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FROM THE COVER

Burkholderia species are ancient symbionts of legumes

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Abstract

***Burkholderia* has only recently been recognized as a potential nitrogen-fixing symbiont of legumes, but we find that the origins of symbiosis in *Burkholderia* are much deeper than previously suspected. We sampled 143 symbionts from 47 native species of *Mimosa* across 1800 km in central Brazil and found that 98% were *Burkholderia*. Gene sequences defined seven distinct and divergent species complexes within the genus *Burkholderia*. The symbiosis-related genes formed deep *Burkholderia*-specific clades, each specific to a species complex, implying that these genes diverged over a long period within *Burkholderia* without substantial horizontal gene transfer between species complexes.**

Keywords: biodiversity, Brazil, *Mimosa*, nitrogen fixation, rhizobia

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Introduction

The genus *Burkholderia* belongs to the β-*proteobacteria* class and currently includes more than 50 species that colonize a wide diversity of niches ranging from soil and water to plants and animals. Nevertheless, *Burkholderia* remains mostly known and studied through its various pathogenic representatives. For this reason, the description in 2001 of legume-nodulating *Burkholderia* (Moulin *et al*. 2001) and their subsequent characterization as real symbionts (Chen *et al*. 2003, 2005a,b; Barrett & Parker 2005, 2006, 2006; Andam *et al*. 2007) was a double shock, changing our perceptions of both the ecological capabilities of *Burkholderia* and the taxonomic diversity of legume symbionts.

Legume-nodulating bacteria, collectively called rhizobia, live as saprophytes in the soil and in a facultative symbiosis with plants. They induce the formation of root nodules, within which they fix atmospheric nitrogen and provide it to the plant in exchange for carbon compounds. This mutualistic association occurs in the majority of legumes and constitutes the main terrestrial system of biological nitrogen fixation.

Rhizobia were already known to be taxonomically heterogeneous and polyphyletic, but the known examples were confined to the *Alphaproteobacteria* class ('α-rhizobia') until the identification of nodulating *Burkholderia* and *Cupriavidus* established that there were also 'β-rhizobia' (Chen *et al*. 2001; Moulin *et al*. 2001). So far, five nodulating *Burkholderia* species have been described: *Burkholderia tuberum* (Vandamme *et al*. 2002), *B. phymatum* (Vandamme *et al*. 2002), *B. mimosarum* (Chen *et al*. 2006), *B. nodosa* (Chen *et al*. 2007) and *B. sabiae* (Chen *et al*. 2008). Symbiotic strains were also identified in *B. caribensis* (Chen *et al*. 2003) and *B. cepacia* (Rasolomampianina *et al*. 2005), previously described as nonsymbiotic species, and some isolates represent other species, which are yet unnamed (Barrett & Parker 2006; Parket *et al*. 2007; Garau *et al*. 2009). The only β-rhizobium so

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far described outside the genus *Burkholderia* is *Cupriavidus taiwanensis* (Chen *et al.* 2001, 2003). Although the core genomes of α-rhizobia and β-rhizobia are highly diverged and representative of their respective classes, genes for the symbiotic interaction, such as *nodA*, are no more diverged between the α-rhizobia and β-rhizobia than they are within the α-rhizobia (Moulin *et al.* 2001; Chen *et al.* 2003), strongly suggesting that they have been transferred laterally rather than evolved separately.

So far, the majority of β-rhizobia have been isolated from *Mimosa* species, and the *B. phymatum* type strain can nodulate 30 of 31 tested *Mimosa* species in laboratory conditions (Elliott *et al.* 2007b), suggesting that β-rhizobia may have a particular association with *Mimosa*. However, although *Mimosa* is one of the largest legume genera, with over 500 species, only 11 different *Mimosa* species have been checked for the presence of β-rhizobia and most of these have been growing outside their native range (Chen *et al.* 2003, 2005a,b; Barrett & Parker 2005, 2006, 2006; Andam *et al.* 2007).

We have investigated symbiont diversity of a large number of *Mimosa* species in three regions of central Brazil, which is the native environment and a major diversification centre of the genus *Mimosa*. We identified *Burkholderia* species as the main symbionts of *Mimosa* in Brazil and proved a specific association between these two genera. Sequencing of core and symbiosis genes provides insight into β-rhizobial evolution and interestingly suggests that nodulation is an ancient function within the genus *Burkholderia*.

