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Research

Endemic *Mimosa* species from Mexico prefer alphaproteobacterial rhizobial symbionts

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Summary

- The legume genus *Mimosa* has > 500 species, with two major centres of diversity, Brazil (*c.* 350 spp.) and Mexico (*c.* 100 spp.). In Brazil most species are nodulated by *Burkholderia*. Here we asked whether this is also true of native and endemic Mexican species.
- We have tested this apparent affinity for betaproteobacteria by examining the symbionts of native and endemic species of *Mimosa* in Mexico, especially from the central highlands where *Mimosa* spp. have diversified. Nodules were tested for betaproteobacteria using *in situ* immunolocalization. Rhizobia isolated from the nodules were genetically characterized and tested for their ability to nodulate *Mimosa* spp.
- Immunological analysis of 25 host taxa suggested that most (including all the highland endemics) were not nodulated by betaproteobacteria. Phylogenetic analyses of *16S* rRNA, *recA*, *nodA*, *nodC* and *nifH* genes from 87 strains isolated from 20 taxa confirmed that the endemic Mexican *Mimosa* species favoured alphaproteobacteria in the genera *Rhizobium* and *Ensifer*: this was confirmed by nodulation tests.
- Host phylogeny, geographic isolation and coevolution with symbionts derived from very different soils have potentially contributed to the striking difference in the choice of symbiotic partners by Mexican and Brazilian *Mimosa* species.

Introduction

Bacteria called 'rhizobia' form nodules on the roots of many legumes (Fabaceae) (Graham, 2008; Sprent, 2009) and are recognised as the main contributors of biologically-fixed nitrogen to undisturbed terrestrial ecosystems (Cleveland *et al.*, 1999). Until early this century, known rhizobia were confined to a few genera in the order Rhizobiales of the class Alphaproteobacteria (Graham, 2008), but it is now known that some legumes may also form effective nodules with Betaproteobacteria in the genera *Burkholderia* and *Cupriavidus* (Gyaneshwar *et al.*, 2011). Most studies so far have been carried out on the genus *Mimosa* (tribe Mimoseae, subfamily Mimosoideae). Approximately 500 species are native to the tropical and subtropical New World, but there are two Old World centres in Madagascar/East Africa (*c.* 30 spp.) and Asia (6 spp.) (Simon *et al.*, 2011). Species vary in habit from tall trees and shrubs to vines and herbs. They are found in a wide variety of habitats from wet to dry, growing on many different soils, including those that are very low in nutrients and organic matter, and low in pH. *Mimosa* is particularly abundant and diverse in the Cerrado and Caatinga biomes of Brazil, where there are many endemics, particularly at elevations above 1000 m a.s.l. (Barneby, 1991; Simon & Proença, 2000; Simon *et al.*, 2011). Almost all of the > 100 species that have been examined have been found to be nodulated, and thus it appears that nodulation is a generic character (Chen *et al.*, 2005a; dos Reis Junior *et al.*, 2010; Gehlot *et al.*, 2013; Lammel *et al.*, 2013). In terms of their symbionts, most work has been on widespread and/or invasive species. Mainly betarhizobial strains, particularly in the species *C. taiwanensis*, *B. mimosarum* and *B. phymatum*, have been isolated from the three major invasive *Mimosa* weed species (*M. diplotricha*, *M. pigra* and *M. pudica*) in many Southeast Asian tropical regions, such as Taiwan (Chen *et al.*, 2001, 2005b), India (Gehlot *et al.*, 2013), northern Australia (Parker *et al.*, 2007), Papua-New Guinea (Elliott *et al.*, 2007, 2009), southern China

(Liu *et al.*, 2012), the Philippines (Andrus *et al.*, 2012) and New Caledonia (Klonowska *et al.*, 2012).

Although we have learnt much about the symbionts of these three aggressive invasive species, are they representative of the vast majority of *Mimosa* species, most of which are highland endemics with a highly restricted range and distribution (Simon & Proença, 2000; Simon *et al.*, 2011)? In order to address this, Bontemps *et al.* (2010) and dos Reis Junior *et al.* (2010) examined the symbionts of *Mimosa* spp. native to the largest centres of radiation – the Cerrado and Caatinga biomes in central Brazil (together containing *c.* 250 spp.). They found that almost all of the 70 (mostly endemic, but also some widespread) species examined were exclusively nodulated by *Burkholderia*. Regardless of their degree of endemism, all the *Mimosa* species nodulated with *Burkholderia* strains that were genetically similar to each other, but the widespread ones were also capable of nodulating with other symbiont types, such as promiscuous strains of *Burkholderia* and *C. taiwanensis* (dos Reis Junior *et al.*, 2010). This suggests not only that the environment in which they have evolved is of great importance for the selection of *Mimosa* rhizobial symbionts, but also that their restriction to very particular localities has meant that the endemic Brazilian species have become very specialized in their selection of symbionts, whereas the widespread ones have remained capable of nodulating with a more diverse range of rhizobia (Elliott *et al.*, 2009; Bontemps *et al.*, 2010; Melkonian *et al.*, 2014).

The general aim of the present study was to investigate further the relationships between rhizobial symbionts and their *Mimosa* hosts, but in this case in the second largest centre of radiation of the genus, Mexico, which houses *c.* 100 species (Barneby, 1991; Grether *et al.*, 1996; Simon *et al.*, 2011). As in Brazil, native Mexican *Mimosa* species are a mixture of widespread and endemic species, with many of the latter residing in the central highlands/altiplano at altitudes above 1000 m a.s.l. (Supporting Information Fig. S1) (Martínez-Bernal & Grether, 2006; Grether *et al.*, 2007; Martínez-Bernal *et al.*, 2008). The widespread Mexican species are also found throughout the tropical New World, including Brazil, but the central Mexican endemics, which are closely related to each other, are confined to particular clades that are quite distant from those containing, for example, the central Brazilian endemics (Simon *et al.*, 2011). It is possible that these Mexican endemics have selected different rhizobial symbionts as a result of their geographic and taxonomic separation from the Brazilian endemics, and their subsequent evolution in a different environment (e.g. within neutral–alkaline rather than acid soils). Indeed, one of the few earlier studies conducted on the symbionts of Mexican *Mimosa* showed that a common Mesoamerican species, *M. affinis* (Grether, 2001), was nodulated by *Rhizobium etli* sv mimosae, a close relative of symbionts of common bean (*Phaseolus vulgaris* L.) (Wang *et al.*, 1999), although a more recent study has shown that the Mexican native species *M. occidentalis* was nodulated by *Burkholderia* (Ormeño-Orrillo *et al.*, 2012).

The present study had the following specific aims: to study nodulation of *Mimosa* species in the central and western Mexican highlands/altiplano; to isolate rhizobia from *Mimosa* nodules collected from plants growing in their native environments and/or grown in soil collected from their rhizospheres, and genetically characterize the rhizobial isolates by comparing sequences of some of their 'housekeeping' and symbiosis-essential genes with those in the databases; and to perform cross-inoculation studies to determine the symbiotic preferences and host range of representative Mexican *Mimosa* isolates.

Materials and Methods

Sampling of nodules, seeds and soils

Species of *Mimosa* were sampled from various locations in central and western Mexico in September 2007 and in October 2008 (Tables 1, 2, S1; Figs S1, S2). Strain CCGE1002 was isolated in 2006 from a field in Nayarit in western Mexico (Ormeño-Orrillo *et al.*, 2012) (Tables 1, S1). Voucher specimens were taken for all species and deposited in the herbarium at UAM-Iztapalapa (UAMIZ), Mexico City, and the locations from where they were sampled can be seen using Google Earth© (Notes S1). As many of the species are rare and nodule harvesting is destructive, we minimised the number of plants taken. Seeds were collected if present. In both expeditions, nodules (if present) were collected and preserved in silica gel for later bacterial isolation. Some nodules (3–4 per plant) were also cut in half to determine if they were potentially active and effective by the appearance of a pink colouration due to 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 microscopical analysis. Soil was also taken for rhizobial 'trapping' experiments using seedlings of the species that was originally found in that soil. Soil characteristics are listed in Table S2. The trapping experiments were conducted at CCG, UNAM, Cuernavaca, Mor., Mexico. Seeds of *Mimosa* spp. were germinated according to Elliott *et al.* (2007), and were placed in the appropriate rhizosphere soil in small pots (300 ml). Seeds of Mexican species that did not have soil particular to them were rooted in a mixture of all the soils. Nodules were sampled 3 months after the seeds were sown, and treated as for field-collected nodules.

