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Nodulation and nitrogen fixation by *Mimosa* spp. in the Cerrado and Caatinga biomes of Brazil

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Summary

• An extensive survey of nodulation in the legume genus *Mimosa* was undertaken in two major biomes in Brazil, the Cerrado and the Caatinga, in both of which there are high degrees of endemicity of the genus.

• Nodules were collected from 67 of the 70 *Mimosa* spp. found. Thirteen of the species were newly reported as nodulating. Nodules were examined by light and electron microscopy, and all except for *M. gatesiae* had a structure typical of effective *Mimosa* nodules. The endosymbiotic bacteria in nodules from all of the *Mimosa* spp. were identified as *Burkholderia* via immunolabelling with an antibody against *Burkholderia phymatum* STM815.

• Twenty of the 23 *Mimosa* nodules tested were shown to contain nitrogenase by immunolabelling with an antibody to the nitrogenase Fe- (*nifH*) protein, and using the $\delta^{15}N$ (^{15}N natural abundance) technique, contributions by biological N_2 fixation of up to 60% of total plant N were calculated for Caatinga *Mimosa* spp.

• It is concluded that nodulation in *Mimosa* is a generic character, and that the preferred symbionts of Brazilian species are *Burkholderia*. This is the first study to demonstrate N_2 fixation by beta-rhizobial symbioses in the field.

Introduction

Until recently, 'rhizobia' were considered to consist of a limited number of genera in the family Rhizobiales in the Alpha-Proteobacterieaceae (Graham, 2008), but evidence has accumulated to show that legumes, particularly those in the genus *Mimosa*, but also other mimosoids and some papilionoids, such as *Cyclopia* (Elliott *et al.*, 2007a) and *Rhynchosia* (Garau *et al.*, 2009), may also form effective nodules with beta-proteobacteria, the so-called 'beta-rhizobia' (Elliott *et al.*, 2007b). Although beta-rhizobia have been isolated in many tropical regions from invasive *Mimosa* species, such as *M. diplotricha* (often cited as *M. invisa*), *M. pigra* and *M. pudica* (Chen *et al.*, 2001, 2005b;

Verma *et al.*, 2004; Barrett & Parker, 2005, 2006; Parker *et al.*, 2007), and selected strains of beta-rhizobia, such as *Burkholderia phymatum* STM815, *Burkholderia mimosarum* PAS44 and *Cupriavidus taiwanensis* (syn. *Ralstonia taiwanensis*) LMG19424, have shown a particular ability to nodulate *Mimosa* spp. (Elliott *et al.*, 2007b, 2009), little is known about the symbionts of *Mimosa* species in their native environments.

Mimosa is a large and complex genus with over 500 species and is mainly native to the New World (Barneby, 1991; Simon & Proença, 2000). Species vary in habit from tall trees and shrubs to vines and herbs, and they are found in a wide variety of habitats from wet to dry, growing on many different soils. *Mimosa* is considered by Barneby (1991) to have 'differentiated profusely in tropical and warm temperate savanna habitats', but is particularly abundant and diverse in the Cerrado and Caatinga biomes of Brazil (Lewis, 1987; Barneby, 1991; Simon & Proença, 2000; Mendonça *et al.*, 2008; de Queiroz, 2009). The Cerrado has *c.* 200 *Mimosa* species, most of them endemic to either the Cerrado biome as a whole or to very specific localities within it (Simon & Proença, 2000). Similarly, the semi-arid Caatinga biome in the northeast of Brazil is home to 38 species of *Mimosa* (de Queiroz, 2009), plus 15 species that grow in the highlands of the Chapada Diamantina (Lewis, 1987; de Queiroz, 2009). As with the Cerrado, many of these species are endemic.

The present study aimed to:

- determine the extent of nodulation of *Mimosa* species in the Cerrado and Caatinga. Additional collections were made in the Pantanal, a wetland biome neighbouring the Cerrado, in which nodulated *Mimosa* spp. have previously been recorded (James *et al.*, 2001);
- identify the microsymbionts '*in situ*' using antibodies specific either to the genus *Burkholderia* or to the species *C. taiwanensis*;
- determine whether nodulated species fix nitrogen in the field by immunolabelling nodules with an antibody against the nitrogenase *nifH* (Fe-) protein, and by applying the $\delta^{15}N$ (^{15}N natural abundance) technique to *Mimosa* spp. using neighbouring nonnodulated plants as references.

Materials and Methods

Cerrado and Caatinga: vegetation types, soils and precipitation

The collection sites are indicated in Fig. 1 and Supporting Information, Table S1. The Cerrado biome covers an area of 2×10^6 km^2 (Ratter *et al.*, 1997). Its soils are dystrophic, with low cation exchange capacity, and acidic with pH values of *c.* 4.0–5.5 (Adámoli *et al.*, 1986), and they support a mainly savannah vegetation, dominated by grasses, which is frequently subjected to natural burning (Felfili *et al.*, 2004; Simon *et al.*, 2009). The Caatinga biome, which has an area of 850 000 km^2, is typified by Fe-rich soils and xeric scrub vegetation (de Queiroz, 2009). Both biomes contain areas of campo rupestre, which is a type of vegetation (mainly herbaceous with shrubs and small trees) that occurs above 900 m in litholic soils that are acidic and low in fertility (Ribeiro & Walter, 2008). Precipitation in the Cerrado is highly seasonal, but generally higher than 1000 mm yr^{-1}, whereas that in the Caatinga is < 800 mm yr^{-1} (classified as semi-arid) and is concentrated in only a very short and sometimes unpredictable wet season of 2–3 months' duration. Precipitation in the high-altitude campo rupestre is generally higher than in the surrounding Cerrado or Caatinga, but the rocky soils within them have low water retention capacity (Ribeiro & Walter, 2008).

Collection of plant material

Species of *Mimosa* were collected from various locations (mainly highland regions) in the Cerrado in September

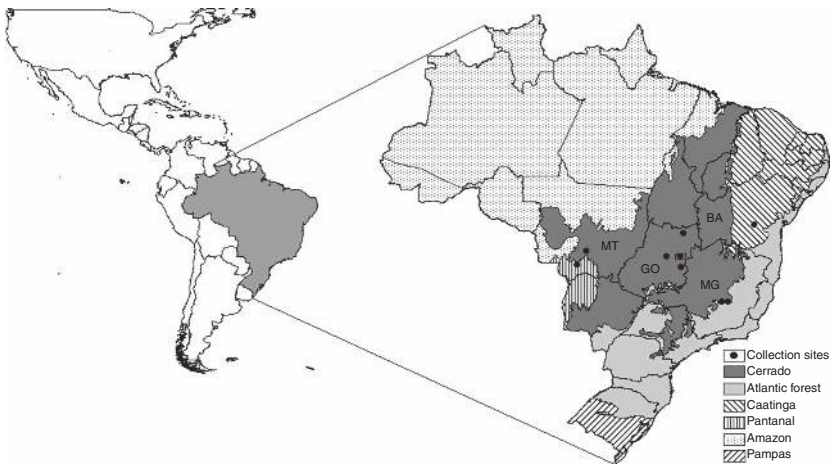

Fig. 1 The locations of the *Mimosa* nodule-collecting sites in the various biomes of central Brazil. Two-letter codes are Brazilian states: BA, Bahia; GO, Goiás; MT, Mato Grosso; .