Materials and methods

Sampling, strain isolation and plant tests

Nodules were collected from the roots of 47 different *Mimosa* species growing spontaneously at 67 locations in the Brazilian states of Bahia, Goiás, Distrito Federal and Mato Grosso (Table S1, Supporting information). The coordinates of each location were recorded using a Global Positioning System receiver and can be viewed in Google Earth using File S1 (Supporting information). The outline map in Fig. 2 is based on http://commons.wikimedia.org/wiki/File:Brazil_states_blank.png by Golbez. The nodules were dried in tubes containing silica gel for transport to the laboratory. Bacteria were axenically isolated from single nodules, purified from single colonies and cultivated on YM medium (Vincent 1970), which yielded 143 isolates, each from a separate nodule. Symbiotic effectiveness of 114 strains was tested using nodulation tests with *M. pudica* (those not tested had gene sequences identical to others from the same plant). *Mimosa pudica* seeds were germinated (Chen *et al.* 2003) and inoculated (Elliott *et al.* 2007b) along with uninoculated plants as negative controls. Where seed was available, strains were also tested for nodulation of the original host species of isolation. For competition experiments, plants were coinoculated with equal volumes of overnight cultures of the two isolates.

PCR amplification

PCR amplifications were performed using the GoTaq kit (Promega) with genomic DNA extracted as described in Moulin *et al.* (2004) or with the GoTaq kit (Promega). For all strains, a nearly full length 16S rRNA gene was amplified and sequenced with primers AGAGTTTGATCCTGGCTCAG and AAGGAGGTGATCCAGCC (Weisburg *et al.* 1991). For *Burkholderia*, 869 bp of *recA* was amplified and sequenced with primers GATCGARAAGCAGTTCGGCAA and TTGTCCTTGCCCTGRCCGAT (Payne *et al.* 2005) and a 658 bp of *nifH* with primers CGCIWTYTACGGIAARGGIGG and GGIKCRTAYTSGATIACIGTCAT (Chen *et al.* 2005b). For SC7 strains, a 300-bp *nodC* fragment was amplified with primers GTNGGNAARMGNAARGC and CANGGVCCRCARCARCACA (Bontemps *et al.* 2005). For other symbiotic *Burkholderia*, a 635-bp *nodC* fragment was amplified with primers ACTSATACTYAACGTMGAYTC and GMRAAYCCRAGAAATCGAAG for 30 cycles of 30 s at 95 °C, annealing for 30 s at 58 °C and extension at 72 °C for 45 s, with a final 5 min extension at 72 °C. For α-rhizobia, 930-bp *nodC* and 783-bp *nifH* gene fragments were amplified with primers AYGTHGTYGAYGACGGTTC and CGYGACAGCCANTCKCTATTG, and TACGGNAARGGSGGNATCGGCAA and AGCATGTCYTCSAGYTCNTCCA respectively (Laguerre *et al.* 2001).

Phylogenetic analyses

Nucleotide alignments were constructed with Clustal_X 1.83, imported into BioEdit 4.8.4 (Hall 1999) and manually corrected. Phylogenetic trees in Fig. 1 were constructed with sequence alignments of 1250 nt of 16S concatenated with 625 nt of *recA*, 569 nt of *nifH* and 633 nt of *nodC*. The Phylo_win software (Galtier *et al.* 1996) was used to build neighbour-joining trees using Kimura's 2-parameter distance correction. Maximum likelihood analyses with a fully estimated GTR model were performed with the PhyML software (Guindon *et al.* 2005). For neighbour-joining, support for the tree branches was estimated with 1000 bootstrap replicates and with 100 for maximum likelihood. A maximum likelihood NodC peptide tree (Fig. 3) was constructed utilizing amino acids 143–360 by using RAxML 7.0.4 (Stamatakis *et al.* 2008) with JTT matrix substitution model, gamma rate distribution ($\alpha = 0.704$) plus invari-