Microscopy and *in situ* detection of microsymbionts

Pink nodules collected in the field or from trap experiments 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 (IGL-SE) using antibodies raised against *Burkholderia phymatum* STM815^T and *Cupriavidus taiwanensis* LMG19424^T according to dos Reis Junior *et al.* (2010).

Bacterial strains, DNA extraction and amplification

Rhizobia were isolated from *Mimosa* nodules according to Bontemps *et al.* (2010). Bacteria from glycerol stocks were grown at 28°C for 3 d on TY medium (Beringer, 1974); a single colony for each sample was then transferred to 5 ml of liquid TY medium and grown at 28°C in a shaking incubator for 3 d. As

Table 1 Nodulation of *Mimosa* species collected in central and western Mexico in 2006 and 2007

Nodule sample (Herbarium voucher no.)	Species (E, endemic to Mexico; R, restricted to Mexico & Central America; W, widespread in the Americas)	State and location/vegetation type from where nodules were collected (more details are given in Supporting Information Table S3 and can be viewed on GoogleEarth; Notes S1)	Elevation (m a.s.l.)	Rhizobial isolates obtained (JPY) and their generic identification via sequencing of their *16S* rRNA and *recA* genes (*16S–recA* 'clusters' in parentheses)
UoD 189	*M. affinis* B.L. Rob. (R)	Mor., Cuernavaca – Tepotzlán. Roadside.	1418	None isolated
UoD 191	*M. albida* H. & B. ex. Willd. var. *albida* (W)	Mor., Cuernavaca – Tepotzlán. Roadside.	1418	None isolated
UoD 199	*M. albida* H. & B. ex. Willd. var. *strigosa* (Willd.) B.L. Rob. (W)	Mor., Cuernavaca – Tepotzlan. Roadside.	2019	*Rhizobium* **(5) 1075^{Mp}**
UoD 200	*M. albida* H. & B. ex. Willd. (W)	Mor., Cuernavaca. Roadside.	1907	None isolated
UoD 201	*M. affinis* B.L. Rob. (R)	Pue., Tepexco. Pasture.	1242	None isolated
UoD 207	*M. tricephala* Schltdl. & Cham. var. *tricephala* (E)	Pue., Izucar de Matamoros. Roadside.	1372	*Rhizobium* **(3) 820$^{**, Mp}$** **(6) 810^{Mp},** 811^{nt}
UoD 208	*M. benthamii* J.F. Macbr. var. *malacocarpa* (B.L. Rob.) J.F. Macbr.* (E)	Pue., Izucar de Matamoros. Pasture.	1273	None isolated
UoD 210	*M. mollis* Benth.* (E)	Pue. Izucar – Cuatla. Roadside.	1453	None isolated
UoD 211	*M. lactiflua* Delile ex Benth.* (E)	Pue. Izucar – Cuatla. Roadside.	1288	None isolated
UoD 212	*M. tricephala* Schltdl. & Cham. var. *tricephala** (E)	Pue., Izucar de Matamoros. Roadside.	1323	*Ensifer* **(2) 851$^{**, Mp}$, 996^{Mp}, 998^{Mp}**
UoD 222	*M. albida* H. & B. ex. Willd. var. *strigosa* (Willd.) B.L. Rob. (W)	Mor., Xochicalco. Pasture.	1368	*Rhizobium* **(6) 773$^{**, Mp}$**
UoD 223	*M. depauperata* Benth.* (E)	Qro., Tequisquiapan. Roadside.	1900	None isolated
UoD 224	*M. lacerata* Rose* (E)	Qro., Cadereyta. Roadside.	2174	None isolated
UoD 230	*M. depauperata* Benth. (E)	Qro., Toliman. Roadside.	1735	None isolated
UoD 232	*M. aculeaticarpa* Ortega (E)	Gto., Ranch Santa Ines. Roadside.	2192	None isolated
UoD 233	*M. monancistra* Benth.* (E)	Gto., San Miguel de Allende. Roadside.	1939	*Rhizobium* **(6) 826$^{**, Mp}$**
UoD 236	*M. albida* H. & B. ex. Willd. var. *albida* (W)	Gto., Campuzana. Roadside.	2141	*Rhizobium* **(5) 880^{Mp}, 888^{Mp+}**
UoD 239	*M. tequilana* S. Watson* (E)	Jal., Tequila. Roadside.	1174	*Rhizobium* **(4) 934^{Mp},** 947^{nt} **(6) 936, 940^{Mp}** 924^{nt}, 926^{nt}, 946^{nt}
UoD 244^{Bp}, 246	*M. skinneri* Benth. var. *skinneri* (W)	Jal., Tequila – Tepic. Roadside.	1203	*Burkholderia* **(1) 807^{Mp+}** *Rhizobium* **(4) 794^{Mp},** 785^{nt}, 792^{nt} **(5) 877^{Mp+}** **(6) 783^{Mp+},** 740^{nt}
UoD 245^{Bp}	*M. somnians* H. & B. ex. Willd. (W)	Jal., Tequila – Tepic. Roadside.	1203	*Burkholderia* **(1) 681^{Mp+}, 690^{Mp+},** 682^{nt}, 687^{nt}, 694^{nt}, 697^{nt}, 802^{nt}, 804^{nt}
UoD 247	*M. diplotricha* C. Wright ex. Sauvalle var. *diplotricha* (W)	Jal., Tequila – Tepic. Roadside.	1203	None isolated
MFS821	*M. occidentalis* Britton & Rose (R)	Nay., Tepic. Roadside.	716	*Burkholderia* **(1) 655 (CCGE1002)$^{Mp+}$**

New reports of nodulation are indicated by an asterisk after the species, and effective nodulation was confirmed by microscopical examination of the nodules in each case. The *in situ* reaction of the symbionts in the nodules to antibodies against *Burkholderia phymatum* STM815 (Bp) and *Cupriavidus taiwanensis* LMG19424 (Ct) was found to be negative for all samples except for those marked ^{Bp}. Strains isolated from the nodules are also listed, and unless marked otherwise each strain was tested positive for its ability to nodulate *M. affinis* (^{nt}, not tested; **, no nodulation). Strains marked ^{Mp} were also tested for their ability to nodulate *M. pudica*, and those marked $^{Mp+}$ nodulated it. Bold indicates that strains have been tested for nodulation on *M. affinis*.

