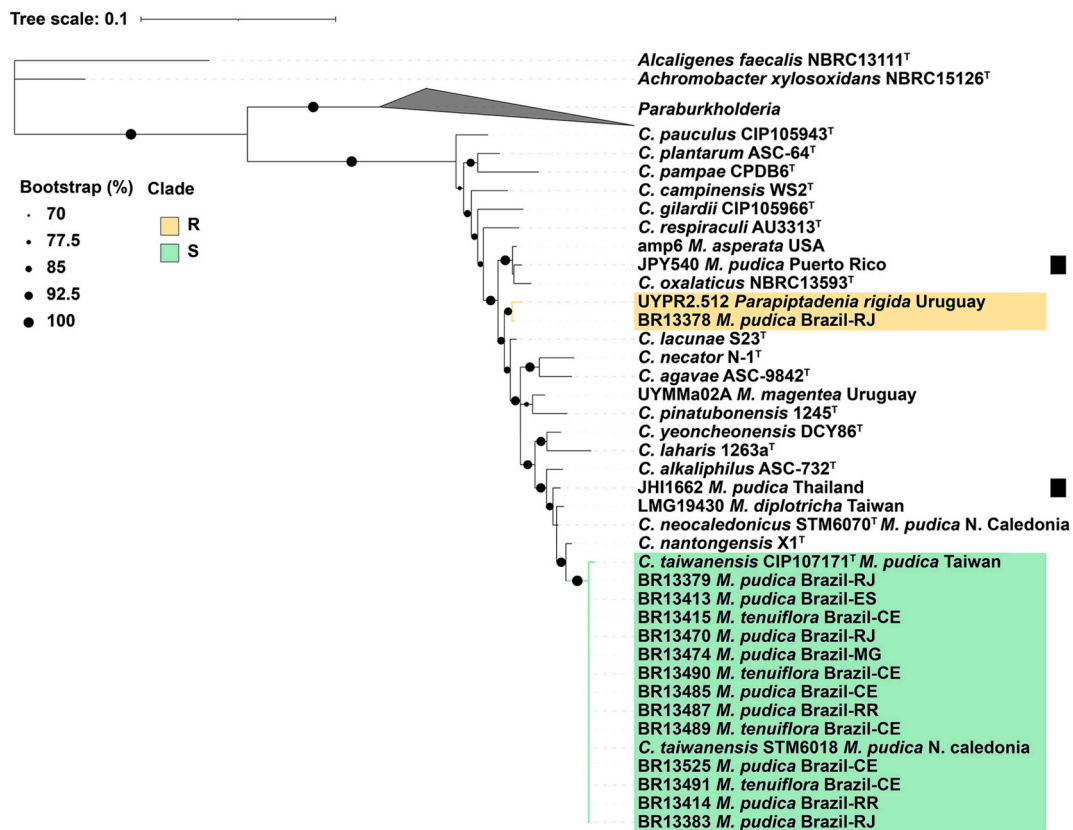


Fig. 2. 16S rRNA-*recA* *Cupriavidus*. Phylogenetic tree showing *Cupriavidus* clades R and S. The tree was constructed as described in the legend of Fig. 1, but in this case, the genus *Paraburkholderia* was collapsed to facilitate visualization of phylogenetic relationships within *Cupriavidus*. Black squares indicate strains from the present study with complete genome sequences. Abbreviations: S. Africa = South Africa, Fr. Guiana = French Guiana, N. Caledonia = New Caledonia, *Pipt. gonoacantha* = *Piptadenia gonoacantha*, *Mach. Lunatum* = *Machaerium lunatum*, *H. sophoroides* = *Hypocalyptus sophoroides*, *L. ambigua* = *Lebeckia ambigua*. Details in Table S1.

parvifolia in Uruguay (Langleib et al., 2019), which was closest to the non-symbionts *P. antibiotica* and *P. sejongensis*; JPY584 isolated from *Piptadenia stipulacea* in BA plus EG181B from *M. filipes* in BA and BR10910 and BR10914 from *M. pudica* in RR which were all closest to *P. franconis*, *P. diazotrophica*, *P. piptadeniae* and to **Group G**; finally, JPY368 and JPY385 from *Mimosa* spp. in BA clustered together, but were divergent from *P. youngii*.

The 16S rRNA-*recA* phylogeny suggested at least six potential new species among the BR-JPY *Paraburkholderia* strains. To further investigate this, we sequenced the whole genomes of representative strains from **Groups G, J, K, L, M and O** (strains from **Groups N and P** were unavailable for genome sequencing at this time) and performed an ANI analysis on them compared with the closest type strains in the 16S rRNA-*recA* phylogeny. This analysis (Table S2a) demonstrated that the **Group G** strain JPY565 from *L. viridiflorum* (Bournaud et al., 2013) shared low similarities (<94 % ANI) with all type strains in the *P. diazotrophica*-*P. franconis*-*P. piptadeniae* Species Complex or SC (equivalent to SC2–3 of Bontemps et al., 2010), the **Group J** strain JPY481 isolated from *M. debilis* in the Pantanal wetlands of MT was very closely related to strains from MA and AM (97–99 % ANI); JPY451 plus the three strains from *M. monacensis* in MA shared slightly >95 % ANI with *P. atlantica* and *P. youngii*, while the *M. pudica* strain from AM, EG304, was

dissimilar to either of these species having an ANI value of just over 93 % with both. There were several genome-sequenced strains from various states in the *P. mimosarum*-*P. nodosa*-*P. guartelaensis* SC (SC4–5 of Bontemps et al., 2010). Notable examples of these were, JPY167 and BR10936 (**Group K**, *M. paludosa* and *M. radula*), and JPY164 (**Group O**, *M. setosissima*, GO) plus 10 strains in this group from endemic *Mimosa* species in GO, which all shared slightly >94 % ANI with the type strains of *P. nodosa* and *P. guartelaensis*, but are closely related to the **Group K** strains, e.g., with JPY164 and JPY167 sharing >98 % ANI. In the same SC, five strains from *M. filipes*, *M. misera* and *M. paludosa* in BA (**Group L** and a singleton; EG287B), plus JPY452 and BR10937 (**Group M**, *M. clausenii* and *M. radula*, GO), shared >96 % ANI with each other, but <95 % ANI with *P. nodosa* and *P. guartelaensis*. Strain JHI869 isolated from *Calliandra haematocephala* nodules at the Royal Botanic Gardens Edinburgh (RBGE), Edinburgh, UK, was a singleton in the 16S rRNA-*recA* phylogeny, and its genome shared only 92 % ANI with the closest type strain, *P. nodosa*, while the genome of JHI2823, which was isolated from the widespread neotropical species *M. somnians* sampled in northern Argentina, shared >98 % ANI with *P. mimosarum*. Finally, the genome of JPY584 from *Piptadenia stipulacea* in BA shared <94 % ANI with *P. diazotrophica*, *P. franconis* and *P. piptadeniae*, while that of EG181B from *M. filipes* in BA shared slightly >94 % ANI with

P. piptadeniae. The ANI results thus suggest that **Group G**, **Group J**, **Groups K + O**, and **Groups L + M**, may represent four new *Paraburkholderia* species.