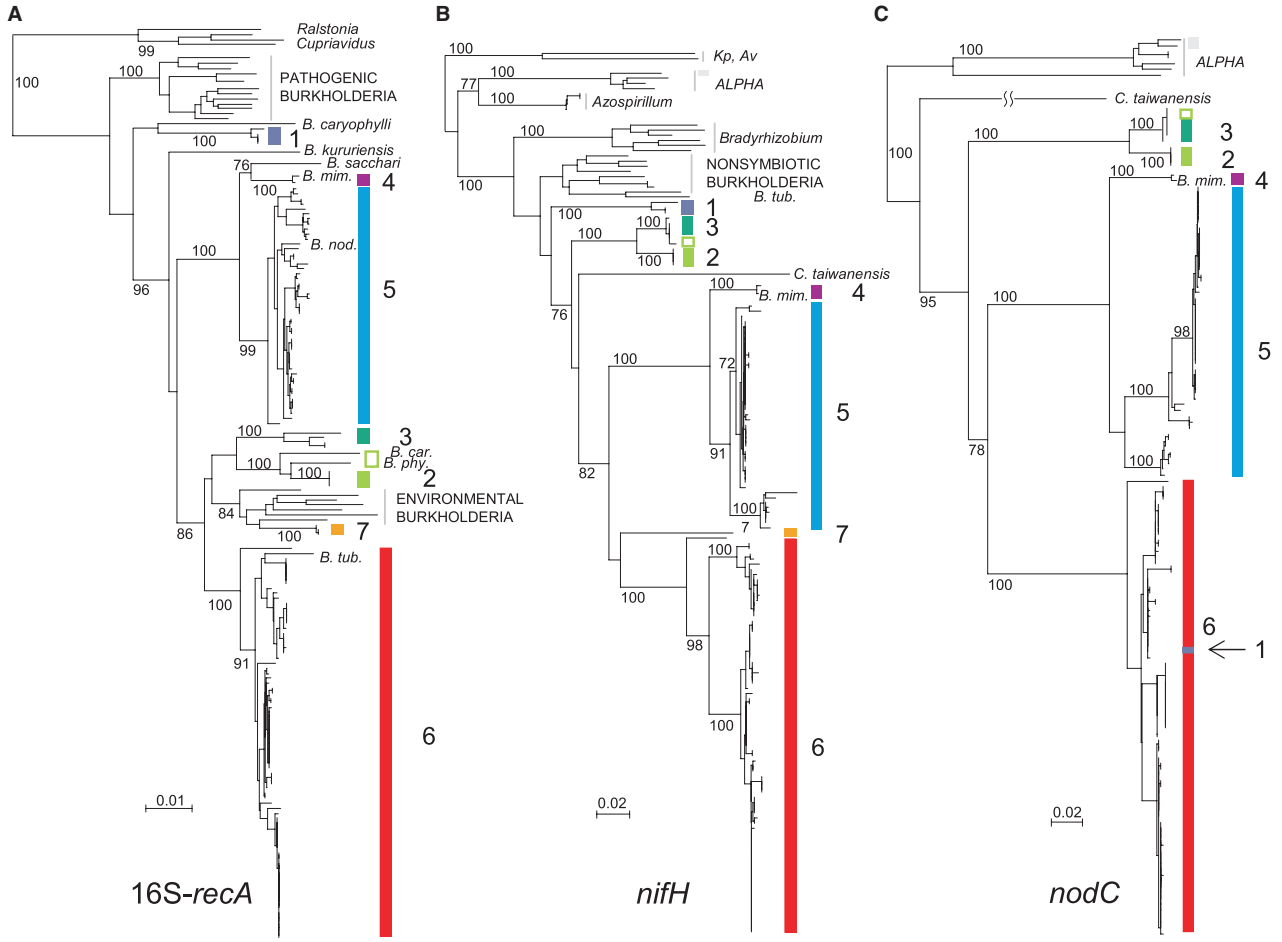

Fig. 1 Phylogenies of genes in *Burkholderia* strains isolated from *Mimosa* nodules. (A) Concatenated 16S rRNA gene and *recA* sequences; (B) *nifH*; (C) *nod*C. Phylogenies were constructed using a maximum likelihood method, and percentage bootstrap support (100 replicates) is shown if >70%. Scale represents mutations per nucleotide. Colours denote clades in A and are used consistently for the same isolates in B and C. The 'pathogenic' group includes *B. cepacia*, *B. mallei*, *B. gladioli* and related species. The 'environmental' group includes *B. graminis*, *B. phenazinium*, *B. xenovorans* and related species. Full sequence identifiers are given in Figs S1–S4 (Supporting information).

able sites (0.191) estimated from data and 100 rapid CAT bootstraps. Tree was displayed using Mega 4.0 (Tamura *et al.* 2007).

Results

The preferred symbionts of the genus Mimosa *in its native environment are* Burkholderia

Nodule symbiont diversity was surveyed for an extensive and representative range of *Mimosa* species in their native environment. Symbionts were isolated from 143 nodules (one isolate per nodule) on 47 different *Mimosa* species (Table S1, Supporting information) that represent a significant part of the phylogenetic diversity of the genus (Simon *et al.* 2009). Sequences of 16S rRNA and *recA* genes showed that 141 isolates were strains of *Burkholderia*. Part of the symbiosis-related *nodC* gene was amplified and sequenced for all isolates, except seven for which amplification could not be achieved. Six of these seven induced effective (i.e. nitrogen-fixing) nodules on *M. pudica* or the original host, suggesting that they probably had *nodC* genes but with sequences too diverged to amplify. The majority of the other isolates were also tested on host plants and demonstrated effective nodulation ability. All isolates might, therefore, be considered as genuine symbionts, apart from one *Burkholderia* that failed for both *nodC* amplification and nodulation. Two alphaproteobacterial strains were isolated from *M. xanthocentra* in Mato Grosso and identified as *Rhizobium* spp.