2005 (dry season) and in the Cerrado, Caatinga and Pantanal in March 2006 (wet season). Additional collections were made in the Cerrado in Minas Gerais and in the Caatinga in Bahia during the wet season of 2009 (Fig. 1, Table S1). Voucher herbarium specimens were taken for all species and deposited in UB, HUEFS, BHCB and FHO (herbarium abbreviations follow Holmgren *et al.*, 1990). In both expeditions, nodules (if present) were collected and preserved in silica gel for later bacterial isolation. Some nodules (three to four per plant) were also cut in half to determine if they were potentially active and effective by the appearance of a pink coloration resulting from the presence of leghaemoglobin (Lb), and these were then placed into vials containing 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.5) for microscopic analysis. Since many of the plants in the September 2005 dry season expedition only had dried nodules on their roots, these were used solely for bacterial isolation (see Bontemps *et al.*, 2010); nodules sampled in the March 2006 wet season were generally sufficiently fresh for both bacterial isolation and microscopy. *Mimosa* seeds were collected whenever they were found, but this was generally restricted to the dry season. Leaf material was collected in the wet season for $\delta^{15}N$ analysis (see the section on $\delta^{15}N$ analysis for details), and soil samples were collected in the Caatinga for pH analysis.

Microscopy and *in situ* detection of microsymbionts

All pink nodules collected in the field were prepared and sectioned for light microscopy to determine general nodule structure, and then were further analysed by *in situ* immunogold labelling (plus silver enhancement) using antibodies raised against *B. phymatum* STM815^T and *C. taiwanensis* LMG19424^T according to Chen *et al.* (2005b) and Elliott *et al.* (2007b). The cross-reactions of the antibodies against a wide range of bacteria, including both alpha- and beta-proteobacteria (Table S2), were first tested with the enzyme-linked immunosorbent assay (ELISA) according to Gyaneshwar *et al.* (2001). Two preliminary experiments established the ability of the antibodies to detect defined *Burkholderia* and *Cupriavidus* strains within *Mimosa* nodules.

Seedlings of various *Mimosa* spp. collected in the Cerrado and Caatinga were inoculated with either *B. phymatum* STM815 or *C. taiwanensis* LMG19424 according to Elliott *et al.* (2007b).

Seedlings of *M. setosissima* and *M. decorticans*, both Cerrado endemics, and *M. blanchetii*, a campo rupestre endemic from Chap. Diamantina, were inoculated, respectively, with rhizobial strains JPY164, JPY297 and JPY578, which had originally been isolated from dry nodules of these hosts, and have been identified via their 16S rDNA and *recA* sequences as belonging to the genus *Burkholderia* (Bontemps *et al.*, 2010).

In both experiments, the plants were harvested at 6 wk after inoculation, tested for nitrogenase activity via acetylene reduction assays (ARAs) according to Chen *et al.* (2003), and any nodules formed were sampled for microscopy and immunogold labelling as for the field samples.

Pre-immune sera were used as negative controls in all of the immunogold assays. The reactions of the test samples were compared visually with the corresponding negative and positive controls to determine which of the antibodies had reacted with each nodule, and these were then scored as being positive for either *B. phymatum* ('Bp') or *C. taiwanensis* ('Ct'), or giving no reaction with either antibody ('NR').

Western blots and *in situ* detection of nitrogenase *nifH* protein

A polyclonal antibody raised against the Fe-protein (*nifH*) of the nitrogenase enzyme complex from *Rhodospirillum rubrum* (Norén *et al.*, 1997) was used to detect the presence of nitrogenase in some of the nodules collected from the Cerrado and the Caatinga according to Chen *et al.* (2003). The specificity of the *nifH* antibody was tested by Western blots of nodule extracts of *M. pudica* infected with either *B. phymatum* STM815^T or *C. taiwanensis* LMG19424^T (Elliott *et al.*, 2007b) according to Rubio *et al.* (2009). The *nifH* antibody was used at a dilution of 1 : 5000, and extracts of nodules from alfalfa (*Medicago sativa*) infected with *Sinorhizobium meliloti* 102F78 were used as positive controls. The ability of the *nifH* antibody to detect the nitrogenase enzyme via immunogold silver enhancement in *Mimosa* nodules collected in the field was first tested on sections of nodules of Cerrado species inoculated with *B. phymatum* STM815^T or *C. taiwanensis* LMG19424^T that were positive or negative for nitrogenase (acetylene reduction) activity, as well as on nodules of *M. setosissima* (+ *Burkholderia* strain JPY164) and *M. blanchetii* (+ *Burkholderia* strain JPY578) from actively N_2-fixing plants (see previous section). In addition to immunogold silver enhancement for light microscopy, more detailed analyses using transmission electron microscopy (TEM) with both the *B. phymatum* and *nifH* antibodies were performed on nodules from selected field-collected *Mimosa* spp. according to Elliott *et al.* (2007b) and Chen *et al.* (2005a). Nonimmune serum was used as a negative control in all immunogold assays.

$\delta^{15}N$ analysis

Leaf samples were taken from *Mimosa* spp. for analysis of their $\delta^{15}N$ content in the 2006 wet season expedition (in which nodules were more likely to be actively fixing N_2)

according to Sprent *et al.* (1996). Samples were only taken from sites that were apparently undisturbed (e.g. pristine Cerrado, Caatinga or campo rupestre), and not from agricultural land or roadsides where the $\delta^{15}N$ signal can be greatly altered by human activities (Unkovich *et al.*, 2008). Sites were effectively delineated by the presence of at least three individuals of each endemic or biome-restricted *Mimosa* sp. within 5 m of each other, and leaves were taken from three separate nodulated plants at each site. Where available (i.e. within a 1 m radius of the test plant, but not immediately adjacent to it), samples were also taken from three replicate plants of three nonnodulated dicotyledonous reference species (preferably nonnodulating legumes, if present), as recommended by Boddey *et al.* (2000). Samples of test and reference plants were dried, ground to a fine powder, and their $\delta^{15}N$ enrichment was determined using the methodology of Teixeira *et al.* (2006). A '*B*-value' for a defined *Mimosa* symbiosis was obtained according to Unkovich *et al.* (2008) by growing seedlings of *M. caesalpiniifolia* inoculated with *B. sabiae* strain Br3407 (Chen *et al.*, 2008) in a mixture of vermiculite/perlite without any added N. The plants were harvested at 6 months and were assessed for the presence of root nodules and nitrogenase activity using ARA. The aerial parts of those plants with active N_2-fixing nodules were dried and ground and their $\delta^{15}N$ content determined. Aerial parts had a mean (± SEM, $n = 6$) $\delta^{15}N$ enrichment of $-1.24 \pm 0.22‰$, which was within the range of reported *B*-values for other (albeit nonwoody) legumes (Unkovich *et al.*, 2008), and thus it was used to determine the proportion of N derived from air (%Ndfa) according to the equations given by Unkovich *et al.* (2008).