The 16S rRNA-*recA* phylogeny also suggested that the nodulating strains in **Group C** belonged to *P. azotifigens*. This species was isolated from paddy field soil in South Korea (Choi and Im, 2018) and was previously considered to be a free-living diazotroph with no capacity to nodulate legumes. This was tested by comparing the genomes of the **Group C** strains JPY530 and JPY534 (isolated from *M. pigra* and *M. pudica*, respectively, in Kenya), JPY105 (AMAC11–3) (isolated from the mimosoid tribe Ingeae species *Jupunba macradenia* in Panama; Barrett and Parker, 2005), and UYPR1.413 (isolated from the Piptadenia group species *Parapiptadenia rigida* in Uruguay; Taulé et al., 2012; De Meyer et al., 2015b) with that of the type strain of *P. azotifigens*, NF5-2-3^T. This comparison showed these genomes shared >98 % ANI (Table S2a).

Most of the 14 *Cupriavidus* strains isolated in this study (prefixed BR) were from nodules on the widespread species *M. pudica* and *M. tenuiflora* sampled in CE, ES, MG, RJ and RR, and all but one was placed in **Group S** with *C. taiwanensis* in the 16S rRNA-*recA* phylogeny (Fig. 2). The exception was BR13378 from *M. pudica* in RJ, which was placed in **Group R** together with UYPR2.512 from *Parapiptadenia rigida* nodules in Uruguay (Taulé et al., 2012; De Meyer et al., 2015a); the type strain of *C. lacunae* was closest to this pair of strains. Other divergent strains were UYMMa02A from *M. magentea* in Uruguay (Platero et al., 2016; Iriarte et al., 2016), which was closest to *C. pinatubonensis*, JPY540 and amp6 from *M. pudica* in Puerto Rico and from *M. asperata* in Texas, respectively, which were closest to each other and to *C. oxalaticus*, and JH11662 from *M. pudica* nodules sampled in northern Thailand, which was related to “*C. neocaledonicus*”, a symbiont of *M. pudica* in New Caledonia (Klonowska et al., 2020). The genomes of both JPY540 and JH11662 were sequenced for this study, so their relatedness to these type strains could be tested using ANI (Table S2b); this revealed that JPY540 shared <91 % ANI with *C. oxalaticus*, while JH11662 shared <93 % ANI with *C. neocaledonicus*.

The final multi-strain genotype (**Group T**) was *Rhizobium* (Fig. S3). These fifteen strains were all isolated from *M. pudica* in CE, DF, MG, RJ and RR. The ten DF strains were previously described as belonging to the new species *R. altiplani* (Baraúna et al., 2016). Of the remaining five strains, BR13443 was isolated from seedlings grown in soil from MG, and it clustered with *R. pisi* and *R. etli*, while strains BR10892 (CE) and BR13410 (RJ) were grouped with *R. tropici* and *R. multihospitium*; the two strains from RR, BR10891 and BR10904, were close to *R. mesoamericanum*.

A single strain of *Trinickia*, EG282A, isolated from *M. misera* in BA was shown by ANI to be >97 % similar to the *T. symbiotica* type strain, JPY345^T, as well as two other *T. symbiotica* strains, JPY347, and JPY581 (Table S2c), indicating that it most likely belongs to this species.

3.2. Symbiosis-essential genes

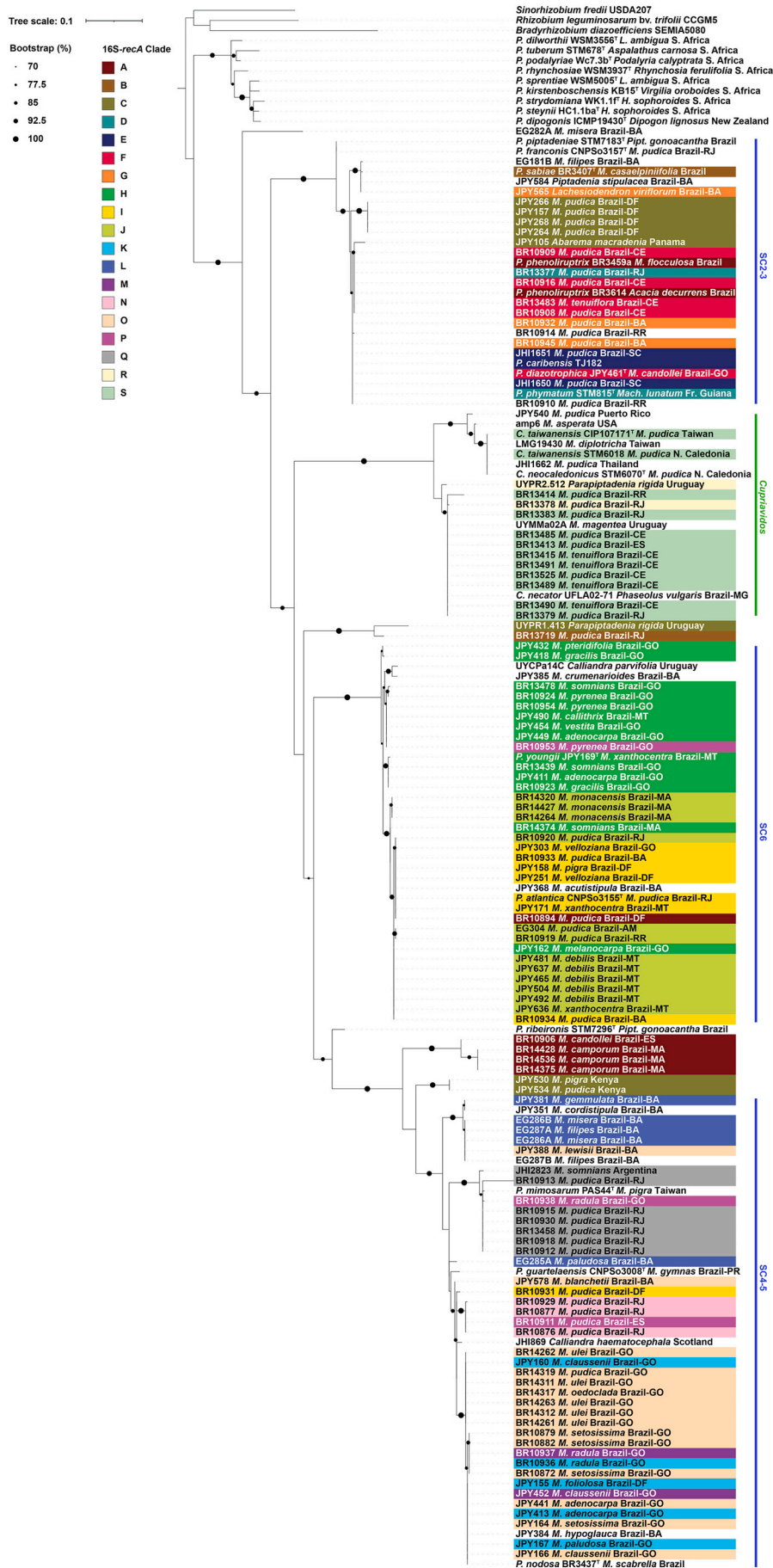
Two symbiosis-related genes, *nodC* and *nifH*, were examined in this study (Figs. 3, 4). For the *Paraburkholderia* strains, both genes were generally congruent with each other and with the 16S rRNA-*recA* phylogeny, forming groups that coincided with each of the three largest SC identified by Bontemps et al. (2010) for *Mimosa*-nodulating (*Para*)*burkholderia* i.e., SC2–3 comprising *P. phymatum*-*P. sabiae*-*P. diazotrophica*-