> 700 isolates were obtained from the nodules, it was necessary to reduce these to a more manageable number for detailed analysis. Potential rhizobia were selected visually according to their colony morphology on yeast mannitol broth (YMB) + Congo Red agar plates (Vincent, 1970); most of the isolates from individual nodules appeared to be very similar, and so only one or two were selected for further analysis. DNA extractions were carried out according to Chomczynski & Sacchi (1987). Amplifications were performed with GoTaq® (Promega) according to the manufacturer's instructions using the primers shown in Table S3. DNA was amplified using a standard temperature profile with an initial DNA denaturation step at 95°C for 5 min followed by 30 cycles

Table 2 Nodulation of *Mimosa* species in rhizobial trapping experiments using soil collected in central and western Mexico in 2007 and 2008

Nodule sample (Herbarium voucher no.)	Species tested (E, endemic to Mexico; R, restricted to Mexico & Central America; W, widespread in the Americas)	State and location/vegetation type from where soil was collected (more details are given in Table S3 and can be viewed on GoogleEarth; Notes S1)	Elevation (m a.s.l.)	Rhizobial isolates obtained (JPY) and their generic identification via sequencing of their *16S* rRNA and *recA* genes (*16S–recA* clusters in parentheses)
na	*M. biuncifera* Benth. (R)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Ensifer* **(2) 1210** *Rhizobium* **(5)1206** **(6) 1209**
na	*M. borealis* A. Gray (R)#	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Ensifer* **(2) 1220^{Mp}, 1226^{Mp}, 1228, 1229** *Rhizobium* **(6) 1225**
na	*M. dysocarpa* Benth. (R)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Ensifer* **(2) 1260** *Rhizobium* **(5) 1252^{Mp}, 1263**
na	*M. orthocarpa* Spruce ex. Benth. (W)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	No nodules
na	*M. robusta* R. Grether* (E)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Rhizobium* **(5) 1283** **(6) 1269**
UoD 215, 216	*M. luisana* Brandg. (E)	Pue., Tehuacan. Pasture.	1632	*Ensifer* **(2) 1111, 1123, 1165**
UoD 217	*M. polyantha* Benth. (E)	Pue., Tehuacan. Pasture.	1144	*Ensifer* **(2) 1114, 1118, 1132****
UoD 219	*M. luisana* Brandg. (E)	Mor., Xochicalco. Pasture.	1282	*Ensifer* **(2) 1088^{Mp}, 1091^{Mp}**
UoD 224	*M. lacerata* Rose (E)	Qro., Cadereyta. Roadside.	2174	*Ensifer* **(2) 1139^{Mp}**
UoD 227	*M. similis* Britton & Rose* (E)	Qro., Cadereyta. Roadside.	1545	*Ensifer* **(2) 1142**
UoD 239	*M. tequilana* S. Watson (E)	Jal., Tequila. Roadside.	1174	*Rhizobium* **(4) 1153** **(5) 1145^{Mp}, 1152^{Mp}** **(6) 1151, 1154**
UoD 325	*M. polyantha* Benth. (E)	Mor., Sierra de Huautla. Roadside.	1021	*Rhizobium* **(6) 1198^{Mp}, 1201, 1202**
UoD 326	*M. goldmanii* B.L. Rob.* (E)	Mor., Sierra de Huautla. Roadside.	1021	*Rhizobium* **(6) 1300, 1301, 1321, 1322, 1323**
UoD 328	*M. albida* H. & B. ex. Willd. (W)	Mor., Sierra de Huautla. Roadside.	1148	*Ensifer* **(2) 1168**** *Rhizobium* **(6) 1166, 1170, 1171, 1172, 1385, 1388, 1389, 1390**
UoD 333	*M. albida* H. & B. ex. Willd. (W)	Mor., Sierra de Huautla. Roadside.	1060	*Rhizobium* **(5) 1403, 1404, 1405**
UoD 335	M. benthamii J.F. Macbr. (E)	Mor., Sierra de Huautla. Pasture.	1234	*Rhizobium* **(6) 1359, 1363**, 1367**
UoD 336	*Mimosa* sp. X* (E)	Mor., Sierra de Huautla. Roadside.	1043	*Ensifer* **(2) 1431, 1432** *Rhizobium* **(5) 1429**

R#, restricted to southern USA. New reports of nodulation are indicated by an asterisk after the species, and effective nodulation was confirmed by microscopical examination of the nodules in each case. The *in situ* reaction of the symbionts in the nodules to antibodies against *Burkholderia phymatum* STM815 (Bp) and *Cupriavidus taiwanensis* LMG19424 (Ct) was found to be negative for all samples. Strains isolated from the nodules are also listed, and unless marked otherwise each strain was tested positive for its ability to nodulate *M. affinis* (**, no nodulation). Strains marked ^{Mp} were also tested for their ability to nodulate *M. pudica*, and those marked $^{Mp+}$ nodulated it.

for the *16S* rRNA and *recA* genes or 40 cycles for *nifH, nodA* and *nodC* consisting of 30 s at 95°C, 30 s of primer annealing and 30 s of DNA amplification (or 1 min 30 s for *16S* rRNA) at 72°C. Annealing temperatures were 63°C for *recA* and *nifH*, 50°C for *nodA* and *nodC* and 56°C for *16S* rRNA. Amplifications were finished with a final extension step at 72°C for 7 min.

For *Burkholderia* isolates, PCR of these genes was performed as above, but with modifications according to Bontemps *et al.* (2010).

Restriction fragment length polymorphism analysis and sequencing

PCR-amplified *16S* rRNA, *recA* and *nodC* genes were digested with the restriction enzymes HinfI and MspI in order to classify the isolates into groups (Laguerre *et al.*, 1994; Chen *et al.*, 2003, 2005a,b). Five microlitres of each PCR product was incubated with 5 units of enzyme and the appropriate buffers at 37°C for a minimum of 3 h. The digestion products were separated on a 2% gel for 2.5 h at 80 V and visually compared. In order to better establish their taxonomic position, profiles of *16S* rRNA and *recA* were combined, and the isolates were considered to be similar when profiles were identical for both genes with both enzymes. The efficiency of the restriction fragment length polymorphism (RFLP) grouping was then checked by sequencing several isolates from each group. The PCR-amplified products were sequenced in both directions by Macrogen Inc. (Seoul, Korea) or by the sequencing service at the James Hutton Institute, Dundee (UK). The sequences were aligned with the MAFFT software (Katoh *et al.*, 2009) and their quality checked with BioEdit (Hall, 1999). Accession numbers are given in Table S1.

Phylogenetic and statistical analyses

Nucleotide alignments and phylogenetic trees were constructed and edited with Mega6 (Tamura *et al.*, 2013) using a maximum-likelihood (ML) method based on a GTR + G + I model. Support for the tree branches was estimated with 100 bootstrap replicates and all positions with < 80% site coverage were eliminated for the *16S* rRNA, *recA* and *nifH* genes, whereas all positions containing gaps and missing data were eliminated for the *nodA* and *nodC* genes. A total of 1074 positions were used for the concatenated *16S* rRNA-*recA* phylogenetic tree, 737 for the *16S* rRNA, 337 for the *recA*, 285 for the *nodA*, 424 for the *nodC* and 479 for the *nifH* phylogenetic trees. Canonical discriminant analysis (CDA) was applied to assess the plant host and ecological preferences of the different rhizobial genera (*Burkholderia*, *Ensifer* and *Rhizobium*) according to different qualitative (plant clade, site, plant-status) and quantitative (elevation) variables with XLSTAT. The distribution of the genera was summarized by their centroids. The plant status was used and defined according to the plant distribution within the Americas (W, widespread in the Americas; R, restricted to certain parts of Central and North America; E, Endemic to Mexico). The different plant clades are those defined in the *Mimosa* phylogeny of Simon *et al.* (2011). The locations refer to the sampling locations that can be found together with their elevations in Tables 1, 2 and S1.