Results

Nodulation of *Mimosa* species in the Cerrado and Caatinga

Seventy species of *Mimosa* were examined for nodulation in the Cerrado and Caatinga (Tables S3, S4). Of these, 59 were shrubs or small trees (2–3 m in height), such as *M. decorticans* (Fig. S1a), *M. splendida* (Fig. S1b) and *M. crumenarioides* (Fig. S1c), five were herbs (*M. debilis*, *M. lewisii*, *M. pudica*, *M. skinneri*, *M. ursina*; Fig. 2d), and six were trees (*M. acutistipula*, *M. caesalpiniifolia*, *M. dominarum*, *M. hexandra*, *M. ophthalmocentra*, *M. tenuiflora*). The species were categorized according to Simon & Proença (2000) as either 'endemic' (E), 'biome-restricted' (R) or 'widespread' (W) (i.e. occurring in more than one biome). The majority of the endemic and biome-restricted species (29 in the Cerrado, nine in the Caatinga) were found at high altitude (> 800 m) mainly in pristine vegetation (often growing in campo rupestre on rocky outcrops at elevations above 1000 m; Fig. S1a–c), whereas the widespread species were generally found at lower altitudes alongside roads or on disturbed ground close to roads (Fig. S1d), and sometimes in urban areas (Tables S3, S4). In total, nodules were found on 67 separate species (84 taxa); 44 of these had not previously been reported as nodulating before these expeditions to the Cerrado and the

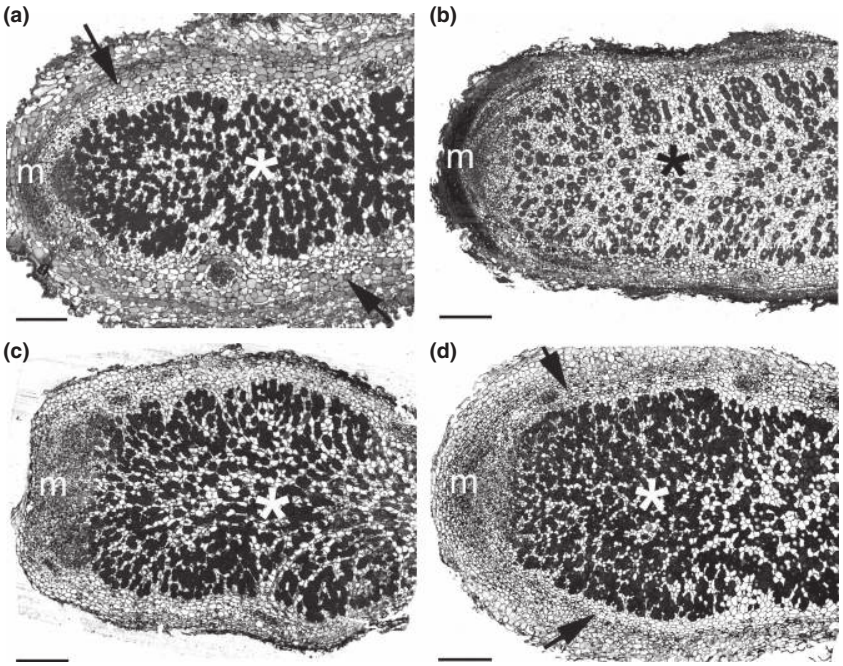

Fig. 2 (a) Light microscopy of a section of a N_2-fixing nodule formed on the Cerrado endemic, *Mimosa setosissima*, after inoculation with a *Burkholderia* strain (JPY164) which was originally isolated from this species. Note that it has a structure typical of an effective nodule, with an apical meristem and a large infected zone which contains the N_2-fixing symbionts. (b–d) Light microscopy of sections of nodules collected in the Cerrado and the Caatinga biomes. Nodules from *Mimosa callithrix* (b), endemic to Chapada dos Guimarães in the western Cerrado (Mato Grosso); *Mimosa cordistipula* (c), an endemic of campo rupestre in Chapada Diamantina; and *Mimosa pseudosepiaria* (d), an endemic of the Caatinga. Arrows indicate thick cortex in *M. setosissima* (a) and *M. pseudosepiaria* (d). *, N_2-fixing infected zone; m, meristem. Bars, 200 μm.

Caatinga, but as effective rhizobia were isolated from 31 of them in our parallel study from the same expeditions (Bontemps *et al.*, 2010; see Tables S3, S4), the present study has completely new reports on nodulation by the following 13 species: *M. calodendron*, *M. campicola*, *M. dominarum*, *M. gatesiae*, *M. irrigua*, *M. pseudosepiaria*, *M. pyrenea*, *M. regina*, *M. setuligera*, *M. skinneri*, *M. speciosissima*, *M. ulbrichiana* and *M. verecunda*.

Soil samples taken from the rhizosphere of the *Mimosa* spp. confirmed the characteristic low pH of the Cerrado soils; for example, those near Brasilia had a pH of 4.9, whereas those from the western Cerrado (Chap. dos Guimarães) had an average pH of 5.1. The pH of the *Mimosa* rhizosphere soils in Chap. Diamantina (Caatinga) were similarly very low, with an average pH of 4.7.

Nodule structure and *in situ* identification of microsymbionts

As only fresh nodules could be analysed using microscopy, most of the nodules examined were those collected in the 2006 and 2009 wet seasons. The only exceptions from the dry season expedition were the nodules on *M. decorticans*, and those on some widespread species in Chap. dos Guimarães (Table S3). The majority of the nodules on all the species examined from both biomes (49 species in total) were found to be effective in appearance when examined under the light microscope (Tables S3, S4, Fig. 2). To ascertain whether nodules collected in the field could be regarded as likely to be 'effective' in terms of N_2 fixation they were compared anatomically with actively N_2-fixing (i.e. ARA positive) nodules on Cerrado/Caatinga species that had been grown under controlled (N-free) conditions after inoculation of seedlings with strains of *Burkholderia* isolated from nodules collected in the field (Bontemps *et al.*, 2010). The example shown in Fig. 2(a) is from an effective nodule on *M. setosissima*, a species endemic to Serra dos Pirineus in Goiás that was inoculated with the *Burkholderia* strain JPY164. This can be compared with nodules on field-collected nodules on the endemics *M. callithrix* (Fig. 2b), *M. cordistipula* (Fig. 2c) and *M. pseudosepiaria* (Fig. 2d). In all cases the field-collected nodules have the typical appearance of effective *Mimosa* nodules with an apical meristem, and an elongated infected zone containing enlarged host cells packed with N_2-fixing symbiotic bacteroids (cf. Chen *et al.*, 2005a; Elliott *et al.*, 2007b). In some cases (but not all, e.g. Fig. 2b,c), such as *M. setosissima* (Fig. 2a) and *M. pseudosepiaria* (Fig. 2d), the nodules had thick cortices with layers of tannin-containing 'corky' cells external to the endodermis which may help the nodules to retain moisture in a semi-arid or seasonally dry environment (Brown & Walsh, 1994; Gross *et al.*, 2002). *Mimosa gatesiae* from the Cerrado was the only species examined by microscopy in which all the nodules examined appeared to be senescent, although ineffective/senescent nodules were also observed on some plants of *M. adenocarpa* and *M. lewisii* (Table S4).