P. caribensis-*P. azotifigens* (**Groups B–G** in the present study), SC4–5 comprising *P. mimosarum*-*P. nodosa*-*P. guartelaensis* (**Groups K–P** in the present study), and SC6 comprising the ex-*P. tuberosum* sv. *mimosae* species *P. atlantica* and *P. youngii* (**Groups H–J** in the present study).

Some strains showed incongruity in that they were placed in a *nodC* **Group** that was different from their 16S rRNA-*recA* SC/**Group**. These included a *P. mimosarum*-like strain from the GO endemic *M. pyreneae*, BR10953 (16S rRNA-*recA* SC4–5/**Group P**), that was nested in a *nodC* lineage that corresponds to 16S rRNA-*recA* SC6/**Groups H–J** (*P. atlantica*-*P. youngii*); and the *P. sabiae*-like strain BR13719 (16S rRNA-*recA* SC2–3/**Group B**) isolated from *M. pudica* in RJ whose *nodC* sequence was distinct from all the SC/**Groups**, being most closely related to UYPR1.413 isolated from *Parapiptadenia rigida* in Uruguay (Taulé et al., 2012). Moreover, the *P. azotifigens*-like strains JPY530 and JPY534 (16S rRNA-*recA* SC2–3/**Group C**) from Kenya, were divergent from all the SC/**Groups** in their *nodC* phylogenies, but loosely affiliated with SC4–5, while the *nodC* sequence of the *P. atlantica* strain BR10931 from *M. pudica* in DF (16S rRNA-*recA* SC6/**Group I**) grouped closer to *P. guartelaensis* (16S rRNA-*recA* SC4–5).

The *Paraburkholderia nifH* phylogeny (Fig. 4) gave a similar picture to the *nodC* phylogeny (Fig. 3), but there were some minor differences, and particular mention should be made about the symbiotic strains in **Group A**. These are closely related or belonging to *P. phenoliruptrix*, which was originally described as a non-symbiont, and is closer to non-symbionts in the 16S rRNA-*recA* phylogeny than any of the other *Mimosa*-nodulating *Paraburkholderia* groups (Fig. 1). Two **Group A** strains, BR3614 from the introduced Australian legume *Acacia decurrens*, and BR3459b from *M. flocculosa* in southern Brazil, possessed *nodC* and *nifH* genes that placed them in SC2–3 (Figs. 3, 4). This contrasts with other strains in this group. For example, the *nodC* of strain BR10906, which was isolated from *M. candollei* in ES, placed it in a group close to SC4–5 with three *M. camporum* strains from MA, while the *nodC* of the *M. pudica* symbiont BR10894 from DF was in SC6. Furthermore, in the *nifH* phylogeny these five Brazilian *P. phenoliruptrix* strains aligned with a pair of Kenyan *P. azotifigens* strains, JPY530 and JPY534, forming two diverged *nifH* lineages, neither of which could be placed in *nifH* lineages that correspond to any of the 16S rRNA-*recA* SC/**Groups**. Strain BR10894 was, therefore, incongruent across all three phylogenies (16S rRNA-*recA*, *nodC* and *nifH*).

The *nodC* and *nifH* sequences of all but one of the BR *Cupriavidus* strains were grouped with a *Mimosa*-nodulating *C. necator* strain (UFLA02-71) isolated from nodules of common bean (*Phaseolus vulgaris*) and *Leucaena leucocephala* grown in soil sampled from a pasture in MG, Brazil (da Silva et al., 2012). It was a substantial incongruity given that all the BR strains (except for the *C. lacunae*-like BR13378) were placed in *C. taiwanensis* according to the 16S rRNA-*recA* phylogeny, including BR13474 (Fig. 2) that was isolated from *M. pudica* growing in the same field from which the soil that was used to trap UFLA02-71 was sampled (James and de Faria, unpublished). Strain UYMMa02A from *M. magentea* in Uruguay and the *C. lacunae*-like BR13378 were also clustered in this *nodC/nifH* group, but UYPR2.512 from *P. rigida* (Taulé et al., 2012; De Meyer et al., 2015a) was slightly divergent from it. The *nodC* and *nifH* sequences of the *C. oxalaticus*-like strains, JPY540 and amp6, isolated, respectively, from *M. pudica* in Puerto Rico (this study) and *M. asperata* in Texas (Andam et al., 2007; De Meyer et al., 2015c), were both slightly divergent from *C. taiwanensis*. The only *nifH*-specific incongruity in the *Cupriavidus* collection was that of two *C. taiwanensis* strains isolated from



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Fig. 3. *nodC* Phylogenetic tree built using IQ-TREE, with ultrafast bootstrap analysis (1000 iterations) and the 'HKY + F + I + G4' best-fit model according to Bayesian Information Criterion. The alignment contained 155 sequences, 318 nucleotide positions and 157 parsimony-informative sites of *nodC* genes from strains of genera *Paraburkholderia*, *Cupriavidus* and *Trinickia*. Plant host species and geographic origin of relevant strains are indicated. For Brazilian strains and when available, the state of origin is also indicated using two-letter abbreviations (e.g., Brazil-DF). Strains are colour-coded with respect to the 16S-*recA* clades from Figs. 1 and 2. *Paraburkholderia* species clusters (SC), as described in Bontemps et al. (2010), as well as *Cupriavidus* strains are indicated by blue and green vertical lines to the right of the phylogram. Abbreviations: S. Africa = South Africa, Fr. Guiana = French Guiana, N. Caledonia = New Caledonia, *Pipt. gonoacantha* = *Piptadenia gonoacantha*, *Mach. Lunatum* = *Machaerium lunatum*, *H. sophoroides* = *Hypocalyptus sophoroides*, *L. ambigua* = *Lebeckia ambigua*. Details in Table S1.

