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Brazilian species of *Calliandra* Benth. (tribe Ingeae) are nodulated by diverse strains of *Paraburkholderia*

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ABSTRACT

The Chapada Diamantina in NE of Brazil is a biodiversity hotspot and a center of radiation for many Neotropical legume genera, such as *Calliandra* and *Mimosa*. The present study aimed to evaluate nodulation in *Calliandra* species endemic to various environments, and to characterize the diversity of their symbiotic rhizobia using housekeeping (16S rRNA, *recA*) and plasmid-borne, symbiosis-related (*nifH* and *nodC*) genes. The nodulation ability of selected isolates was assessed. All of the 126 bacterial isolates from 18 *Calliandra* species collected in six different vegetation types were identified as *Paraburkholderia* according to their housekeeping and symbiosis gene phylogenies. They were grouped in seven clades in relation to the dominant vegetation type in their native environments. The majority, particularly those from highland "campo rupestre" vegetation, were similar to *Paraburkholderia nodosa*, but had *nodC* genes identical to the *Mimosa* symbiont *Paraburkholderia tuberum* sv. mimosae. The other smaller groups were related to *Paraburkholderia diazotrophica* and *Paraburkholderia sabiae*, and some single strains were not close to any known species. The symbionts of *Calliandra* spp. in NE Brazil are *Paraburkholderia* strains closely-related to *Mimosa* symbionts from the same region. NE Brazil is a reservoir of symbiotic *Paraburkholderia* that have an affinity for genera in the Mimosoid clade.

Introduction

Calliandra is a large genus in the tribe Ingeae, subfamily Caesalpinoideae of the Leguminosae (Fabaceae). In his monograph on the exclusively neotropical genus, Barneby [3] divided the 132 species into five sections, mainly defined by the architecture of their inflorescences [63]. The distribution of *Calliandra* is restricted to three centres of diversity: one in North America (southern United States and Mexico to Central America) with 35 species, another in north western South America with 29 species, and the third in eastern Brazil with 46 species [3,29,63,64]. Most Brazilian *Calliandra* species occur in "campo rupestre" (loosely translated as open rocky fields, often at altitudes >1000 m), savannas ("Cerrado") and seasonally dry tropical forests (SDTFs), but a few species are native to wet forests or to subtropical grasslands in southern South America. The Chapada Diamantina in the interior of the north eastern Brazilian state of Bahia is the main center of diversity of the genus, and houses 46 *Calliandra* species of which 36 are endemic [64]. *Calliandra* species range in form from small shrubs to small trees. They are often used as ornamentals because of their flowers with their colourful and eye-catching stamens, but are also used for forestry restoration, forage [35], green manure, and for controlling soil erosion [11,42]. Some species, such as *Calliandra houstoniana* var. *calothyrsus* (Meisn.) Barneby (*ex. Calliandra calothyrsus*), have been introduced into other tropical regions (*e.g.* parts of Africa) for all or some of these uses [2].

Like most species in the tribe Ingeae, *Calliandra* forms mutualistic associations with nitrogen-fixing bacteria resulting in the formation of root nodules [1,35,36,65,66]. Legume-nodulating nitrogen-fixing bacteria, were originally considered to belong exclusively to the order Rhizobiales in the Alphaproteobacteria [49], but over the last 15 years several members of the order

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Burkholderiales in the Betaproteobacteria have been isolated from legume nodules and confirmed as symbionts [28,68]. So far, confirmed legume symbionts in the Betaproteobacteria, also termed "Beta-rhizobia", have been identified as belonging only to the genera *Paraburkholderia* (formerly *Burkholderia* — see Beukes et al. [6] and *Cupriavidus*). Beta-rhizobia are currently only known as symbionts of legumes in the tropics and the sub-tropics, with there being two main centres of radiation: South America and South Africa [28,34,66]. In South and Central America Beta-rhizobia are widely associated with native and endemic species in the large mimosoid genus *Mimosa* (tribe Mimosae) [4,7,13,44,50,51,53] and its close relatives in the "Piptadenia Group" [9,70]. *Paraburkholderia* symbionts are more commonly isolated from *Mimosa* than *Cupriavidus*, although the latter may dominate in some locations, especially non-native ones, depending upon soil conditions and/or host preferences [12,14,21,31,34,43,44,51]. A particular centre of diversification for legume-nodulating *Paraburkholderia* strains is the state of Bahia (BA) in NE Brazil [7,9,53,58,59], which houses an enormous variety of endemic legumes, including several *Mimosa*, *Piptadenia* and *Calliandra* species [37].