An attempt was then made to identify the symbionts in all of the nodules prepared for microscopy. This involved immunolabelling them with antibodies raised against two common *Mimosa* symbionts, *B. phymatum* STM815^T and *C. taiwanensis* LMG19424^T (Chen *et al.*, 2005b; Elliott *et al.*, 2007b). It was first necessary, however, to check the specificity of the antibodies, and an ELISA confirmed that the *B. phymatum* antibody strongly recognized *B. phymatum* STM815^T, as well as all the other *Burkholderia* strains that were tested, including legume-nodulating strains, plant-associated diazotrophs and environmental isolates (Table S1), but it did not react with other bacteria, including *Cupriavidus*, *Ralstonia* and *Rhizobium* strains. The *C. taiwanensis* antibody was more specific, reacting only with *C. taiwanensis* strains, and giving no cross-reaction even with other *Cupriavidus* or *Ralstonia* species. Preliminary immunolabelling tests showed that the antibodies could detect *B. phymatum* and *C. taiwanensis* within Cerrado and Caatinga *Mimosa* hosts (Table 1), but also that they did not cross-react with nodules containing the 'alternative' strain (see Elliott *et al.*, 2007b for more details). In a second preliminary experiment, the *B. phymatum* antibody could detect *Burkholderia* within symbiotic nodules from Cerrado and Caatinga endemic *Mimosa* species inoculated with strains originally isolated from them (Bontemps *et al.*, 2010), for example, *M. blanchetii* inoculated with JPY578 (Fig. 3a), but also *M. decorticans* inoculated with JPY297 and *M. setosissima* inoculated with JPY164 (not shown). Sections of nodules treated with pre-immune serum gave no reaction (Fig. 3b). Finally, when the field-collected nodules were examined, it was shown that almost all of them (i.e. 118 of the 126 *Mimosa* nodules from 49 species) reacted positively with the *B. phymatum* antibody, with the antibody reaction coinciding with the infected, N_2-fixing zone in each case (Tables S3, S4, Fig. 3c,d). There was no reaction with the pre-immune serum negative controls that were run concurrently with each sample. These results were confirmed under the TEM for selected samples, in which it was shown that the *B. phymatum* antibody mainly recognized antigens on the bacteroid surfaces (Fig. 3e,f). No nodules reacted with the antibody against *C. taiwanensis*, and the eight samples that did not react with either antibody included one nodule from *M. misera* (Caatinga), one from *M. verecunda* (Chap. dos Veadeiros), and six nodules from three separate plants of the widespread species *M. xanthocentra* (Chap. dos Guimarães). It should be noted, however, that all the other samples of *M. cordistipula*, *M. verecunda* and *M. xanthocentra* that were tested reacted positively with the *B. phymatum* antibody (Table S4).

Table 1 Acetylene reduction activity and nodule structure of *Mimosa* species from the Cerrado and the Caatinga inoculated with *Burkholderia phymatum* STM815 or *Cupriavidus taiwanensis* LMG19424

	B. phymatum STM815		*C. taiwanensis* LMG19424	
	ARA (nmol C_2H_4 per plant h^{-1})	Nodule structure[a]	ARA (nmol C_2H_4 per plant h^{-1})	Nodule structure
M. albolanata (R)	0–34	Eff	0	In
M. callithrix (E)	0	In	0	In
M. claussenii (R)	0	In	0	In
M. cordistipula (E*)	10	Eff	0	In
M. debilis (W)	1562–44640	Eff	0–10	Eff
M. decorticans (E)	0	In	0	In
M. densa (R)	0	In	0	In
M. foliolosa (R)	0–106	Eff	0	In
M. melanocarpa (R)	0	In	0	In
M. setosa (W)	551–5554	Eff	0	In
M. setosissima (E)	0	No nodules	0	No nodules
M. ursina (W)	250–1399	Eff	0	In
M. velloziana (W)	271–1232	Eff	0	In
M. xanthocentra (W)	94–1489	Eff	0–41	Eff

Nodules from all species/strain combinations were confirmed to contain the strain with which they were inoculated using antibodies against *B. phymatum* STM815 or *C. taiwanensis* LMG19424 (Elliott *et al.*, 2007b). E, endemic to the Cerrado; E*, endemic to the Caatinga; R, restricted to the Cerrado; W, widespread (Simon & Proença, 2000).
[a]Eff, effective in appearance; In, ineffective in appearance.

Nodulation of Cerrado/Caatinga species with common beta-rhizobial strains

The 'standard' *Mimosa*-nodulating beta-rhizobial strains, *B. phymatum* STM815^T and *C. taiwanensis* LMG19424^T, nodulated the five widespread species (*M. debilis*, *M. setosa*, *M. ursina*, *M. velloziana*, *M. xanthocentra*) highly effectively, but nodulated Cerrado-restricted species either much less effectively (*M. albolanata*, *M. foliolosa*) or ineffectively (*M. claussenii*, *M. densa*, *M. melanocarpa*), and the Cerrado endemic species either ineffectively (*M. callithrix*, *M. decorticans*) or not at all (*M. setosissima*) (Table 1). By contrast, *C. taiwanensis* LMG19424^T could only form slightly effective nodules on *M. debilis* and *M. xanthocentra*, and ineffective nodules on all the other species, except for *M. setosissima*, which it could not nodulate. No Caatinga endemics were tested, but *Burkholderia phymatum* STM815^T, but not *C. taiwanensis* LMG19424^T, was also capable of forming slightly effective nodules on *M. cordistipula*, the only Caatinga endemic tested (Table 1).

In situ immunolocalization of *nifH* protein in field-collected nodules

Nodules from 23 species were examined for nitrogenase expression by immunogold labelling with an antibody raised against the Fe-protein (*nifH* protein) of nitrogenase (Table 2). When possible, the plants from which the nodules were sampled were the same as those from which leaves were collected for $\delta^{15}N$ analysis (Table 3). The specificity of the *nifH* protein antibody was demonstrated in Western blots of extracts of *M. pudica* nodules that were infected with either of the 'standard' beta-rhizobial strains, *B. phymatum* STM815^T and *C. taiwanensis* LMG19424^T (Fig. 4). The symbioses formed by *M. pudica* with either of these strains are known to be highly effective in terms of N_2 fixation (Elliott *et al.*, 2007b). The *nifH* antibody recognized a strong single immunoreactive band of an approximate molecular mass of 36 kDa in both kinds of nodules and also a single band of 37 kDa in alfalfa nodules used as positive controls. In preliminary tests on sections of nodules from plants grown under controlled-environment conditions and which had tested positive for nitrogenase activity, nodules from *M. debilis* and *M. ursina* inoculated with *B. phymatum* STM815^T (Table 1), *M. blanchetii* plants inoculated with *Burkholderia* sp. JPY578 (Table 2) and *M. setosissima* plants inoculated with *Burkholderia* sp. JPY164 (Table 2) all gave a positive immunogold reaction with the antibody (Fig. 5a), whereas nodules from nonN_2-fixing associations (e.g. *M. himalayana* inoculated with *C. taiwanensis* LMG19424^T; Elliott *et al.*, 2007b) gave no signal (not shown, but similar to Fig. 3b), thus demonstrating that the antibody could be used to probe nodules collected in the field. Fifteen of the 17 Cerrado species and five of the six Caatinga species gave a positive signal with the *nifH* protein antibody (Table 2), and the reaction coincided only with regions of the infected zone that were nonsenescent (Fig. 5b,c). The specificity of the antibody only to bacteroids was confirmed under the TEM for *M. caesalpiniifolia* (Fig. 5d,e) and *M. regina* (both from the Cerrado), and for *M. cordistipula* (from *campo rupestre* in Chap. Diamantina)