Nodulation tests

Seeds were not available for most of the *Mimosa* species to test for their ability to nodulate with their potentially symbiotic isolates, so *M. affinis* was chosen as a 'model' host, as it is widespread in Mexico and Central America, is herbaceous and fast growing, and has an ability to nodulate with a wide range of rhizobial types, both Alpha and Beta (Wang *et al.*, 1999; Elliott *et al.*, 2007, 2009). Out of the 87 strains used in the phylogenetic analysis, 74 strains from 17 *Mimosa* species, as well as reference type strains, were tested on *M. affinis*. Similar tests were also conducted on the pan-tropical species *M. pudica* with 28 strains (all of which were also tested on *M. affinis*), as this species has been used in several studies from South America as a model host for *Mimosa* symbionts, particularly Betaproteobacterial ones (Chen *et al.*, 2005a; Bontemps *et al.*, 2010; Mishra *et al.*, 2012). The *M. affinis* and *M. pudica* plants were grown hydroponically in a sterile solution of Jensen's medium (quarter strength), and they were inoculated according to Elliott *et al.* (2009). The plants were harvested 6 wk after inoculation and were scored for the presence of nodules. *Rhizobium etli* sv mimosae strain Mim-1 served as a positive control with *M. affinis*, and *C. taiwanensis* LMG19424 with *M. pudica*. Cross-inoculation tests were performed with selected isolates on various *Mimosa* spp. native to Mexico and/or to South America, as well as on common bean cv Negro Jamapa. The seeds were sourced and germinated according to Elliott *et al.* (2007), and the tests were performed under sterile conditions in glass tubes (70 ml volume) that were quarter-filled with an autoclaved mixture of vermiculite and perlite, and fed with Jensen's N-free medium. The plants were inoculated according to Elliott *et al.* (2009). The mimosas were harvested at 6–8 wk and the beans at 3 wk after inoculation, when they were scored for the green colour of their aerial parts and the presence of pink nodules, which are indications of effective nitrogen fixation. Nodules were also taken for microscopical analysis.

Additional nodulation tests were performed at CCG-UNAM, Cuernavaca on a range of *Mimosa* spp. inoculated with *R. etli* sv mimosae Mim-1, which was originally isolated from *M. affinis* by Wang *et al.* (1999). In this case the plants were rooted in agar made with Jensen's medium inside enclosed tubes according to Chen *et al.* (2003), and were harvested at 8 wk after inoculation. Uninoculated plants served as controls in all the experiments.

Results

Nodules on endemic Mexican *Mimosa* spp. do not contain betaproteobacteria

In 2007, nodules were obtained directly from 21 separate *Mimosa* plants in the field, representing 15 separate taxa (Table 1; Fig. S2). Eight of the endemic species (*M. benthamii*, *M. biuncifera*, *M. depauperata*, *M. lactiflua*, *M. monancistra*, *M. mollis*, *M. tequilana* and *M. tricephala*) are new reports of nodulation (Table 1; Fig. S3). Nodules that were prepared and sectioned for microscopy had a structure typical of *Mimosa* nodules and were effective in appearance (see Fig. S3 for representative examples). None of the nodules on the endemic and Central American species reacted with the specific betaproteobacterial antibodies using IGL-SE, but nodules from the widespread species *M. skinneri* and *M. somnians* reacted with the

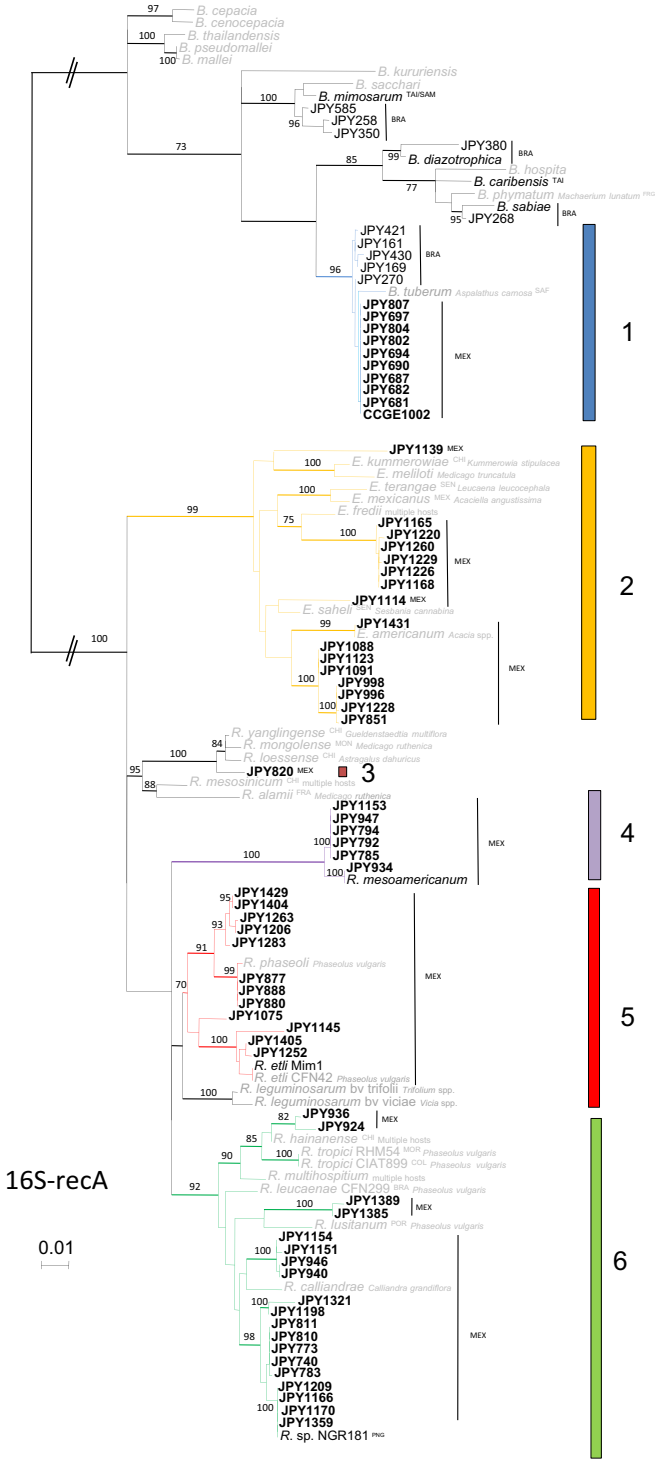


Fig. 1 Phylogenetic relationships of strains isolated from *Mimosa* nodules in this study, and reference strains, based on a 1074 nucleotide 16S rRNA-*recA* concatenated sequence. The tree was built using a maximum-likelihood method and heavy lines indicate branches supported by bootstrap values > 70% (100 replicates). The scale represents mutations per nucleotide. *Mimosa* symbionts are in black, and those isolated in this study are in bold. Taxa in grey are nonsymbiotic bacteria or symbionts of other hosts that are indicated beside the strain name. Full sequence identifiers, accession numbers and strain numbers can be found in Supporting Information Fig. S3 and Table S3. Coloured bars indicate clusters 1–6 that are discussed in the main text. When known, geographical origins of the strains are indicated as follows: BRA, Brazil; CHI, China; COL, Colombia; FRA, France; FRG, French Guiana; MEX, Mexico; MON, Mongolia; MOR, Morocco; PNG, Papua New Guinea; POR, Portugal; SAM, South America; SAF, South Africa; SEN, Senegal; TAI, Taiwan. B, *Burkholderia*; E, *Ensifer*; R, *Rhizobium*.

B. phymatum STM815 antibody (Table 1). Nodules were also harvested from *Mimosa* spp. grown as 'trap plants' in rhizosphere soils, as well as from four Mexican *Mimosa* species grown in a mixture of all the rhizosphere soils. A fifth species, *M. orthocarpa*, did not form any nodules (Table 2). In total, these trap experiments yielded nodules on a further 15 taxa, including 10 that were different from those of the field samplings. There were new reports of nodulation by the endemic species *M. goldmanii*, *M. robusta*, *M. similis* and by another endemic species from Sierra de Huautla, which is still awaiting a formal description (*Mimosa* sp. X; Fig. S2f). Sections of all of the nodules from the trap experiments showed that the nodules were effective (Fig. S3), but none reacted with either antibody using IGL-SE (Table 2).

Endemic Mexican *Mimosa* spp. are specifically associated with alphaproteobacteria

After genetic analyses and nodulation tests, the survey of Mexican *Mimosa* symbiont (MMS) diversity resulted in 87 isolates from single nodules from 26 plants in 18 locations. These represented potential symbionts of 19 *Mimosa* taxa (17 species), 33 of which came from nodules collected from eight *Mimosa* taxa in the field, and 54 from 13 *Mimosa* taxa grown in the trap experiments (Tables 1, 2, S1). According to their *16S* rRNA and *recA* sequences, isolates from the field-sampled nodules were classified in both Alphaproteobacteria (*Rhizobium*, *Ensifer*) and Betaproteobacteria (*Burkholderia*), with the latter being almost confined to the widespread species *M. somnians*, although another widespread species *M. skinneri* also yielded some *Burkholderia* isolates amongst its largely alphaproteobacterial microbiota. All isolates from the trap experiments were alphaproteobacteria, and belonged to either *Rhizobium* or *Ensifer*.