M. pudica in RR (BR13414, BR13487); these were paired together and were substantially divergent from all the other strains.

3.3. Effects of host species, geography and edaphic factors on rhizobial genotype

There were some potential relationships evident between the 16S rRNA-*recA* Groups and their (mainly) *Mimosa* host plants (Fig. 5). For example, **Group N** comprises only four strains isolated from *M. pudica* sampled in RJ, while **Group Q** (*P. mimosarum*) also contains only *M. pudica* strains from RJ with the exception of a single strain from an Argentinian *M. somnians* nodule. On the other hand, although *M. pudica* strains were present in 12 out of the 17 *Paraburkholderia* Groups (**Groups A–G, I, J, N–Q**), there were none in **Groups H, K, L, and M**; these Groups only contained strains (including *P. youngii*) mainly isolated from *Mimosa* spp. endemic to the highlands of GO, MT and BA (Simon and Proença, 2000; dos Reis Junior et al., 2010). As mentioned previously, *Cupriavidus* (**Groups R and S**) and *Rhizobium* (**Group T**) were only associated with the widespread species *M. pudica*, although **Group S** was also isolated to a lesser extent from *M. tenuiflora*, another widespread species.

The geographical distribution of the 16S rRNA-*recA* Groups was challenging to determine with any precision, as there were substantial differences in sampling, and there was also some sampling bias, especially in states where particular locations harboring *M. pudica* were more densely sampled (e.g., *Rhizobium altiplani* in DF; Baraúna et al., 2016) (Fig. 6). However, if we look at the states in which many endemic *Mimosa* spp. were sampled, such as BA and GO, it can be seen that the dominant 16S rRNA-*recA* **Groups** were those mostly associated with endemic *Mimosa* spp., e.g., *Paraburkholderia* **Groups H, K–M, and O**, but also in MA and MT in which *Paraburkholderia* **Group J** was consistently isolated (albeit not exclusively) from native/endemic *Mimosa* spp. These states thus highlight the tight links between the endemic plants, their location, and their microsymbionts. On the other hand, in another of the more densely sampled states, RJ, but which harbors no endemics, there was also a wide diversity of 16S rRNA-*recA* genotypes, albeit ones more commonly isolated throughout the neotropics and within the invasive pantropical range of *M. pudica*; these were *Cupriavidus* (**Group R and S**), *P. mimosarum* (**Group Q**), *P. phymatum*, and *Rhizobium* (**Group T**). Contrastingly, however, RJ was also the only Brazilian state that yielded *Paraburkholderia* **Group N** strains (all isolated from *M. pudica*).

The effect of soil characteristics on the genotype and distribution of symbionts of *M. pudica* and other widespread species was tested via an RDA. It used the 62 nodule isolates for which soil data were available from the sites where their hosts were excavated (Table S1). Of the total variation occurring in the biological data, the first two axes (RDA1 and RDA2) could explain 37.8 % of the total variation, whereas the remainder (62.2 %) corresponded to residual variance (Table 1 and Fig. 7). It can be seen in Fig. 7 that the soil variables pH and Ca are located on the right of the first ordering axis (RDA 1) together with the genus *Rhizobium*, indicating that the occurrence of *Rhizobium* correlated positively with soil pH and Ca. On the negative side of RDA 1, the variable Al³⁺ positively correlated with the genus *Paraburkholderia* but negatively correlated with soil pH and Ca. *Paraburkholderia* did not correlate with P, K and Mg. Concerning RDA 2, *Cupriavidus* was positively correlated with K, Mg and P. Therefore, in soils with higher pH and Ca values, there is a lower chance of finding *Paraburkholderia* and a higher chance of finding *Rhizobium*. More acidic and aluminum rich soils

tend to favor *Paraburkholderia*, while mildly acidic soils with higher K, Mg and P tend to favor the genus *Cupriavidus*.