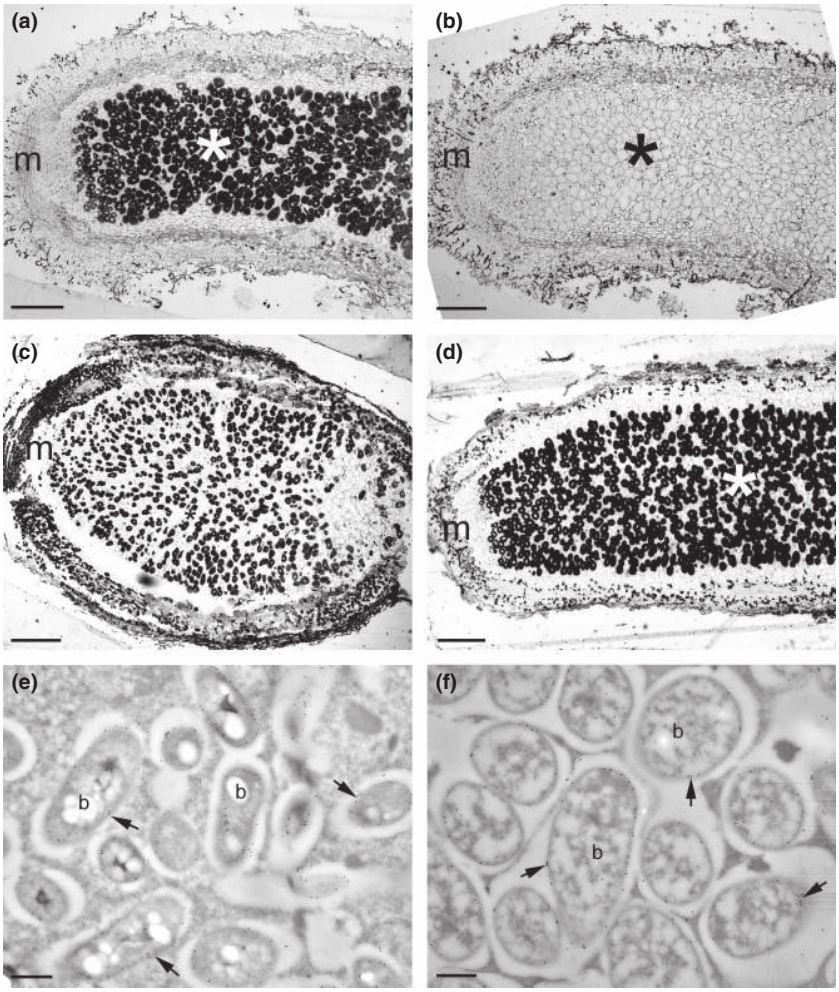

Fig. 3 Light microscopy (a–d) and transmission electron microscopy (TEM) (e, f) of sections of *Mimosa* nodules immunogold-labelled either with an antibody against *Burkholderia phymatum* STM815 (a, c–f) or with pre-immune serum (b). (a, b) Nodules from *M. blanchetii* inoculated with *Burkholderia* sp. JPY578. Note the strong reaction with the STM815 (a, c–f) or with pre-immune serum (b). (c, d) Light microscopy of nodules from the Cerrado endemic *M. manidea* (c) and the widespread species *M. xanthocentra* var. *subsericea* (d). (e, f) TEM of the Cerrado endemic *M. regina* (e) and the Caatinga endemic *M. cordistipula* (f). Note that the surfaces of the bacteroids in both nodules are strongly labelled (arrows), but not the bacteroid cytoplasm. *, N_2-fixing infected zone; b, bacteroids; m, meristem; s, senescent zone. Bars, 200 μm (a–d); 500 nm (e, f).

(Fig. 5f) and *M. ulbrichiana* (from the Caatinga) (not shown). Interestingly, the three samples that were negative (*M. adenocarpa*, *M. gatesiae* and *M. lewisii*) had previously been identified as having nodules that were senescent and/or ineffective in appearance (Table S4).

δ^{15}N content of *Mimosa* spp.

Leaf samples taken from vegetation growing in undisturbed areas of the Cerrado had significantly lower δ^{15}N values than those from corresponding Caatinga vegetation. This is well illustrated by the *Mimosa* spp. (Fig. 6), but is also clearly evident in a whole range of dicotyledonous species from both biomes. The mean δ^{15}N signals of the nonlegumes, nonnodulated legumes (all *Bauhinia* spp.) and *Mimosa* spp. in the Cerrado were similar to those reported previously by Sprent *et al.* (1996) and Bustamante *et al.* (2004), and the significantly lower values of the *Mimosa* spp. compared with the nonnodulated plants (Fig. 6) suggest that the mimosas were probably fixing N_2 (Unkovich *et al.*, 2008). However, on a site-to-site basis, the highly variable and rather low δ^{15}N signals in the Cerrado made it impossible to apply the δ^{15}N technique to estimate the amounts of N fixed by the nodulated *Mimosa* spp., as the differences between the 'fixing' plants and the immediately neighbouring 'nonfixing' plants were not significant (data not shown). On the other hand, in the Caatinga, although the δ^{15}N signals of the nodulated *Mimosa* spp. were much higher than those from the Cerrado, on average the neighbouring nonlegume reference plants had δ^{15}N values that were > 3‰ greater than the corresponding nodulated legumes (Fig. 6), and in many cases the difference between them was even greater (up to 5‰ for *M. gemmulata* and *M. ulbrichiana*; Table 3), and so the technique could be applied to those species that were significantly different from their nearest nonnodulated neighbours (Table 3). Thus, using a *B*-value obtained from *M. caesalpiniifolia* plants inoculated with *B. sabiae* Br3407, %Ndfa values of 38% for *M. blanchetii*, 51% for *M. gemmulata*, and 60% for *M. ulbrichiana* were obtained. However, for others, such as *M. hypoglauca*, *M. lewisii*, *M. misera* and *M. polydidyma*, there were no significant differences between the *Mimosa*

Table 2 *In situ* immunogold reaction of nodules from Cerrado and Caatinga *Mimosa* species with an antibody against the *nifH* protein (Fe-protein) of the nitrogenase enzyme complex

Species	*nifH* antibody reaction
Cerrado	
M. albolanata	+
M. adenocarpa (i)[a]	–
M. caesalpiniifolia	+
M. callithrix	+
M. claussenii	+, +
M. cyclophylla	+
M. densa (i)	–
M. foliolosa	+
M. laniceps	+
M. manidea	+
M. melanocarpa	+
M. radula	+
M. regina	+
M. speciosissima	+
M. splendida	+
M. ulei	+
M. venatorum	+
M. setosissima	+
+ *Burkholderia* sp. JPY164[c]	
Caatinga	
M. blanchetii[b]	+
M. cordistipula	+
M. gemmulata[b]	+
M. lewisii (i)	–
M. misera[b]	+
M. ulbrichiana[b]	+
M. blanchetii	+
+ *Burkholderia* sp. JPY578[c]	

[a]All nodules examined had previously been identified as being effective in anatomical appearance with the exception of those marked as ineffective (i).
[b]Samples from plants showing significant contributions by biological N_2 fixation using the $\delta^{15}N$ assay (Table 3).
[c]Nodules from actively N_2-fixing plants inoculated with homologous *Burkholderia* strains giving mean acetylene reduction assay (ARA) values ± SEM (n = 4) of 1152 ± 422 nmol C_2H_4 per plant h^{-1} (*M. setosissima* + JPY164) and 420 ± 77 nmol C_2H_4 per plant h^{-1} (*M. blanchetii* + JPY578).

spp. and their neighbouring nonlegumes (Table 3), and so their %Ndfa was assumed to be negligible.