The taxonomic positions of the 87 isolates were assessed by a phylogenetic tree based on concatenated *16S* rRNA and *recA* sequences (Fig. 1). According to bootstrap values and reference strain positions, five clusters and a single-strain lineage (JPY820) were defined in the concatenated tree and in the *16S* rRNA and *recA* trees (Fig. S4a,b). Nine closely related isolates (Cluster 1) belonged to *Burkholderia*; these were isolated from the widespread species *M. skinneri* and *M. somnians* and they grouped with *B. tuberum* strains isolated from Brazilian *Mimosa* spp. (JPY161–JPY430; Bontemps *et al.*, 2010) and from *M. occidentalis* (CCGE1002). Cluster 2 encompassed *Ensifer* isolates from 10 *Mimosa* species. The remaining Clusters (3–6) belonged to the genus *Rhizobium*. Cluster 3 grouped one isolate (JPY820 from *M. tricephala*) with the reference species *R. loessense*, *R. mongolense* and *R. yanglingense*, Cluster 4 was closely related to *R. mesoamericanum*, Cluster 5 grouped with reference strains already known to nodulate *Mimosa* or *P. vulgaris*, and Cluster 6 grouped with the *R. tropici* (CIAT899^T),

R. leucaenae (CFN299^T) and *R. calliandrae* (CCGE524^T) type strains, as well as the Papua New Guinea *Mimosa* strain NGR181 (Elliott *et al.*, 2009). In addition, Cluster 6 also contained JPY491 (Fig. S4a), one of only two *Rhizobium* strains that were isolated from central Brazil by Bontemps *et al.* (2010), both from the widespread species *M. xanthocentra*.

Mexican *Mimosa* symbionts have diverse, but specific, stable and ancient nodulation genes

The symbiotic phenotype was confirmed for all 87 isolates at the molecular level by amplification and sequencing of the symbiosis-related genes, *nodA*, *nodC* and *nifH*, and/or by nodulation tests (Tables 1, 2, S1). Both *nodA* and *nodC* were sequenced from 45 strains, *nodA* from 10 strains and *nodC* from a further 35 strains; *nifH* was sequenced from 34 strains. Phylogenetic trees were constructed for all three genes (Fig. 2). Similar clusters as for *16S* rRNA/*recA* were generally observed, that is Cluster 1 (*Burkholderia*), Cluster 2 (*Ensifer*) and Clusters 3–6 (*Rhizobium*). Clusters 2–6 also frequently encompassed common bean symbionts and, more rarely, other mimosoid symbionts that are also capable of nodulating *P. vulgaris*. The clusters of MMS strains defined in the 16S/*recA* phylogeny were also seen in the phylogenies of the symbiosis-related genes, and groupings within the major clusters 2, 5 and 6 were largely conserved. However, there were four strains that had *nodA* sequences typical of Cluster 6 rather than of their own cluster (Fig 2): the Cluster 4 *Rhizobium* strains JPY1153 (from *M. tequilana*) and JPY785 (from *M. skinneri*), the Cluster 5 *Rhizobium* strain JPY1429 (from *Mimosa* sp. X), and the Cluster 2 *Ensifer* strain JPY1168 (from *M. albida*). JPY1153 also had a Cluster 6 *nodC* sequence, whereas the *nifH* sequence of JPY1168 was in Cluster 2. It thus seems that these four strains may be the recipients of *nod* genes, but not necessarily *nif* genes, from donors in Cluster 6. Further evidence for horizontal transfer of nodulation genes is that JPY996 and JPY998 (both from *M. tricephala*) formed a separate lineage for *nodA* (but not *nodC*) from the other *Ensifer* strains in Cluster 2; these *nodA* sequences are more closely related to those of the *Rhizobium* strains in Cluster 3, which also contains a *M. tricephala* symbiont (JPY820).

Relationship between rhizobial type, plant host and location

On the one hand, there are no obvious differences between MMS from field-collected nodules and those obtained from trap plants. Soils in which the plants were growing were relatively similar, at least in pH (Table S1), which is often the major determinant of bacterial diversity in the soil, including that of *Burkholderia* (Stopnisek *et al.*, 2014). On the other hand, 18 sites were sampled across 600 km (Fig. 3) and there are evident geographic trends: symbiotic *Burkholderia* strains were isolated only in the west (from the widespread species *M. skinneri* and *M. somnians* and the Mexican–Central American-restricted species *M. occidentalis*), whereas *Ensifer* strains were most prominent in the centre and the east of Mexico (Fig. 3a). The results of a CDA test indicated that the different genera were, indeed, not randomly distributed (Fig. 3b). One of two main factors explaining this distribution was the sampling location (Fig. 3c). The other factor was the plant phylogeny based upon the *Mimosa* clades defined by Simon *et al.* (2011), and presented in selected form in Fig. 4. In Mexico, there were preferential associations of: *Burkholderia* with widespread *Mimosa* spp. from clades L, M and N; *Rhizobium* with *Mimosa* spp. from clades R, T and V, where the studied species have a more restricted geographical range (except for *M. albida* and *M. skinneri*); and *Ensifer* with *Mimosa* spp. from clade B that are mainly endemic to Mexico (Figs 3c, 4).

Endemic Mexican *Mimosa* species have a preference for nodulating with alphaproteobacteria

Mimosa affinis was nodulated effectively by the four *Burkholderia* strains (CCGE1002, JPY681, JPY690, JPY807) and all but six of the 70 alphaproteobacterial strains tested (as well as *R. etli* sv mimosae Mim7-4) (Tables 1, 2, S1). Those that did not nodulate *M. affinis* included the single strain in Cluster 3 (JPY820), and some strains from Clusters 2 and 6. By contrast, *M. pudica* was only nodulated (partially) effectively by the *Burkholderia* strains, whereas *Rhizobium* strains JPY783 (Cluster 6) and JPY877 (Cluster 5) from *M. skinneri* and JPY888 (Cluster 5) from *M. albida* formed ineffective nodules. The other *Rhizobium* and *Ensifer* strains did not nodulate this host (Tables 1, 2, S1). Of the reference strains, *R. etli* CFN42^T, *R. leucaenae* CFN299^T and *R. tropici* CIAT899^T nodulated *M. affinis* ineffectively and failed to nodulate *M. pudica* (Table S1). No uninoculated control plants nodulated.

Cross-inoculation tests (Table 3; Fig. 5) showed that all species had the capacity to nodulate effectively with most *Rhizobium* strains, but there were also differences in host range between *Mimosa* species: those in the southern USA–Mexican Clade B (*M. borealis*, *M. dysocarpa*, *M. luisana* and *M. polyantha*) were less capable of nodulating effectively (or at all) with betarhizobia. This contrasts with the four species in Clade T (including the Mexican endemic *M. tequilana*,) and *M. orthocarpa* in Clade M, which were all capable of nodulating effectively with *B. phymatum* STM815 (Table 3, this study; Elliott *et al.*, 2007; dos Reis Junior *et al.*, 2010). Finally, all of the alphaproteobacterial strains tested, JPY1220 (Cluster 2), JPY820 (Cluster 3), JPY934 (Cluster 4), Mim-1 (Cluster 5) and JPY940 and 1198 (both Cluster 6) nodulated *P. vulgaris* effectively (Table S1), but *Burkholderia* sp. CCGE1002 (Cluster 1) only formed occasional ineffective nodules on this host.

Discussion

Betaproteobacteria are not the usual symbionts of Mexican *Mimosa* spp.