In the case of *Calliandra*, in spite of the wide diversity of species and their ecological importance in various vegetation types, there are few studies concerning the occurrence of nodulation in the genus and/or studies characterizing the rhizobia associated with it. Earlier studies focused on testing *Rhizobium* strains that were isolated from nodules of introduced *C. houstoniana* var. calothyrsus (Meisn.) Barneby (*ex. C. calothyrsus*) grown in Costa Rica, Ethiopia, Kenya, Cameroon and New Caledonia as an agroforestry/green manure species [1,35,36]. It is only in the past few years that studies on *Calliandra* symbionts isolated from plants in their native environments have been published *e.g.* three new *Rhizobium* spp. have been recently described nodulating the medicinal legume *Calliandra grandiflora* in Mexico [54]. Interestingly, and in contrast to the Mexican species, potential *Paraburkholderia* symbionts were isolated from nodules on *Calliandra germana* and *Calliandra luetzelburgii* collected in the Caatinga in NE Brazil by Santini et al. [56] raising the possibility that *Calliandra* nodulates with Beta-rhizobia in Brazil. Furthermore, Moulin et al. [46] have shown that the promiscuous Beta-rhizobial strain, *Paraburkholderia phymatum* STM815, which can nodulate more than 40 *Mimosa* species [19,53], was also capable of nodulating three out of five *Calliandra* species tested. In this context, the aim of the present study was to determine nodulation of the numerous *Calliandra* species which are endemic or native to diverse vegetation types in the state of Bahia and to assess the diversity of rhizobia that nodulate them.

Material and methods

Nodule collection and rhizobial isolation

Root nodules were sampled from 18 different *Calliandra* species native to the state of Bahia in NE Brazil from December 2012 to August 2014.

Nodules were stored in glass tubes containing silica gel. Voucher specimens with inflorescences and fruits (when present) were collected, pressed and deposited in herbaria at the Universidade Estadual de Santa Cruz (HUESC) and Universidade Estadual de Feira de Santana (HUEFS), wherein they were identified under the following HUESC registration numbers: 16312-16322, 16389-16391, 18354-18357, 18413-18416, 19920, 19921, 19924-19929, 19938-19949.

For isolation of rhizobia the nodules were hydrated with sterile distilled water for 24 h, surface disinfected with 70% ethanol for 30 s and with commercial sodium hypochlorite (2.5%) for 5 min, followed by six washes with sterile distilled water. Microorganisms were isolated from the interior of the nodules and were cultivated at 28–30 °C on solid Medium 79 [23], which is otherwise known as yeast mannitol agar (YMA).

All bacteria isolated were morphologically characterized and Gram-stained to select pure cultures of potentially rhizobial isolates. Of the Gram-negative isolates, those having particular phenotypical characteristics known to be indicative of rhizobia from legumes native to Brazil, such as an ability to modify (usually increase) the pH of the medium and a relatively slow growth rate (taking longer than one day to form colonies after initial streaking on YMA plates) were selected for the molecular analysis. The isolates were maintained in yeast mannitol broth (YMB) with 50% (w/v) glycerol at −80 °C for long-term storage in the Bank of Rhizobia of UESC (BRUESC).

DNA extraction, amplification, sequencing and phylogenetic analysis

Isolates were initially grouped and selected according to similarities in their phenotypical characteristics. Genomic DNA from these selected isolates was extracted according to Santos et al. [57]. The genomic material was amplified using primers against four different genes. The 16S rRNA gene was amplified with the same primers and PCR conditions as used by Santos et al. [57]. For the *recA nifH* and the *nodC* genes the primers used and the PCR conditions were as described by Bontemps et al. [7]. The amplified genes were sequenced directly by ACTGene Co. (Alvorada, Rio Grande do Sul, Brazil) using a ABI-PRISM 3100 sequencer.

The acquired sequences were aligned using the ClustalW program [71] and compared with sequences in the NCBI-Genbank database using the Megablast tool [45]. Phylogenetic trees were constructed with the Mega 6 software [69] was used to build Neighbour-joining trees using Kimura 2 parameter to correct the distance. All the sequences obtained in this study were deposited in the GenBank database and their Accession numbers are listed in Supplementary information. Sequences with <95% similarity were considered different species in the 16S rRNA and *recA* phylogenies.

Nodulation tests

The selection of isolates for nodulation tests was determined according to the different groups discernible after analysis of their symbiosis-related genes (*nifH*, *nodC*), so that at least one strain per *nodC* group was tested for nodulation on all three legume species. Tests were conducted in a greenhouse under natural light and temperatures for three months to check for nodulation of *Calliandra macrocalyx* Harms, *Mimosa pudica* (L.) and *Mimosa bimucronata* (L.). Seeds were surface sterilized in 70% ethanol for 1 min and commercial sodium hypochlorite (2–2.5%) for 5 min. After initial germination, 4 d seedlings were transferred aseptically to pots containing 2 kg of autoclaved substrate (sand and vermiculite, 1:1 v/v), that was fertilized without addition of nitrogen. The substrate surface was covered with washed and autoclaved gravel to reduce cross-contamination, and the pots were arranged in a completely randomized design. Throughout the experiment the plants were watered with sterile distilled water. The seedlings were inoculated four days after they were sown; each strain to be tested was grown in YMB until log phase and 1 ml was inoculated onto each seedling (4–5 seedlings of each species were tested with each strain). The plants were harvested after three months, nodules were counted, and their rhizobia were re-isolated and characterized. Strains were considered to be effective at forming a N-fixing symbiotic relationship if the host plant had green leaves and nodules were pink in their interior due to the presence of the symbiosis-essential protein leghaemoglobin (Lb). Ineffective strains were those that nodulated

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