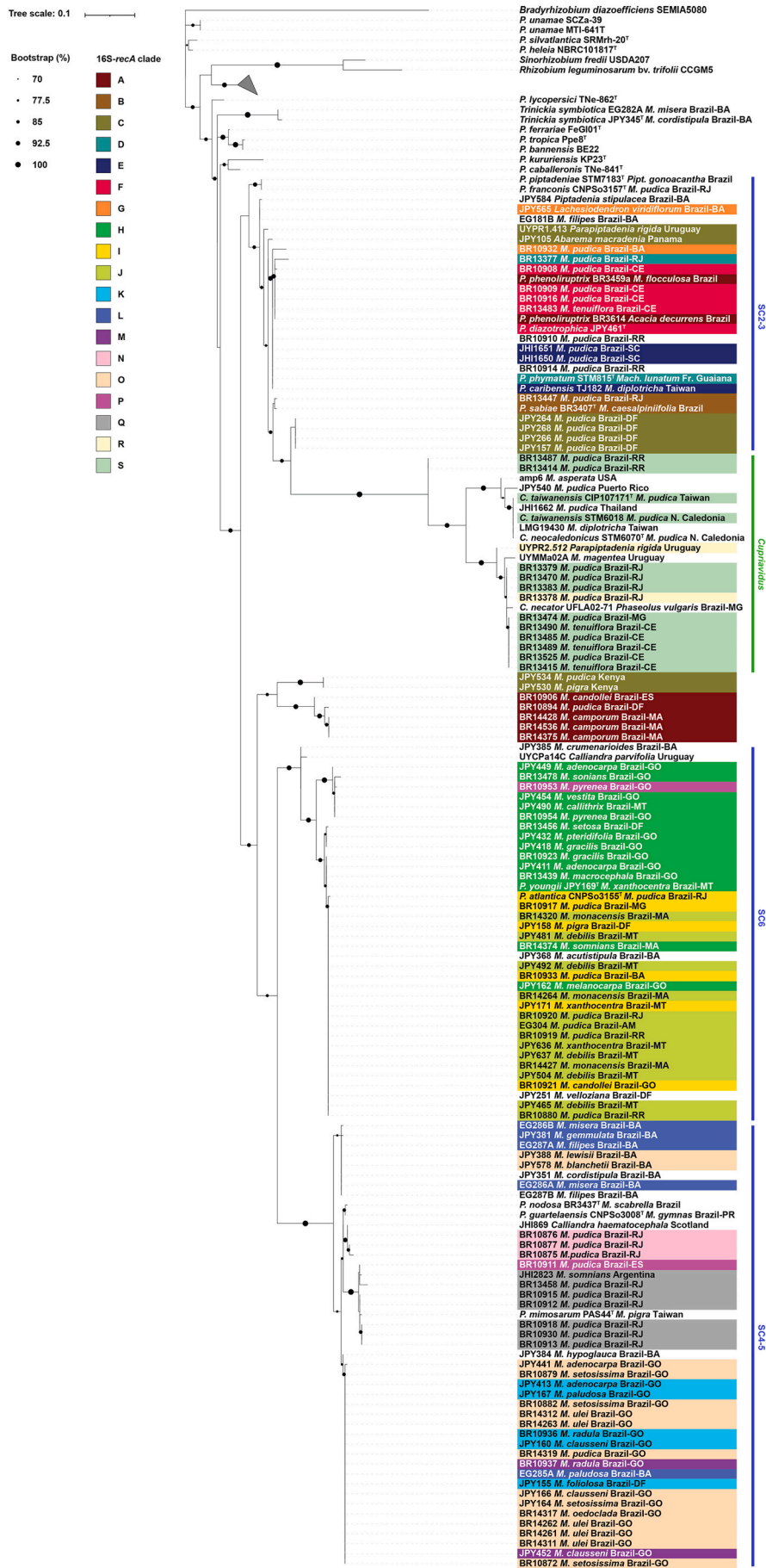
4. Discussion

4.1. *Paraburkholderia nodulating Mimosa in Brazil is contained within three large species complexes*

In the present study, we took a different approach towards uncovering the range of symbionts of the speciose genus *Mimosa* by not focusing so much on the enormous variety of endemic Brazilian species but rather on the symbionts of widespread species that have become pantropical weeds because of centuries of human-mediated dispersal, particularly *M. pudica*. We then combined the dataset from the present study with that of the earlier study of Bontemps et al. (2010) on mainly endemic *Mimosa* spp. to establish if and how the symbionts of these two groups of *Mimosa* differ and if they interact with each other's hosts. Taken together, these samples covered 13 Brazilian states and all five national regions. Surprisingly, given that it is now >15 years since their study was conducted, the Species Complexes (SCs) demonstrated by Bontemps et al. (2010) for *Mimosa*-nodulating *Paraburkholderia* were confirmed by this new combined dataset. No novel SCs were revealed either in this study or in others published since 2010, with only the species *P. ribeironis*, which was isolated from *Piptadenia gonoacantha* (Bournaud et al., 2013, 2017), being divergent from the already known SCs. On the other hand, the various species that comprise the SCs of Bontemps et al. (2010) are now being progressively described. These include *P. diazotrophica* (SC2–3), *P. franconis* (SC2–3), *P. guartelaensis* (SC4–5), and the ex- "*B. tuberosum* sv. *mimosae*" species *P. atlantica* and *P. youngii* (SC6) (Sheu et al., 2013; Paulitsch et al., 2019a, 2019b, 2020a; Mavima et al., 2021, 2022). In addition, based on groups without any type strains closely affiliated to them, the combined 16S rRNA-*recA* dataset from the present study suggested that there might be at least another four new species (not including single strain lineages). This was reinforced by the ANI analysis using full genomes of representative strains from four of the unaffiliated 16S rRNA-*recA* groups, i.e., Groups G, J, K, and L + M + O, representing all three of the large SCs. The other two potentially new species suggested by the 16S rRNA-*recA* phylogeny were based on Groups N and P (both SC4–5), but further progress with these awaits a reference strain from each group having its full genome sequenced.

The other important discovery arising from our combined dataset is that the "minor" SC2 and SC3 of Bontemps et al. (2010), which we have combined into SC2–3, is now revealed to be at least as large as the two "major" SCs, SC4–5 and SC6. The expansion of SC2–3 from that first reported by Bontemps et al. (2010) is a consequence of including so many symbionts of widespread lowland *Mimosa* species that were not investigated by this earlier study of mainly endemic species. SC2–3 now comprises *P. phymatum*, *P. sabiae*, *P. diazotrophica*, *P. franconis*, and *P. piptadeniae*, all of which are frequently isolated from *M. pudica* and other widespread *Mimosa* spp. in Brazil and elsewhere in the tropics, but also from non-*Mimosa* mimosoids like *Piptadenia*, *Lachesiodendron*, *Parapiptadenia* and *Jupunba* (*Abarema*) in Brazil (Bournaud et al., 2013), Uruguay (Taulé et al., 2012), and Panama (Barrett and Parker, 2005), confirming the cosmopolitan nature of this SC.

Another striking aspect of the newly expanded SC2–3 was the apparent abundance of two previously described non-symbiotic and non-diazotrophic species as *Mimosa*-nodulating bacteria, i.e.,



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Fig. 4. *nifH* Phylogenetic tree built using IQ-TREE, with ultrafast bootstrap analysis (1000 iterations) and the 'TN + F + I + G4' best-fit model according to Bayesian Information Criterion. The alignment contained 166 sequences, 246 nucleotide positions and 97 parsimony-informative sites of *nifH* genes from strains of genera *Paraburkholderia*, *Cupriavidus* and *Trinickia*. Plant host species and geographic origin of relevant strains are indicated. For Brazilian strains and when available, the state of origin is also indicated using two-letter abbreviations (e.g., Brazil-DF). The collapsed clade represented by a gray triangle contains *P. xenovorans* LB400^T, *P. dioscoreae* Msb3^T, *P. aromaticivorans* BN5^T, *P. strydomiana* WK1.1f^T, *P. podalyriae* WC7.3b^T, *P. sprentiae* WSM5005^T, *P. tuberum* STM 678^T, *P. steynii* HC1.1ba^T, *P. dipogonis* ICMP19430^T, *P. kirstenboschensis* Kb15^T, *P. dilworthii* WSM3556^T, *P. rhynchosiae* LMG27174^T. *Paraburkholderia* species clusters (SC), as described in Bontemps et al. (2010), as well as *Cupriavidus* strains are indicated by blue and green vertical lines to the right of the phylogram. Abbreviations: S. Africa = South Africa, Fr. Guiana = French Guiana, N. Caledonia = New Caledonia, *Pipt. gonoacantha* = *Piptadenia gonoacantha*, *Mach. Lunatum* = *Machaerium lunatum*, *H. sophoroides* = *Hypocalyptus sophoroides*, *L. ambigua* = *Lebeckia ambigua*. Details in Table S1.