In this study, we have confirmed nodulation of the Mexican *Mimosa* species studied by Elliott *et al.* (2007), but have also presented evidence that several other species are capable of nodulation, with new reports of nodulation for 12 Mexican endemics.

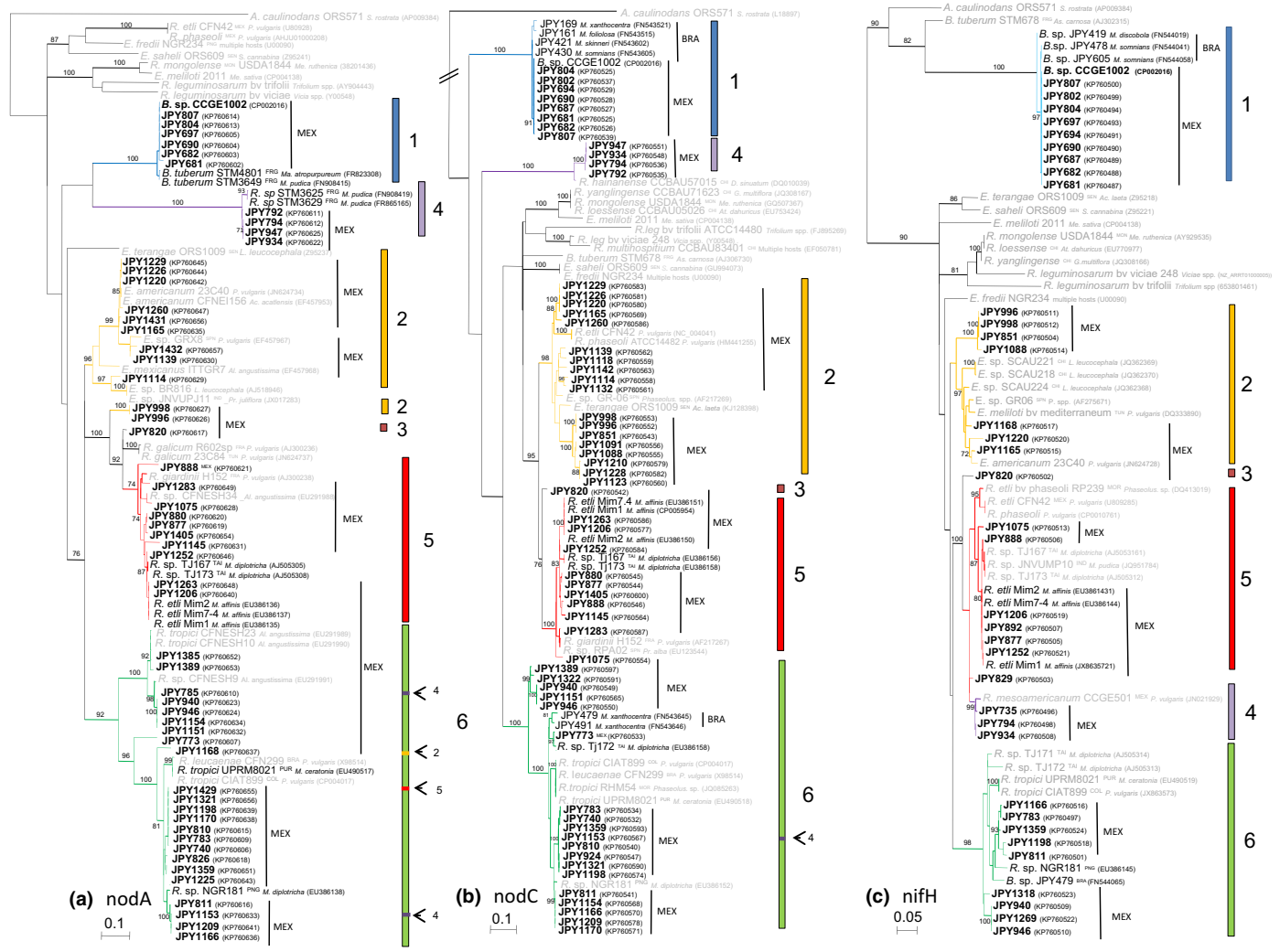
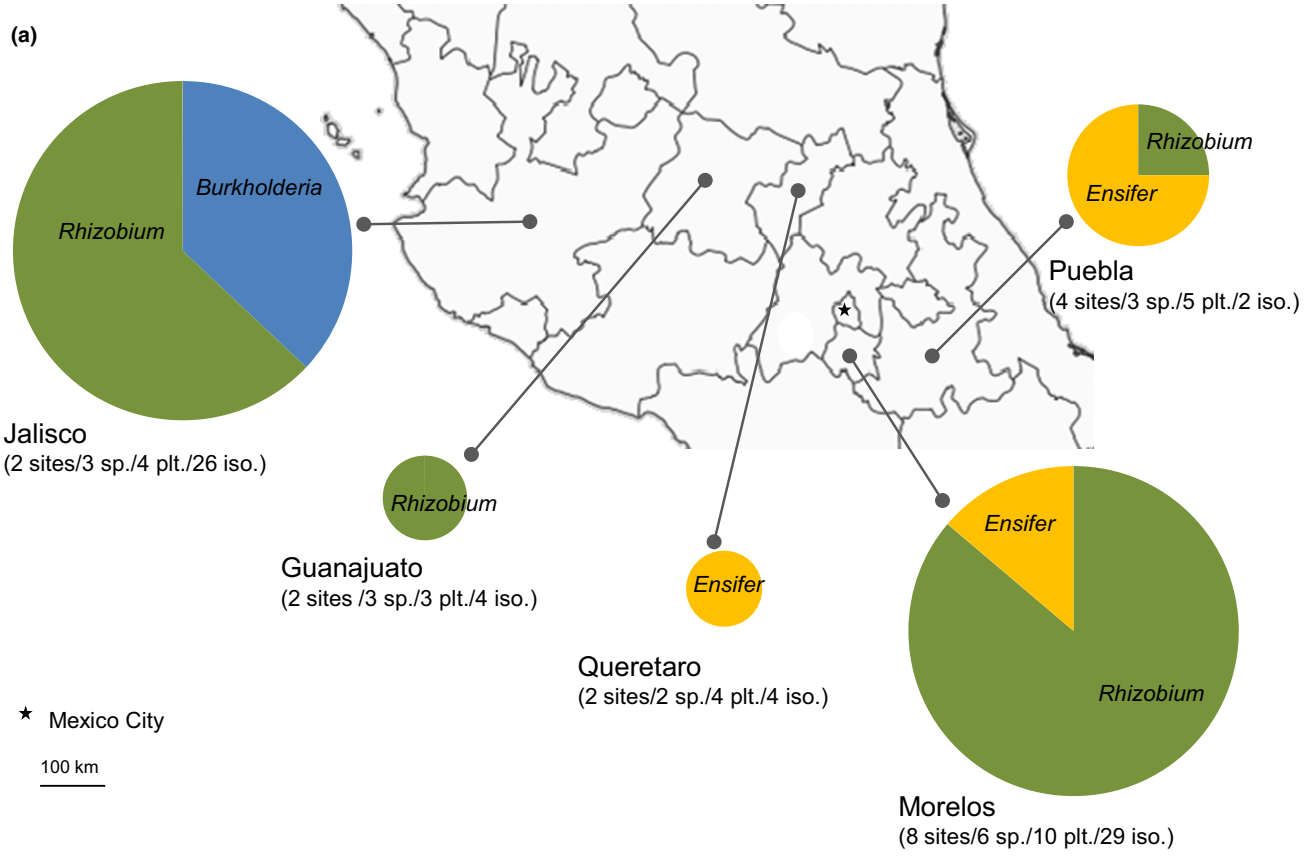
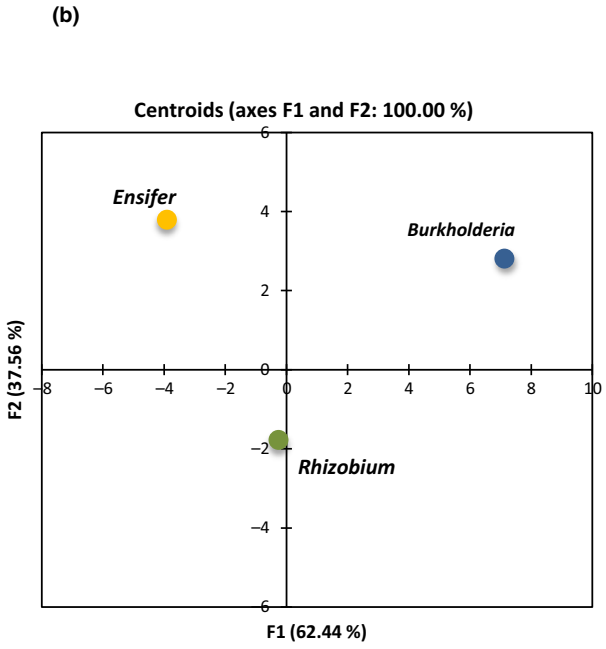