Discussion

Nodulation is a generic characteristic of *Mimosa*

This study, together with the parallel study of Bontemps *et al.* (2010) which examined the molecular phylogeny of the bacteria isolated from many (but not all) of the species in the present study, has shown that 67 species of *Mimosa* native to Brazil are nodulated and that 44 of these are new reports of nodulation. Sprent (2001) listed 54 species as being nodulated, and so the present study, together with those of Elliott *et al.* (2007b) and Lammel *et al.* (2007), brings the total number of *Mimosa* spp. reported to nodulate to 109, which is > 20% of this large genus of > 500 species (Barneby, 1991; Simon & Proença, 2000; Du Puy *et al.*, 2002). In the majority of cases (47 species), nodulation was confirmed in the present study not just by visual inspection of roots for nodule-like outgrowths, which when used alone has the potential to generate erroneous reports of nodulation (Sprent, 2001), but also by detailed anatomical analyses. These have shown that all of the samples (except for those from *M. gatesiae*) were typical of effective, N_2-fixing *Mimosa* nodules, being very similar in longitudinal profile to nodules reported in studies in which N_2 fixation was also measured and/or the nitrogenase enzyme was localized (compare Fig. 3 with Chen *et al.*, 2005a,b; Elliott *et al.*, 2007b). For the other 20 species on which only dry nodules were sampled, their symbiotic ability was confirmed in several cases via isolation of effective *Burkholderia* symbionts (Bontemps *et al.*, 2010) (e.g. *M. setosissima*; Fig. 3a). No nodules could be found on three species in the present study (i.e. *M. echinocaula*, *M. polydidyma*, *M. modesta*), but given the apparently generic nodulation trait in *Mimosa* that this study and others have shown (Elliott *et al.*, 2007b), it is unlikely that they are nonnodulating, and their nodulation was probably inhibited by the dry conditions in which they were found (Sprent, 2009).

Burkholderia are the predominant symbionts of *Mimosa* in the Cerrado and the Caatinga

In situ immunolocalization of 128 separate field-collected *Mimosa* nodules with an antibody against *B. phymatum* STM815^T (Chen *et al.*, 2005b; Elliott *et al.*, 2007b) has demonstrated that the symbionts of 47 species of *Mimosa* in important centres of diversity of this major legume genus are *Burkholderia*. This is also supported by sequences of the 16S rRNA and *recA* genes of 148 strains that were isolated from nodules of 47 *Mimosa* species (Bontemps *et al.*, 2010), many of which were from among the same species examined by microscopy in the present study. Indeed, by using two techniques of symbiont identification from nodules collected in the field, the present study and that of Bontemps *et al.* (2010) have brought the total number of South American *Mimosa* spp. known to have *Burkholderia* as symbionts to > 60 (including those reported by Chen *et al.*, 2005a).

As with nodulation, the degree of endemism apparently had no effect on the general choice of symbiont by the *Mimosa* spp., but it should be noted that the *B. phymatum* antibody used by the *in situ* immunolocalization technique does not allow for detailed identification of the *Burkholderia* symbionts beyond genus level, and so differences between *Mimosa* spp. in their selection of particular *Burkholderia* species/strains cannot be excluded by using

Table 3 $\delta^{15}N$ (‰ ± SEM) values of nodulated *Mimosa* spp. in the Caatinga biome together with those of the immediately neighbouring non-legume reference plants

Test plant	$\delta^{15}N$	Reference plants	$\delta^{15}N$	%Ndfa
M. blanchetii	5.16 ± 0.72	*Sebastiana* sp. (Euphorbiaceae)	9.45 ± 0.12*	38
		Tibouchina sp. (Melastomataceae)	8.76 ± 0.68*	
M. gemmulata	5.58 ± 0.78	*Microtea maypurensis* (Phytolaccaceae)	13.29 ± 1.05*	51
		Unknown dicot.	11.85 ± 0.29*	
M. hypoglauca	0.59 ± 0.28	*Byrsonima* sp. (Malpighiaceae)	1.0 ± 0.25^{ns}	nd
		Croton sp. (Euphorbiaceae)	0.56 ± 0.16^{ns}	
M. lewisii	1.24 ± 0.33	*Eriope latifolia* (Lamiaceae)	2.76 ± 0.44^{ns}	nd
		Sebastiania corniculata (Euphorbiaceae)	2.51 ± 0.46^{ns}	
M. misera	8.51 ± 0.96	*Croton campestris* (Euphorbiaceae)	11.65 ± 0.42^{ns}	nd
		Sida cordifolia (Malvaceae)	10.88 ± 0.29^{ns}	
M. polydidyma	3.83 ± 1.98	*Eugenia* sp. (Myrtaceae)	4.99 ± 0.33^{ns}	nd
		Stigmaphyllon paralias (Malpighiaceae)	3.38 ± 0.74^{ns}	
M. ulbrichiana	3.00 ± 1.22	*Glischrothamnus ulei* (Molluginaceae)	11.03 ± 1.06*	60
		Simaba ferruginea (Simaroubaceae)	8.83 ± 0.52*	
		Copaifera coriacea (Caesalpinioideae)	8.57 ± 0.14*	

Proportion of N derived from air (%Ndfa) was calculated using a '*B*-value' of –1.24 $\delta^{15}N$ for those plants that were significantly different from their neighbouring reference plants.
*Significantly different at the 5% level from value obtained from neighbouring *Mimosa* sp. using a two-tailed *t*-test; ns, not significantly different; nd, not determined.

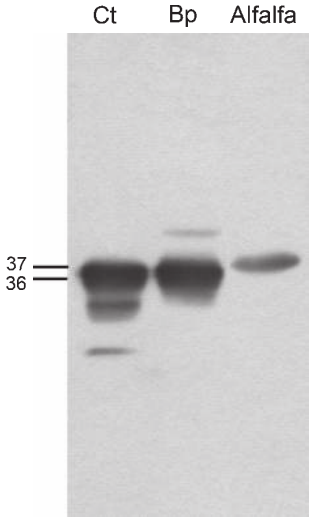

Fig. 4 Western blot of extracts from *Mimosa pudica* nodulated with *Cupriavidus taiwanensis* LMG19424 (Ct) or with *Burkholderia phymatum* STM815 (Bp) probed with an antibody against the iron protein (*nifH* protein) of nitrogenase. A third lane contains extracts from alfalfa nodules infected with *Sinorhizobium meliloti* 102F78.

this technique alone. Indeed, Bontemps *et al.* (2010), after examining *Burkholderia* strains that were isolated from nodules collected during the same expeditions, have shown that one particular species complex ('SC5') predominated at altitudes above 800 m. This suggests that although endemism *per se* is not a factor governing symbiont selection, it is possible that endemics, most of which have evolved at higher altitudes, such as *M. decorticans* and *M. setosissima* (Simon & Proença, 2000; Simon & Hay, 2003), have selected symbionts of a particular type that live predominantly in the soils at these altitudes. Another indicator that high-altitude endemics are selective in their choice of symbionts was the fact that the promiscuous *Mimosa* symbiont, *B. phymatum* STM815^{T} (Elliott *et al.*, 2007b), could not nodulate any of the Cerrado endemics effectively, and, in the case of *M. setosissima*, not at all.