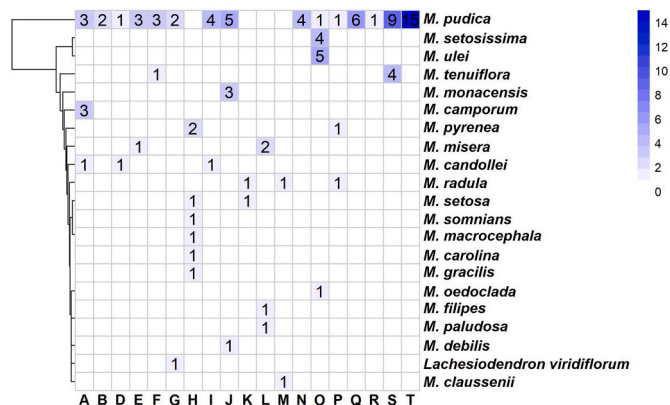


Fig. 5. Heatmap showing the relationship of phylogenetic clades with host-plant origin. Absolute numbers of strains are indicated in heatmap cells. Clade numbers are indicated under the columns. Rows were clustered using hierarchical clustering based on Euclidean distances.

P. azotifigens and *P. caribensis*; these two species join with *P. phenoliruptrix*, which is in a clade divergent from all those containing *Mimosa* symbionts. *Paraburkholderia caribensis* was first reported to be a symbiont of *Mimosa* in Taiwan by Chen et al. (2003a), but the (non-symbiotic) type strain MWAP64^T was isolated originally from soils in Martinique (Achauak et al., 1999); since then, *P. caribensis* has been isolated from nodules of invasive *Mimosa* species in various locations in Asia, such as China (Liu et al., 2020), but this is the first report of it as a symbiont of *Mimosa* in Brazil. Similarly, *P. phenoliruptrix* was originally isolated from a chemostat (Coenye et al., 2004) and then revealed to be related to a symbiont of *M. flocculosa* in Brazil, as represented by *Burkholderia* strain BR3462 (Chen et al., 2005a), which was later formally described as *P. phenoliruptrix* strain BR3459a (Cunha et al., 2012), and which like BR3614 (this study), possesses *P. phymatum*-type *nod* and *nif* genes (Soares Neto et al., 2022). Thereafter, *P. phenoliruptrix* has been quite frequently isolated as a nodulating symbiont of native *Mimosa* and *Calliandra* species in Brazil, and with widely varying *nod* and *nif* genes (this study), but also of introduced species like *Acacia decurrens* (Zilli et al., 2021; this study). A more recent discovery of nodulating symbionts in a species previously described as non-symbiotic is *P. azotifigens*, originally isolated from rice paddy soils in South Korea by Choi and Im (2018). This species is now revealed to be a common symbiont of widespread *Mimosa* species in Brazil and Kenya, but also of other neotropical mimosoids (*Jupunba*, *Parapiptadenia*). Intriguingly, although the four genome-sequenced symbiotic *P. azotifigens* strains (JPY530, JPY534, JPY105, and UYPR1.413) all harbor *nif* genes (Taulé et al., 2012; De Meyer et al., 2015b; this study), the genome of the non-symbiotic type strain, NF2-5-3^T, despite its epithet, does not (data not shown).

As reported by the earlier study of Bontemps et al. (2010), and now with the addition of 103 new strains, there was relatively little evidence of HGT of symbiosis-related genes between the three main *Mimosa*-nodulating *Paraburkholderia* 16S rRNA-*recA* lineages. Indeed, the *nodC* and *nifH* sequences of most strains were congruent with their 16S rRNA-*recA* sequences, with the three largest SCs (SC2–3, SC4–5, and SC6)

largely reconstructed in their phylogenies; these *nodC*- and *nifH*-derived lineages coincided with the “symbiovars” recently described by Paulitsch et al. (2020b) for *Mimosa*-nodulating *Paraburkholderia*. Nevertheless, there was some HGT of the symbiosis-determining loci observed between the SCs, which is to be expected as they often share the same habitats and even the same host plants, and all strains tested can nodulate at least one “common” host, *M. pudica*, regardless of which SC they belong to (Bontemps et al., 2010; this study). The possibility of inter-SC HGT had already been suggested by a study of invasive *Mimosa* symbionts in China in which *P. mimosarum* strains harbored *P. phymatum nodA* genes (Liu et al., 2012), and by a study of Brazilian *Calliandra* symbionts that were mainly *P. nodosa* in terms of their core genomes, but harbored *P. atlantica*/*P. youngii*-type *nodC* genes (Silva et al., 2018).

4.2. *Cupriavidus* is an important nodulating symbiont of widespread *Mimosa* species in Brazil

Although symbiotic *C. necator*-like strains were isolated from nodules of common bean and *Leucaena* plants used to trap rhizobia from pastures in MG in central Brazil by da Silva et al. (2012), the present study is the first report of Brazilian *Mimosa* species being nodulated in the field by *Cupriavidus*. This is surprising given that *Cupriavidus* has long been a major component of studies of *Mimosa* from elsewhere in its native and invasive range (Chen et al., 2001, 2003a, 2005b; Barrett and Parker, 2005, 2006; Liu et al., 2012; Klonowska et al., 2012; Mishra et al., 2012; Gehlot et al., 2013; Reeve et al., 2015; Platero et al., 2016). However, most studies in Brazil (e.g., Bontemps et al., 2010) have focused primarily on the enormous variety of endemic *Mimosa* species in the highlands of central Brazil within the Cerrado and Caatinga biomes, as well as the *Campo rupestre* environments that arise from these biomes at altitudes above 900 m; the highly acidic and nutrient-poor soils of *Campo rupestre* environments are not only known drivers of endemism (Simon and Proença, 2000), but will also favor acid-tolerant *Paraburkholderia* as potential symbionts (Stopnisek et al., 2014). Therefore, a probable reason for the previous absence of reports of *Cupriavidus* as a *Mimosa* symbiont was that endemic Brazilian *Mimosa* spp. generally do not encounter these bacteria in their natural habitats. They also tend to have a reduced ability to nodulate with *Cupriavidus* strains in ex situ single strain inoculation trials, suggesting that they are symbiotically incompatible partners (Elliott et al., 2007a; dos Reis Junior et al., 2010).