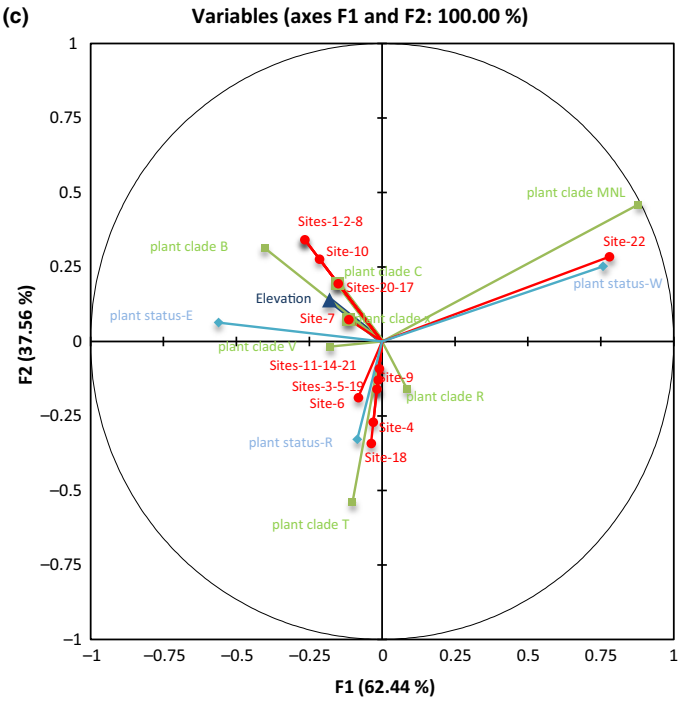
Fig. 2 Phylogenies of symbiosis-related genes in strains isolated from *Mimosa* nodules in this study and reference strains. (a) *nodA*, (b) *nodC* and (c) *nifH*. The trees were built using a maximum-likelihood method with a 285-nt alignment for *nodA*, a 424-nt alignment for *nodC* and a 479-nt alignment for *nifH*. Heavy lines indicate branches supported by bootstrap values > 70% (100 replicates). The scale represents mutations per nucleotide. *Mimosa* symbionts are in black and those isolated in this study are in bold. Symbionts of other hosts are in grey, and their hosts are indicated. For previously published sequences, the host plant is indicated, and the sequence accession number in parentheses. Coloured bars indicate clusters defined in the *16S* rRNA-*recA* phylogeny from Fig. 1. Arrows indicate potential horizontal transfer of symbiosis genes between clusters. When known, geographical origins of isolates are indicated as follows: BRA, Brazil; COL, Colombia; IND, India; MEX, Mexico; MON, Mongolia; MOR, Morocco; FRA, France; FRG, French Guiana; PNG, Papua New Guinea; PUR, Puerto Rico; SEN, Senegal; SPN, Spain; TAI, Taiwan; TUN, Tunisia. A, *Azorhizobium*; B, *Burkholderia*; E, *Ensifer*; R, *Rhizobium*; Ac, *Acacia*; Al, *Acaciella*; As, *Aspalathus*; At, *Astragalus*; G, *Gueldenstaedtia*; L, *Leucaena*; M, *Mimosa*; Ma, *Macroptillium*; Me, *Medicago*; P, *Phaseolus*; Pr, *Prosopis*; S, *Sesbania*.

Fig. 3 (a) Distribution of the bacterial genera found in association with *Mimosa* spp. in each state sampled in Mexico. The area of each circle is proportional to the number of isolates. For each state, the number of sampled sites, the number of sampled *Mimosa* species (sp.), the number of individual plants (plt.), and the number of bacterial isolates (iso.) are indicated. Coloured circles represent the proportion of each genus among the isolates: blue, *Burkholderia*; green, *Rhizobium*; yellow, *Ensifer*. Only confirmed nodulating strains were included in the analysis. Map source: http://en.wikipedia.org/wiki/Template:Location_map_Mexico. (b) Canonical discriminant analysis (CDA) of the distribution of the different bacterial clusters according to qualitative (plant clade, site, plant-status) and quantitative (elevation) variables. The sample distribution for each bacterial genus associated with *Mimosa* in Mexico is summarized by their centroids. The bacterial clusters correspond to those in Fig. 1: *Burkholderia* are in Cluster 1, *Ensifer* in Cluster 2 and *Rhizobium* in Clusters 4–6. Cluster 3, a single strain, was omitted from the analysis. The first axis explains 62.44% of the variation in bacterial cluster distribution and showed a strong difference between *Ensifer* and *Burkholderia* distribution. The second axis explains 37.56% of this variation and also showed a differentiation between *Rhizobium* distribution and those of the two other genera. (c) Correlation circle of the variables on the first factorial plane (F1 × F2) of the CDA. The different plant clades are those defined in the *Mimosa* phylogeny (Fig. 4). Plant status: W, widespread in the Americas; R, restricted to certain parts of Central or North America; E, Endemic to Mexico. The locations and elevations can be found in Tables 1, 2, S3. The distribution of the genus *Burkholderia* appeared to be mainly associated with widespread *Mimosa* found in location 22 and those species that belonged to the Clades L, M and N (see Simon *et al.* (2011) and Fig. 4 (this study)), whereas *Ensifer* distribution was linked more with *Mimosa* clade B (mostly Mexican endemics) in locations 1, 2, 8 and 10. The most explanatory variable for *Rhizobium* distribution appeared to be the *Mimosa* clade T, especially the widespread species *M. albida*.

Betarhizobia were not detected by *in situ* hybridization in nodules from the Mexican native or endemic *Mimosa* species in Clades B, T and V. This is in contrast to the study of Brazilian native and endemic *Mimosa* spp. by dos Reis Junior *et al.* (2010), in which nodules from 67 out of 70 species reacted with the *Burkholderia phymatum* STM815 antibody. It should be noted, though, that nodules from the two species that were common to both studies, *M. skinneri* and *M. somnians*, reacted to this antibody in both Mexico (this study) and Brazil (dos Reis Junior *et al.*, 2010). It is also noteworthy that in neither study were any nodules sampled