The symbiotic character is widespread in the genus Burkholderia

The taxonomic diversity of the *Burkholderia* isolates was assessed by a phylogeny based on concatenated 16S

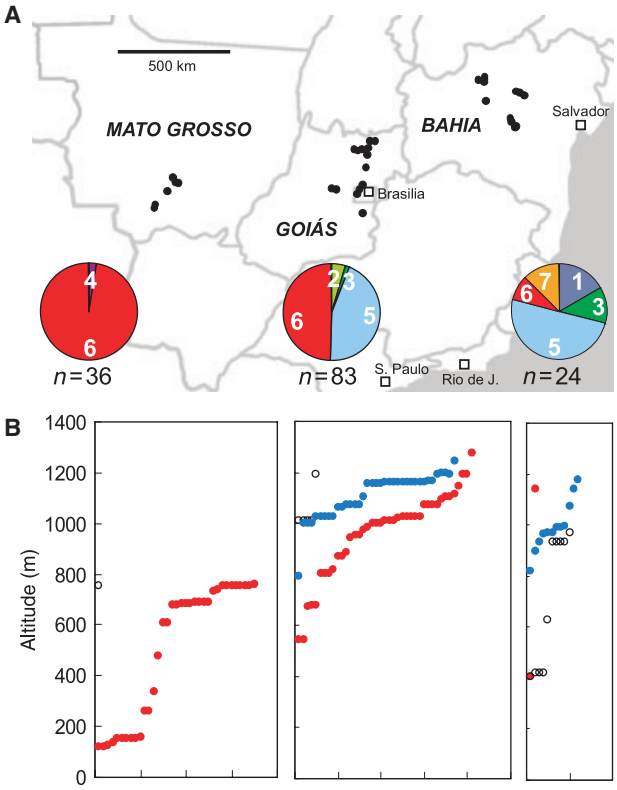

Fig. 2 (A) Sample locations and distribution of *Burkholderia* species complexes. Sample sites are shown (●) and the relative abundance of the species complexes SC1–7 summed for all samples within each of the three states Mato Grosso, Goiás (including Distrito Federal) and Bahia. (B) Altitude of sample sites of the two most frequent taxa SC5 (blue) and SC6 (red), and all other species combined (white), ranked by altitude for each species complex separately.

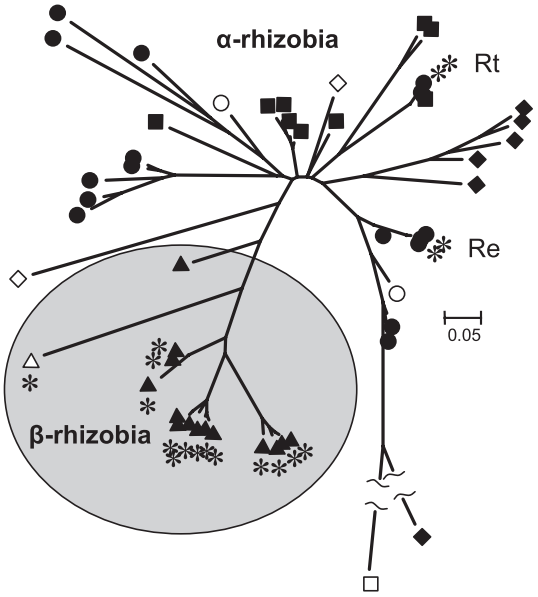

Fig. 3 Phylogeny of partial NodC peptide sequences from representative rhizobia in the α- and β-proteobacteria, illustrating the relative divergence of their symbiosis genes. *Burkholderia* (▲);*Cupriavidus* (△); *Methylobacterium* (◇); *Bradyrhizobium* (◆); *Rhizobium* (●); *Sinorhizobium* (○); *Mesorhizobium* (■); *Azorhizobium* (□); symbionts of *Mimosa* (*). Re, Rt – *Mimosa* symbionts related to *R. etli* and *R. tropici* respectively. Maximum likelihood method with JTT matrix and gamma rate distribution with invariable sites. See Fig. S6 (Supporting information) for full details.

rRNA and *recA* genes (Fig. 1A), as the phylogenies of these genes were largely congruent (Figs S1 and S2, Supporting information). Our isolates were grouped in seven distinct and well-supported clusters (100% bootstrap). The majority were in clusters 6 (54%) and 5 (34%). Sequence identity within each cluster was 98–100% for 16S rRNA and 94–100% for *recA*, similar to that among the closely related species forming the *B. cepacia* complex (Coenye & Vandamme 2003). Thus, each of the clusters defined in this study is likely to represent a complex of closely related species with similar ecological traits. We will refer to the clusters in this study as species complexes SC1–SC7. The isolates in SC1, SC3 and SC7 certainly represent new *Burkholderia* species as their sequences are distant from those of all type strains of the species. Other species complexes include the type strains of named species: *B. mimosarum* is in SC4, *B. nodosa* in SC5 and *B. tuberum* in SC6. *B. phymatum* and *B. caribensis* have the 16S rRNA sequence of SC2 (Fig. S1, Supporting information) but the symbiosis genes of SC3 (Figs S3 and S4, Supporting information). Strains that are effective symbionts of *Mimosa* have previously been reported in all these species (Chen *et al.* 2003, 2005a,b, 2007; Elliott *et al.* 2007b) except *B. tuberum*, which nodulates the papilionoid legume *Cyclopia* (Elliott *et al.* 2007a). We demonstrated that representatives of all seven species complexes formed effective nodules on *M. pudica* and, where seed was available, also on their original host species (Table S1, Supporting information).