Most of the *Cupriavidus* strains in the present study were isolated from *M. pudica*, but also from *M. tenuiflora* and *M. candollei* (syn. *M. quadrivalvis* var. *leptocarpa*), growing in disturbed areas, pastures, and roadsides in the states of CE, ES, MG, RJ, RR. Genotypically, the strains were remarkably uniform, as almost all were *C. taiwanensis*, albeit with *C. necator*-like *nodC* and *nifH* genes like those of the symbionts of endemic Uruguayan *Mimosa* species (Platero et al., 2016; Iriarte et al., 2016). Such an unusual combination of core and symbiosis genes has not been previously observed in any native or invasive environment in which *Cupriavidus* has been isolated as a *Mimosa* symbiont (Chen et al., 2003a, 2005b; Andam et al., 2007; Mishra et al., 2012; Liu et al., 2012, 2020; Andrus et al., 2012; Klonowska et al., 2012; Gehlot et al., 2013; Parker, 2015). Compared to *Paraburkholderia* there are very few species of symbiotic *Cupriavidus*. Furthermore, the nodulating species have a remarkable monophyly of *sym*-genes, with most being close to either *C. taiwanensis* (Chen et al., 2003a, 2005b; Andam et al., 2007;

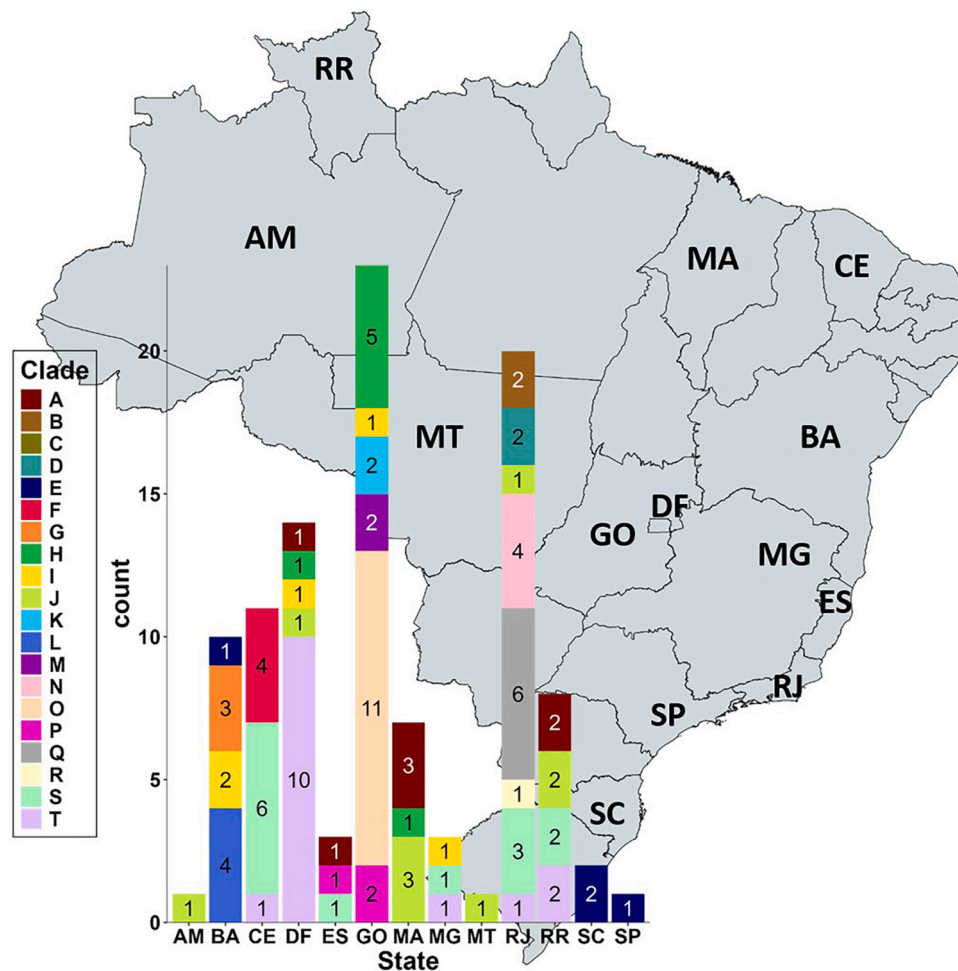


Fig. 6. Geographic distribution of strains from different phylogenetic clades in Brazil. Brazilian states are indicated by two-letter code as in Table S1. The stacked bar plot shows the number of strains for each 16S-*recA* clade per state. Clade colors as in the 16S rRNA-*recA*, *nodC* and *nifH* phylogenies (Figs. 1, 2, 3 and 4). Exact geographic coordinates, if known, are shown in Table S1.

Table 1

Redundancy Analysis (RDA) for soil chemistry data potentially related to the distribution of the bacterial genera *Paraburkholderia*, *Cupriavidus* and *Rhizobium* that were isolated as nodulating symbionts of *Mimosa* spp. growing in Brazilian soils. Canonical axes are significant according to the Monte Carlo permutation test (F = 23.236; P = 0.001, 999 permutations).

Chemical variable	RDA1	RDA2
pH	0.96	0.01
Al ³⁺	-0.65	-0.18
Mg	-0.13	0.51
Ca	0.65	0.13
P	-0.05	0.38
K	-0.11	0.57
Autovalues	0.2491	0.1289
Cumulative % of explained variance		
Variables of soil bacterial genera	24.91	37.80
Variables of soil bacterial genera and chemistry data	65.9	100.0
Sum of all canonical autovalues (%)		37.80

Andrus et al., 2012; Klonowska et al., 2012; Mishra et al., 2012; Parker, 2015) or to *C. necator* (da Silva et al., 2012; Taulé et al., 2012; Platero et al., 2016; this study). *Cupriavidus taiwanensis*, *C. neocaledonicus* and *C. necator* are the only validly named species confirmed as symbionts to date (Chen et al., 2001, 2003b; da Silva et al., 2012; Klonowska et al., 2020), although others are likely to be described. For example, *C. necator*- and *C. pinatubonensis*-like strains were the dominant symbionts of endemic *Mimosa* species from the heavy metal-rich mining area

of Minas in Uruguay (Platero et al., 2016), and these probably represent new species. Meanwhile, the present study suggests a novel *Cupriavidus* species close to *C. lacunae* comprising UYPR2.512, a symbiont of *Parapiptadenia rigida* from Uruguay (Taulé et al., 2012; De Meyer et al., 2015a), and BR13378 from *M. pudica* in RJ (this study). Moreover, JPY540 isolated from *M. pudica* in Puerto Rico is closest to, but divergent from *C. oxalaticus*, and hence may also represent a novel species.

4.3. Factors driving the wide range of Beta-rhizobia in Brazil

Host endemism (altitude, location), and the highly location-specific edaphic factors that are associated with endemism (Simon and Proença, 2000), have most likely driven co-evolution between endemic/biome-restricted *Mimosa* spp. and their symbionts. In the largest center of radiation of *Mimosa* in central Brazil, this is revealed as an almost exclusive association with *Paraburkholderia*, particularly *P. youngii* in SC6, and 16S rRNA-*recA* Groups K, L, M, and O in SC4-5 (Bontemps et al., 2010; this study), or occasionally with the much rarer but related genus, *Trinickia*, which has so far only been isolated from *M. cordistipula* and *M. misera* in BA (Bontemps et al., 2010; Estrada-de los Santos et al., 2018; this study). It contrasts with the second largest *Mimosa* speciation center, Central Mexico, where endemic *Mimosa* spp. are mostly nodulated by *Rhizobium* or *Sinorhizobium* (Bontemps et al., 2016). A significant difference between central Brazil and central Mexico is that soils are highly acidic in the former (dos Reis Junior et al., 2010) and neutral-alkaline in the latter (Bontemps et al., 2016). Low soil pH is a known