Other symbionts of *Mimosa* in the Cerrado and Caatinga

Given that it is very frequently found as a symbiont of the (nonnative) invasive *Mimosa* spp. (*M. diplotricha* and *M. pudica*) in Asian countries, such as India (Verma *et al.*, 2004), Papua New Guinea (Elliott *et al.*, 2009) and Taiwan (Chen *et al.*, 2001, 2005b), as well as in native *Mimosa* spp. in Costa Rica and Texas (Barrett & Parker, 2006; Andam *et al.*, 2007), one of the most striking results of the present study was that no nodules reacted with the antibody against *C. taiwanensis* LMG19424. Bontemps *et al.* (2010) also failed to isolate *Cupriavidus* from any of the *Mimosa* spp. that they examined, and it has been previously shown in comparative studies of nodulation with *B. phymatum* STM815^{T} and *C. taiwanensis* LMG19424^{T} that Cerrado and Caatinga *Mimosa* spp. prefer *Burkholderia* as a symbiont (Table 3; Elliott *et al.*, 2007b). One possible explanation for its apparent absence is that *Burkholderia* is so dominant as a symbiont of *Mimosa* spp. in these biomes that it may out-compete other potential symbionts for nodulation even of species that have a strong affinity for nodulation by them, such as *M. pudica* and *M. diplotricha* (Chen *et al.*, 2003). Indeed, this would be especially true in the

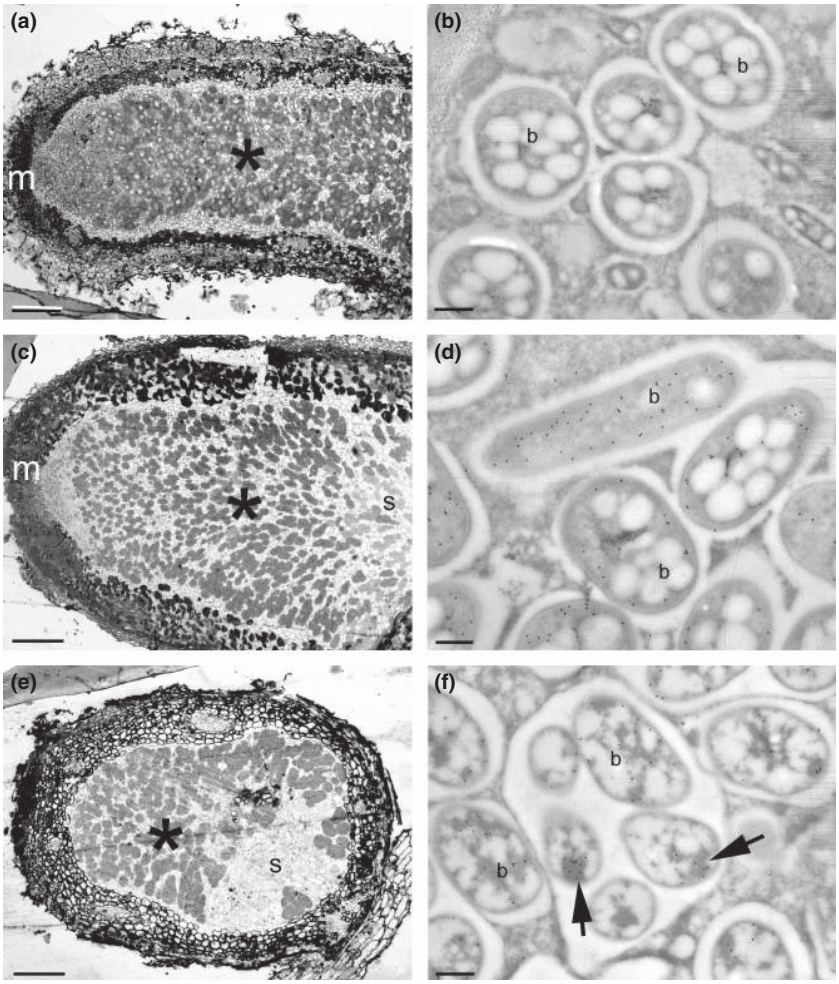

Fig. 5 Light microscopy (a, c, e) and TEM (b, d, f) of sections of *Mimosa* nodules immunogold labeled either with an antibody against the *nifH* protein (a, c - f) or with pre-immune serum (b). (a) Serial section to Figs 4 a, b of a nodule from an actively N_2-fixing *M. blanchetii* plant after inoculation with *Burkholderia* sp. JPY578 showing a positive signal with the *nifH* protein antibody. Nodules from the Cerrado endemic *M. regina* (c) and *M. ulbrichiana* (e) both also show positive reactions, but note that there is no signal in the senescent part of the latter nodule. TEM of bacteroids from nodules on *M. caesalpiniifolia* (d) and *M. cordistipula* (f). Panel (b) was treated with pre-immune serum and shows that there is no non-specific labeling of the bacteroids, whereas the cytoplasm of the bacteroids in (e) and (f) are strongly labelled with the *nifH* protein antibody. Note that the labelling of the bacteroids is largely concentrated within electron dense regions of their cytoplasm (arrows in f). Bars = 200 μm (a, c, e), 500 nm (b, d, f).

very low N-containing soils of the Cerrado and Caatinga, which would give *Burkholderia* a competitive advantage over other potential *Mimosa*-nodulating bacteria (Elliott *et al.*, 2009). The other thing that would favour *Burkholderia* is the low pH of the soils in both the Cerrado (Adámoli *et al.*, 1986) and the Caatinga (e.g. in Chap. Diamantina) (this study; Reis *et al.*, 2004; Garau *et al.*, 2009). On the other hand, there was some evidence that *Rhizobium* was occasionally a symbiont of *M. xanthocentra*, a very widespread species in South America (Simon & Proença, 2000). The six samples from *M. xanthocentra* collected in the Chap. dos Guimarães that failed to react with either the *B. phymatum* or *C. taiwanensis* antibodies did react faintly with an antibody raised against *Rhizobium etli* bv. *mimosae* (EK James & W-M Chen, unpublished), a symbiont commonly found in nodules from *Mimosa* species in Mexico (Wang *et al.*, 1999). In addition, although most were identified as *Burkholderia*, some of the bacteria isolated from the *M. xanthocentra* nodules collected in this location were identified as *Rhizobium* sp. (Bontemps *et al.*, 2010), and hence *M. xanthocentra* was the only species that was shown by both *in situ* immunogold labelling and via identification of bacterial isolates to have symbionts other than *Burkholderia*. Interestingly, it was also the only Cerrado/Caatinga species that tested positively for (slightly) effective nodulation by *C. taiwanensis* LMG19424 (Table 3).