Symbiosis genes in Burkholderia *are diverse and stably inherited*

The *nifH* gene encodes part of the nitrogenase enzyme and is found in all organisms that fix nitrogen, whether free-living or in symbiosis. This gene is therefore not specific to rhizobia, although it is essential for the mutualistic symbiosis. A phylogenetic analysis of *nifH* sequences grouped those of our symbiotic strains with sequences from free-living *Burkholderia* (Fig. 1B). They showed some relationship with the α-rhizobial *Bradyrhizobium* and *Azorhizobium* sequences, which are thought to have acquired their nitrogenase genes from β-proteobacteria (Young 2005), but all the other *Alphaproteobacteria* (including rhizobia) were much more dis-

tant. This supports the suggestion of Chen *et al.* (2003) that the nitrogenase of symbiotic *Burkholderia* may have been present before the acquisition of symbiosis genes, which is consistent with the observation that some of these bacteria, such as *B. phymatum*, can fix nitrogen in the free-living state (Elliott *et al.* 2007b).

Among the symbiotic *Burkholderia*, the *nifH* phylogeny (Fig. 1B) revealed well-supported groups congruent with the species complexes that were defined by 16S–*recA* phylogeny. The only exception was that the *nifH* sequences of SC3 strains were very similar to those of the *B. phymatum* and *B. caribensis* type strains, which were much closer to SC2 according to 16S and *recA*, suggesting a potential *nifH* lateral gene transfer. The generally high congruence of these different markers indicates a rather stable genetic structure with few lateral transfers of nitrogenase genes between the different *Burkholderia* species complexes.

In contrast to *nifH*, *nodC* is a representative of the nodulation genes that are characteristic of, and confined to, rhizobia. The NodC peptide sequences of our *Burkholderia* isolates were similar to those of α-rhizobia, but phylogenetic trees built with extensive diversity (Fig. 3) always grouped *Mimosa*-nodulating *Burkholderia* in a single cluster that excluded any other sequences in the databanks. This monophyletic origin of *nodC* in *Mimosa*-nodulating *Burkholderia* strongly supports a single acquisition of these genes. The phylogeny of *nodC* (Fig. 1C) also separated the different strains in well-supported clusters identical to those observed with *nifH*. The only anomaly was the SC1 strain JPY347, which had a *nodC* sequence typical of SC6. The sequences of the three deep *nodC* clades are highly diverged, with approximately 22% DNA mismatch between SC4/5 and SC1/6, and 28% between these and SC2/3. No *nodC* sequence could be amplified from any other SC1 or SC7 strains; however, they formed nodules, so we presume that they have *nodC* but with diverged sequences, although we cannot rule out the possibility that they have a nodulation mechanism that does not depend on *nodC*, like certain unusual *Bradyrhizobium* symbionts of *Aeschynomene* (Giraud *et al.* 2007). Here again, the almost complete congruence of the *nodC* tree with that of other genetic markers suggests that they all have evolved together with little lateral transfer. Nodulation is, therefore, a stable and important ecological function that has an ancient origin and is found among most *Burkholderia* lineages. The branch depth of the different NodC groups within *Burkholderia* is similar to that observed within different defined α-rhizobia groups, such as *Bradyrhizobium* (Fig. 3). The origin of nodulation within *Burkholderia* could, therefore, be as ancient as in some well-established groups of α-rhizobia, although the known diversity across all α-rhizobia remains higher than that found in β-rhizobia so far.

The *nifH* and *nodC* genes of the two alphaproteobacterial symbionts were related to those previously described from alphaproteobacterial symbionts of *Mimosa* isolated elsewhere, including TJ171 and TJ172 from Taiwan (Chen *et al.* 2003), NGR181 from Papua New Guinea (Elliott *et al.* 2009) and UPRM8021 from Puerto Rico (Zurdo-Piñeiro *et al.* 2004). All these strains are close to *Rhizobium tropici*, but there is another clade of alphaproteobacterial *Mimosa* symbionts, with quite different symbiosis genes, represented by *R. etli* biovar *mimosae* (Wang *et al.* 1999). All these *nifH* and *nodC* sequences are very distant from those of the *Burkholderia* symbionts, implying that the host specificity for *Mimosa* has arisen independently.