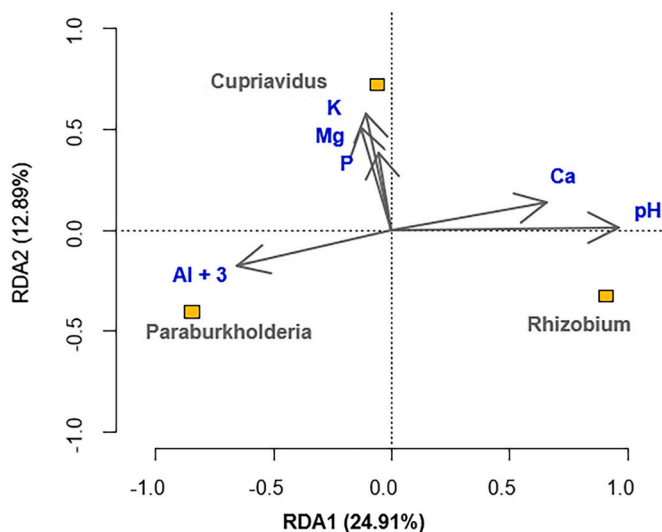


Fig. 7. Ordination plot for the first two axes of a (RDA₁ e RDA₂) resulting from a redundancy analysis (RDA) between microbiological and soil chemical variables. The soil chemistry data are potentially related to the distribution of the bacterial genera *Paraburkholderia*, *Cupriavidus* and *Rhizobium* that were isolated as nodulating symbionts of *Mimosa* spp. growing in Brazilian soils. Canonical axes are significant according to the Monte Carlo permutation test ($F = 23.236$; $P = 0.001$, 999 permutations).

factor in promoting the occurrence of acid-tolerant *Paraburkholderia* (Stopnisek et al., 2014) as symbionts in both the main centers of Beta-rhizobial diversity, central Brazil and the Fynbos biome of South Africa (Garau et al., 2009; Howieson et al., 2013; Lemaire et al., 2015, 2016; de Castro Pires et al., 2018), especially when combined with low soil fertility (Elliott et al., 2009; de Castro Pires et al., 2018; Soares Neto et al., 2022).

Therefore, the pattern that we now see in central Brazil is that native/endemic *Mimosa* spp. are largely nodulated by diverse genotypes of *Paraburkholderia* that they have co-evolved with in these acidic, nutrient-poor and high- Al^{3+} soils (Simon and Proença, 2000), whereas the widespread *Mimosa* spp. that are common in lowland Brazil (but also in disturbed areas in other parts of the country) are nodulated by *Paraburkholderia*, *Cupriavidus* and *Rhizobium* strains, depending on the sampling location. An RDA with *M. pudica*, the most widely sampled *Mimosa* host in the present study, revealed that various edaphic factors like soil pH, Ca, and P were important for separating the three bacterial genera. As stated above, low soil pH (and possibly high levels of potentially toxic aluminum ions, Al^{3+}) was associated with a preference for *Paraburkholderia*. However, although alkaline pH has previously been shown to favor *Cupriavidus* (Mishra et al., 2012), in the present study this was not so obvious, perhaps because none of the sampled soils could be classified as such. Indeed, the soils containing *Cupriavidus* were mildly acidic with none above pH 6.8, suggesting that other edaphic factors are at least as crucial as elevated pH for explaining the presence of *Cupriavidus* symbionts, such as P, Mg, K, and the occurrence of heavy metals (Mishra et al., 2012; Klonowska et al., 2012; Gehlot et al., 2013; Platero et al., 2016; Liu et al., 2012, 2020). That being said, it is clear that above-neutral pH is essential for the presence of *Mimosa*-nodulating *Rhizobium*, as evidenced by studies from Brazil (Baraúna et al., 2016; de Castro Pires et al., 2018; this study), Mexico (Bontemps et al., 2016), and China (Liu et al., 2020), among others. Although the N-contents of non-agricultural soils in Brazil are generally low (de Castro Pires et al., 2018; Soares Neto et al., 2022), further studies of *Mimosa* symbiont diversity should also focus more specifically on the effects of plant-available N, as higher levels were shown to be of importance in the selection of *Cupriavidus* over *Paraburkholderia* for the nodulation of at least three widespread *Mimosa* species by Elliott et al. (2009).

It is also now clear that Brazil and the broader neotropics and neo-subtropics constitute a significant habitat for a diverse range of Beta-rhizobia that are the natural nodulating symbionts of not only *Mimosa*, but also of other mimosoid genera, such as *Anadenanthera*, *Calliandra*, *Jupunba*, *Lachesiodendron*, *Parapiptadenia*, *Piptadenia*, and *Pseudopiptadenia*, and that similar genotypes of *Paraburkholderia* and *Cupriavidus* that nodulate *Mimosa* spp. also nodulate these hosts (Taulé et al., 2012; Bournaud et al., 2013; Silva et al., 2018; Zilli et al., 2021). The recent discovery that the non-mimosoid Caesalpinioideae species, *Chamaecrista eitenorum*, is nodulated effectively by *P. nodosa*-like strains (Casaes et al., 2024) further indicates that it is likely that we are only scratching the surface of the actual diversity of neotropical and sub-neotropical Beta-rhizobial-legume interactions. Indeed, given the propensity of South American *Paraburkholderia* and *Cupriavidus* strains to nodulate effectively with papilionoids like common bean and Siratro (*Macroptilium atropurpureum*) (da Silva et al., 2012; Dall'Agnol et al., 2016, 2017) it is highly probable that Beta-rhizobia will be isolated from more crop legumes, as well as from their diverse native neotropical relatives.

5. Conclusion

There thus appear to be two stories associated with Brazilian *Mimosa* symbionts: that of the *Paraburkholderia* (and *Trinickia*) symbionts of upland endemics that grow in acidic, low nutrient and high Al-containing soils, and that of the *Paraburkholderia*, *Cupriavidus* and *Rhizobium* symbionts of lowland widespread species that grow in highly varied soils, and which select their symbionts according to those that are dominant in any particular soil type and chemistry (pH, organic matter, fertility, nutrient status). It is also not surprising that the pantropical invasive *Mimosa* spp., such as *M. diplotricha*, *M. pigra* and *M. pudica*, are all typical widespread species in their native neotropics, and this might also explain why the same few “typical” symbionts of these species (e.g., *C. taiwanensis*, *P. mimosarum* and *P. phymatum*) are isolated repeatedly in their invasive environments (Chen et al., 2003a, 2005a, 2005b; Andrus et al., 2012; Liu et al., 2012, 2020; Klonowska et al., 2012; Gehlot et al., 2013; Melkonian et al., 2014).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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