Burkholderia fixes N_2 within *Mimosa* nodules in the Cerrado and the Caatinga

Although it is now known that many *Burkholderia* spp. are diazotrophic (Estrada de Los Santos *et al.*, 2001; Martínez-Aguilar *et al.*, 2008), including nodulating strains (Chen *et al.*, 2005b; Elliott *et al.*, 2007b), this is the first study to examine a component of the nitrogenase enzyme complex in *Burkholderia*. Unsurprisingly for such a highly conserved protein, the iron protein (*nifH* protein or dinitrogenase reductase) in both *B. phymatum* and *C. taiwanensis* bacteroids had a similar molecular weight to that of other diazotrophs, such as *Sinorhizobium meliloti*. Moreover, the *nifH* protein in *B. phymatum* and *C. taiwanensis* is probably a

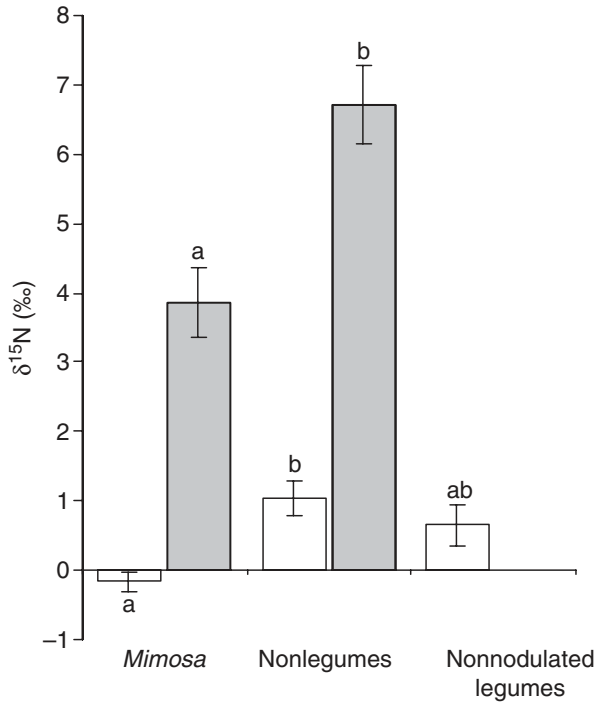

Fig. 6 Mean $\delta^{15}N$ signatures (± SEM) of leaves from *Mimosa* and neighbouring dicotyledonous nonlegumes and nonnodulated legumes (mainly *Bauhinia* spp.) collected in the Cerrado (open bars) and the Caatinga (tinted bars) in March 2006 (wet season). Means differ statistically among plant types for both Cerrado and Caatinga (ANOVA, $F = 9.2$, $P = 0.0002$; *t*-test, $t = -3.7$, $P = 0.0004$; respectively), and a multiple comparison test found that *Mimosa* and nonlegumes are statistically different in the Cerrado (*post hoc* Tukey HSD test, $P < 0.05$).

dimer, as is also the case with *S. meliloti* (Petrova *et al.*, 2002). Sections of nodules from 23 Cerrado and Caatinga *Mimosa* species immunogold-labelled using the *nifH* antibody confirmed that the *Burkholderia* endophytes of 20 of them expressed nitrogenase protein and hence were likely to be symbiotically effective. The high specificity of the antibody to nitrogenase proteins was further illustrated using TEM, which showed that the enzyme was highly localized on electron-dense regions within the bacteroids of *M. caesalpiniifolia*, *M. cordistipula*, *M. regina* and *M. ulbrichiana*.

Further evidence for biological N_2 fixation (BNF) by the *Mimosa–Burkholderia* symbioses comes from $\delta^{15}N$ analysis of leaf material collected from *Mimosa* spp., especially those collected from Chap. Diamantina in the Caatinga of Bahia state. In this biome, although the $\delta^{15}N$ values were significantly higher than those in the Cerrado (this study; Sprent *et al.*, 1996; Bustamante *et al.*, 2004), and are similar to those reported in the Caatinga from the more northerly states of Paraiba and Pernambuco (Freitas *et al.*, 2010), the difference between the mean $\delta^{15}N$ signal of the nodulated legumes (*Mimosa* spp.) and the neighbouring nonlegumes was sufficiently large to apply the $\delta^{15}N$ assay, and a range of %Ndfa values were obtained suggesting very substantial contributions of BNF to the N nutrition of *Mimosa* spp. In the cases of *M. blanchetii*, *M. gemmulata* and *M. ulbrichiana*, evidence for BNF was actually obtained from both techniques (immunogold labelling of *nifH* protein and $\delta^{15}N$), whereas a *Mimosa* sp. that showed no BNF contribution via its $\delta^{15}N$ signal was not nodulated (*M. polydidyma*). Similarly, two other species which showed no BNF contribution via their $\delta^{15}N$ signals had only either dry nodules (*M. hypoglauca*) or senescent ones (*M. lewisii*). In the case of the Cerrado, however, although the mean $\delta^{15}N$ signal of the nonlegumes and the nonnodulating legumes (*Bauhinia* app.) was significantly higher than the nodulated *Mimosa* spp., thus suggesting that the latter were fixing N_2 (Sprent *et al.*, 1996), the high variability of the $\delta^{15}N$ signal at all sites did not allow for the calculation of their %Ndfa in this biome (Gehring & Vlek, 2004; Unkovich *et al.*, 2008).

Concluding remarks

This study has shown that nodulation is a generic character of the genus *Mimosa*, and that *Burkholderia* strains are the predominant symbionts in two of the major centres of diversity of this genus, that is, the Cerrado and the Caatinga. It has also shown that nodulated mimosas, including rare and endangered ones, such as *M. splendida* (Simon & Amaral, 2003), *M. setosissima* and *M. decorticans* (Simon & Hay, 2003), can fix N_2 within their native environments, and therefore that they may make a valuable contribution to the N-cycle of the fragile ecosystems of the Cerrado and the Caatinga. This study is also the first demonstration of BNF by beta-rhizobial symbioses under field conditions.

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Supporting Information

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

Fig. S1 (a) *Mimosa decorticans*; (b) *M. splendida*; (c) *M. crumenarioides*; (d) *M. ursina*.

Table S1 Locations of *Mimosa*-collecting expeditions in the Cerrado and Caatinga dry (September 2005) and wet seasons (March 2006, October 2009).

Table S2 Enzyme-linked immunosorbent assay of various alpha and beta-proteobacterial strains (including rhizobia) using antibodies raised against *Burkholderia phymatum* STM815 and *Cupriavidus taiwanensis* LMG19424.

Table S3 Nodulation, nodule structure and the *in situ* reaction of the symbionts in the nodules to antibodies against *Burkholderia phymatum* STM815 (Bp) and *Cupriavidus taiwanensis* LMG19424 (Ct) of *Mimosa* species collected in the Cerrado and Caatinga in the dry season (September) of 2005.

Table S4 Nodulation, nodule structure and the *in situ* reaction of the symbionts in the nodules to antibodies against *Burkholderia phymatum* STM815 (Bp) and *Cupriavidus taiwanensis* LMG19424 (Ct) of *Mimosa* species collected in the Cerrado and Caatinga in the wet season (March) of 2006 or 2009[#].

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