Burkholderia *and* Mimosa *species exhibit some specificity*

In a number of cases, *Burkholderia* strains belonging to more than one species complex were isolated from the same *Mimosa* species (Table S1, Supporting information). Furthermore, a comparison of the phylogeny of the bacteria (Fig. 1) with that of the host plants (Simon *et al.* 2009) shows that symbionts have frequently switched hosts. This is consistent with our observation that the majority of strains formed nodules on the widespread host *M. pudica* (Table S1, Supporting information). Nevertheless, we did obtain some experimental evidence that all possible partnerships are not equivalent. Seedlings of *M. setosissima*, a species endemic to the Cerrado, were inoculated with a strain isolated from this species (JPY164, a member of species complex SC5) and with a strain isolated from the widespread species *M. quadrivalvis* (JPY461, SC3). Tested separately, each of the strains induced effective nodules, but the level of nitrogen fixation was significantly higher with JPY164. Moreover, when the two strains were coinoculated, all nodules were occupied by JPY164 alone. Hence, in this example, the symbiont isolated from this endemic plant species was significantly more effective and more competitive than one from another host.

SC5 was the most frequently isolated bacterial type (38/60) from host species with an endemic or restricted distribution, but was less commonly found (11/84) on widespread host species (corrected $\chi^2 = 37.1$, $P < 0.001$). In contrast, each of the four SC3 isolates was from a different *Mimosa* species, all widespread.

Symbiotic Burkholderia *species have distinct geographical distributions*

There are two strong patterns in the distribution of the *Burkholderia* strains that we isolated: a spatial gradient in diversity and an altitudinal replacement of species.

We sampled three geographical areas over a distance of 1800 km in Brazil: the Caatinga (xeric shrubland) in Bahia state, the Cerrado (savanna) in Goiás and Distrito Federal (DF) and the Pantanal (wetland) in Mato Grosso. The diversity of symbiotic *Burkholderia* was the highest in the Caatinga, intermediate in the Cerrado (mostly SC5 and SC6) and the lowest in the Pantanal (mostly SC6). Superimposed on this trend is a strong association with altitude: SC5 predominates above 800 m, but is completely absent from lower locations. This is consistent with the absence of this species complex from the Pantanal, which is low-lying, but it is not explained solely by the broader geographical pattern, because the correlation with altitude is seen within Goiás/DF (Fig. 2), and indeed within individual locations in this region (Table S1, Supporting information).

Discussion

Burkholderia *species are the main symbionts of* Mimosa

Our survey of the symbionts of 47 species of *Mimosa* growing in their native environments revealed unambiguously that *Burkholderia* species are the normal symbionts of *Mimosa* in central Brazil, the major centre of *Mimosa* diversity (Simon & Proença 2000). In previous studies, only a small number of host species were sampled, but *Mimosa* species in their native ranges in the Americas have yielded a diversity of symbionts. In Central America, *Burkholderia* strains are the predominant symbionts of *M. pigra*, with occasional *Cupriavidus* (Barrett & Parker 2005, 2006), whereas two close relatives of *M. pigra* that are native to southern Texas were nodulated by either *Cupriavidus* (in the case of *M. asperata*) or *Sinorhizobium* (*M. strigillosa*) (Andam *et al*. 2007). On the contrary, *M. affinis* in Mexico was nodulated by *Rhizobium etli* biovar *mimosae* (Wang *et al.* 1999), whereas *M. pudica* in Costa Rica was associated with a mix of *Burkholderia*, *Cupriavidus* and *Rhizobium* (Barrett & Parker 2006). Although we have shown that *Burkholderia* predominates in the heartland of the genus *Mimosa*, it seems that *Cupriavidus* and α-rhizobia become more important towards the northern margin of the distribution.

Mimosa *symbionts have spread across the world with their hosts*

Burkholderia strains have been found in various places outside the Americas as symbionts of invasive *Mimosa* species of New World origin that were introduced by human activities. These strains are closely related to those described in this study from their native environment. For example, some *Burkholderia* associated with *M. pigra* recently introduced in Australia have 16S rRNA sequences (Parker *et al*. 2007) similar to those of strains of SC3 and SC7 (Fig. S1, Supporting information). This suggests that the symbiotic *Burkholderia* described so far in *Mimosa* may all actually have a neotropical origin, and that central Brazil constitutes a reservoir of *Mimosa*-associated *Burkholderia*. Interestingly, no symbiotic *Cupriavidus* was isolated in this study, although this genus provides the predominant symbionts of the invasive *M. pudica* in Taiwan and indigenous *M. asperata* in Texas. The phylogeny and compact organization of the nodulation genes of *C. taiwanensis* LMG19424 (Amadou *et al.* 2008) suggest that it has evolved as a symbiont by the relatively recent acquisition of nodulation genes from symbiotic *Burkholderia* (Andam *et al.* 2007). It appears that outside their core range, *Mimosa* species may adapt to different local bacterial populations, but they still show a preference for β-rhizobia.

In Taiwan, the predominant symbionts of *M. pigra* were *Burkholderia* SC4 (Chen *et al*. 2005b), whereas those isolated from *M. pudica* and *M. diplotricha* were mostly *Cupriavidus taiwanensis* (Chen *et al*. 2001, 2003). These host species all originate in the New World tropics; *M. pigra* is typically found in wetlands such as the Pantanal.

Symbionts are related to 'environmental' rather than 'pathogenic' Burkholderia

There are two main lineages of *Burkholderia*. One branch of the genus is known largely as pathogens of plants or animals (e.g. the *B. cepacia* complex), whereas the other includes environmental isolates that may form nonpathogenic associations with plants (Perin *et al*. 2006; Suarez-Moreno *et al*. 2008). Although root-nodule symbionts, like pathogens, form an intimate association with their eukaryote hosts, all the symbiotic *Burkholderia* isolates in this study belong to the nonpathogenic branch. In fact, only two nodule isolates have been assigned to the pathogenic branch so far: a strain of *B. cepacia* isolated from *Alysicarpus glumaceus* in Senegal (Vandamme *et al*. 2002), but not proven to nodulate, and a strain from *Dalbergia* that was demonstrated to nodulate and fix nitrogen (Rasolomampianina *et al.* 2005). *Burkholderia* has potential as a model system to understand how pathogenesis and symbiosis have emerged, spread, evolved and segregated within a single bacterial genus.

Burkholderia *species distribution reflects environment rather than host*

When *Mimosa* species from different clades grow together, they share the same set of symbionts. The

phylogenies of the two partners show no clear evidence for cospeciation. It is worth noting that there is normally no vertical transmission of the legume–rhizobium symbiosis: each seedling establishes new partnerships with bacteria in the soil. The opportunity for host switching is therefore always present, subject only to the specificity of the signalling that is mediated by the bacterial nodulation genes and their plant counterparts, and potential host-specific differences in symbiotic performance.

Rather than specializing on host clades, the *Burkholderia* species appear to have distinct niches governed by physical factors. It has been suggested that in comparison with α-rhizobia, *Burkholderia* symbionts are particularly adapted to acid, infertile soils (Garau *et al.* 2009), but our data suggest that there are also ecological differences between *Burkholderia* species. The association of SC5 with higher elevations presumably reflects some factor such as temperature or moisture that varies with altitude, whereas the higher diversity found in the Caatinga may reflect the wide range of elevations sampled in this region and its diverse vegetation, soils and geology. Interestingly, a similar pattern of association with physical factors rather than plant species has been observed in another root symbiosis, that of arbuscular mycorrhizal fungi (Helgason & Fitter 2009).

The Burkholderia–Mimosa *symbiosis is old and stable*

Nitrogen fixation is an ancient function in bacteria and is found in many *Burkholderia* species, symbiotic or not. The phylogenetic congruence implies that the common ancestor of all *Burkholderia* was a diazotroph and that this function has been vertically inherited in most *Burkholderia* species and lost in others. The divergence among *nodC* sequences is unusually wide for rhizobia that share a common host specificity, implying a deep ancestry. Despite the evidence that the *Burkholderia* symbionts themselves and their symbiosis genes have a long evolutionary history, the amount of horizontal gene transfer of symbiosis genes appears to have been very limited. The only clear case involves the sharing of genes between SC2 and SC3; otherwise, each *Burkholderia* species complex retains its own distinctive clade of symbiosis genes. In contrast, horizontal transfer of nodulation genes has been frequent among alphaproteobacterial rhizobia (Fig. 3).

What is the origin of the β-rhizobial symbionts?

We have shown that the *Burkholderia–Mimosa* symbiosis is widespread in a number of senses. It covers a wide geographical area, a wide taxonomic diversity across both partner genera and a wide diversity of symbiosis gene sequences. The existence of a deeply branched clade of nodulation genes confined exclusively to β-proteobacteria (Fig. 3) implies that β-rhizobia have a long independent history and have not simply arisen by recent transfers of nodulation genes from α-rhizobia. Further support is provided by the phylogeny of another nodulation gene, *nodA*, in which β-proteobacterial symbionts of *Mimosa* also form an exclusive deep clade (Elliott *et al.* 2009). It remains true that the great majority of rhizobia characterized so far have been α-proteobacteria, and the α-rhizobia exhibit greater taxonomic diversity, greater nodulation gene diversity and a far greater range of legume host taxa. The known β-rhizobia are all *Burkholderia* or *Cupriavidus*, and their symbiosis falls into two classes. One comprises those that nodulate *Mimosa*, as described in this study, and a small but expanding range of related mimosoid legume genera in the Americas (Chen *et al.* 2003; Barrett & Parker 2005, 2006; Elliott *et al.* 2007b). The other, typified by *B. tuberum* STM678, has quite different nodulation genes and a host range that includes African papilionoid legumes in the tribes Crotalarieae, Podalyrieae and Phaseoleae (Elliott *et al.* 2007a; Garau *et al.* 2009). As it is only a few years since they were first recognized, it is probable that new discoveries will expand the range of known β-rhizobia in the future, but it is unlikely that their true diversity rivals that of the α-rhizobia. The diversity of nodulation gene sequences within α-rhizobia is greater than that between α- and β-rhizobia, implying that horizontal transfer has occurred and is more likely to have been from α- to β-proteobacteria than the reverse. However, this study shows that legume symbiosis is embedded deep within the history of the genus *Burkholderia*.

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The group at York, led by J.P.W.Y., studies the genetic diversity, evolution and genomics of rhizobia and also of arbuscular mycorrhizal fungi; the sequence data were obtained by C.B. (postdoc, now a lecturer at the Université Henri Poincaré, Nancy, France) and R.C.L. (undergraduate project student). E.K.J. is a specialist in microscopy with a research interest in the rhizobium symbiosis; he initiated the project and led the field work, while G.N.E. (postdoc, a molecular microbiologist now developing a novel detection method for microbes) conducted plant tests and other studies, including sequencing. J.I.S. has world-wide expertise on legume nodulation. M.F.S. researches the phylogenetics and distribution of mimosoid legumes; he identified the *Mimosa* species. The other Brazilian teams (F.B.R.J., E.G., N.E.N., M.F.L., S.M.F.) study microbes that benefit the growth of crop plants; they obtained permits, collected samples, cultured rhizobia and carried out plant tests.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of bacterial isolates used in this study. Strains isolated in this study with their host plant, host endemicity group, gene sequence groups, geographical information, result of nodulation tests and sequence accession nos. NT, not tested; +, nodulation; –, no nodulation

Fig. S1 16S rRNA gene phylogeny of strains isolated in this study and reference strains. Tree based on maximum likelihood (ML), and bootstrap percentages over 70% from ML and NJ analyses are listed above and below the branches respectively. The different strain clusters are shown with numbered boxes. Strains isolated in this study are identified by their JPY number. B, *Burkholderia*; C, *Cupriavidus*; R, *Ralstonia*.

Fig. S2 *recA* gene phylogeny of strains isolated in this study and reference strains. Tree based on maximum likelihood (ML), and bootstrap percentages over 70% from ML and NJ analyses are listed above and below the branches respectively. The different strain clusters are shown with numbered boxes. Strains isolated in this study are identified by their JPY number. B, *Burkholderia*; C, *Cupriavidus*; R, *Ralstonia*.

Fig. S3 *nifH* gene phylogeny of strains isolated in this study and reference strains. Tree based on maximum likelihood (ML), and bootstrap percentages over 70% from ML and NJ analyses are listed above and below the branches respectively. The different strain clusters are shown with numbered boxes. Strains isolated in this study are identified by their JPY number. B, *Burkholderia*; C, *Cupriavidus*; R, *Ralstonia*.

Fig. S4 *nodC* gene phylogeny of strains isolated in this study and reference strains. Tree based on maximum likelihood (ML), and bootstrap percentages over 70% from ML and NJ analyses are listed above and below the branches respectively. The different strain clusters are shown with numbered boxes. Strains isolated in this study are identified by their JPY number. B, *Burkholderia*; C, *Cupriavidus*; R, *Ralstonia*.

Fig. S5 Altitudinal differences between species complexes. Sample sites of the two most frequent taxa SC5 (blue) and SC6 (red), and all other species combined (white), ranked by altitude for each species complex separately, within each of the sampled subregions within Goiás and Distrito Federal.

Fig. S6 Phylogeny of partial NodC peptide sequences from representative rhizobia in the α- and β-proteobacteria, illustrating the relative divergence of their symbiosis genes. *Burkholderia* (▲); *Cupriavidus* (△); *Methylobacterium* (◇); *Bradyrhizobium* (◆); *Rhizobium* (●); *Sinorhizobium* (○); *Mesorhizobium* (■); *Azorhizobium* (□). Maximum likelihood method with JTT matrix and gamma rate distribution with invariable sites. Percentage bootstrap support (100 replicates) shown if >70%. This is a detailed version of the phylogeny in Fig. 3. Full accession nos and strain names are included.

File S1 Sampling locations in compressed KML format for visualization in Google Earth.

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