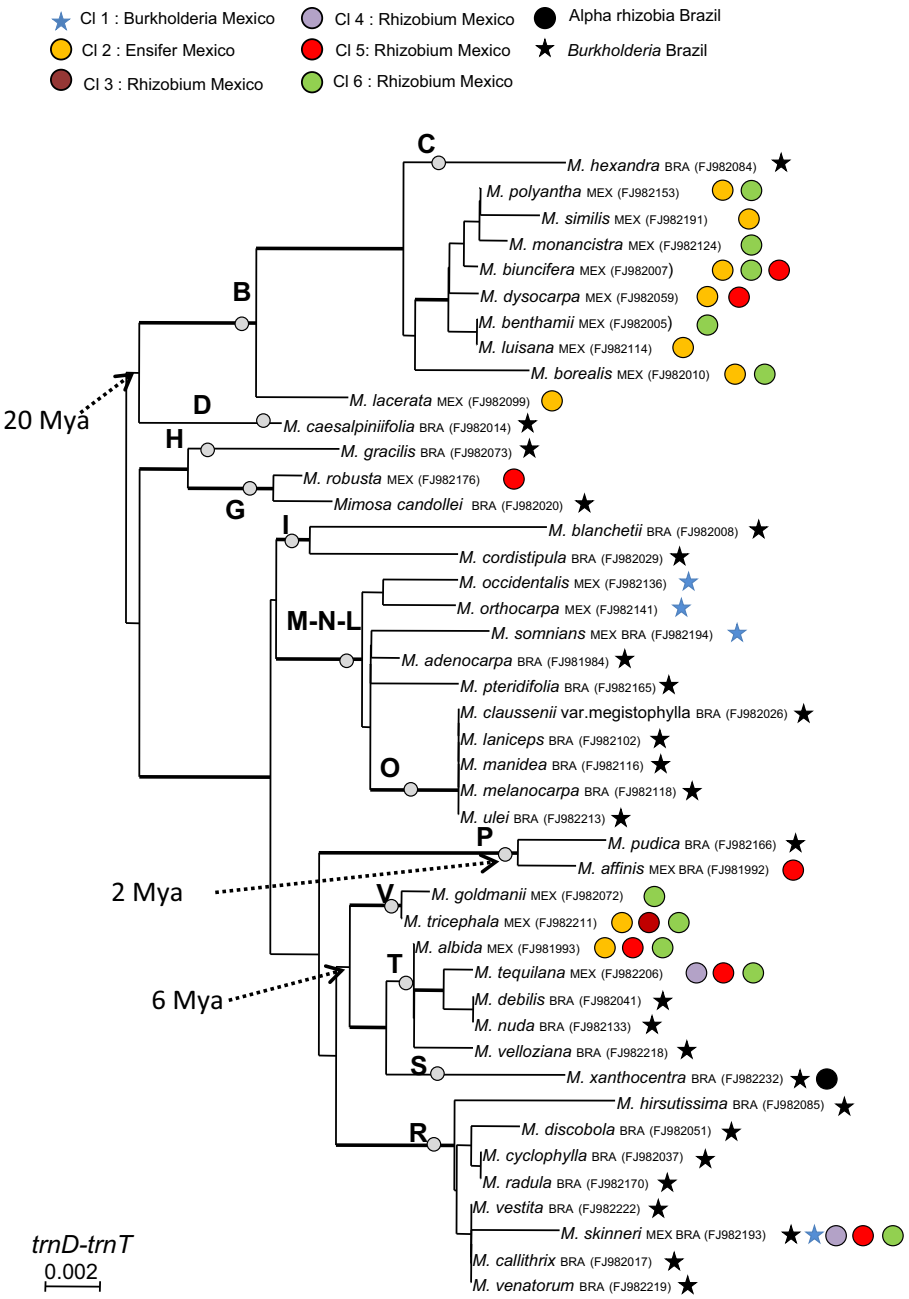


Fig. 4 Phylogeny of *Mimosa* based on DNA sequences of the *trnD-trnT* noncoding plastid locus. Only *Mimosa* species sampled from this study in Mexico, those in Brazil from Bontemps *et al.* (2010), the study of Elliott *et al.* (2007), and the nodulation test species *M. pudica* and *M. affinis* were used to build the tree. The tree was built using the distance (BioNJ) method. Bootstrap values > 75% are indicated with heavy lines (1000 replicates). Letters on branches indicate well-supported *Mimosa* clades defined by Simon *et al.* (2011). The origin of the sampled *Mimosa* species is indicated (MEX, Mexico; BRA, Brazil; MEX BRA, sampled in both locations). Taxonomic groups of associated symbionts are indicated with circles for alpharhizobia and stars for *Burkholderia*. Symbiont data for Brazilian *Mimosa* species are from Bontemps *et al.* (2010), dos Reis Junior *et al.* (2010) and Elliott *et al.* (2007), and those for *M. affinis* from Elliott *et al.* (2009). For Mexican isolates, the associated coloured circle corresponds to the taxonomic clusters defined in Fig. 1. Arrows indicate the ages of nodes as estimated by Simon *et al.* (2011). M, *Mimosa*; Cl, Cluster. Ma, Myr ago.

that reacted with the *Cupriavidus taiwanensis* LMG19424 antibody, even though *C. taiwanensis* is widespread in invasive *Mimosa* spp. in the tropics (see the Introduction section). Furthermore, Elliott *et al.* (2007) found that the Mexican *Mimosa* species in their study, which included some of the Clade B endemics that we also sampled, were not nodulated effectively (and in some cases not at all) by either *B. phymatum* STM815 or *C. taiwanensis* LMG19424.

The *Burkholderia* isolates were confined to two *Mimosa* species, *M. skinneri* and *M. somnians*, nodules from which also reacted positively with the antibody against *B. phymatum* STM815. Both species are widespread in the Neotropics, and were previously reported to be nodulated by *Burkholderia* in Brazil (Elliott *et al.*, 2007; Bontemps *et al.*, 2010; dos Reis Junior *et al.*, 2010). The *Burkholderia* strains from *M. skinneri* and *M. somnians* in Mexico were all very closely related to the sequenced strain CCGE1002 (Ormeño-Orrillo *et al.*, 2012), which was originally isolated from *M. occidentalis*, a Mexican–Central American-restricted species. These Mexican burkholderias in Cluster 1 are most closely related to *B. tuberum* sv mimosae, a species/symbiovar (Rogel *et al.*, 2011) that has been widely isolated from *Mimosa* spp. in South America (Bontemps *et al.*, 2010; Mishra *et al.*, 2012); this is species complex 6, as defined by Bontemps *et al.* (2010). The low diversity of the Mexican *Burkholderia* strains contrasts with the very diverse *Burkholderia* lineages found in the South American studies of Bontemps *et al.* (2010), Mishra *et al.* (2012) and Lammel *et al.* (2013), but the sample is very small, from just two collection sites and three *Mimosa* species, because *Burkholderia* was

Table 3 Cross-inoculation tests with Mexican *Mimosa* rhizobia and *Mimosa* spp. from various locations and clades (Simon *et al.*, 2011)

Mimosa sp. (Clade; Simon *et al.*, 2011)	*Ensifer* sp. JPY1220 (Cluster 2)	*Rhizobium* sp. JPY934 (Cluster 4)	*Rhizobium* sp. JPY1198 (Cluster 6)	*Rhizobium* sp. JPY940 (Cluster 6)	*Rhizobium etli* sv mimosae Mim-1 (Cluster 5)	*Burkholderia* sp. CCGE1002 (Cluster 1)	*B. phymatum* STM815^T	*C. taiwanensis* LMG19424^T
***M. borealis* (B)**	E	E	E	E	N	N	N[a]	N[a]
***M. dysocarpa* (B)**	E	E	E	E	N	N	N[a]	N[a]
***M. biuncifera* (B)**	nt	nt	nt	E	N	N	N[a]	N[a]
***M. luisana* (B)**	E	–	E	E	N	–	–[a]	–[a]
***M. polyantha* (B)**	–	–	–	E	N	N	N[a]	–[a]
***M. tequilana* (T)**	–	E	N	E	N	N	E	N
M. albida (T)	N	E	E	N	N	N	E[a]	N
M. velloziana (T)	nt	N	nt	–	nt	N	E[a]	N[a]
M. debilis (T)	nt	nt	nt	–	nt	nt	E[a]	N[a]
M. pudica (P)	–	–	–	–	N[b]	N	E[a]	E[a]
***M. affinis* (P)**	E	E	E	E	E	N	E[a]	N
M. orthocarpa (M)	nt	nt	nt	nt	nt	N	E	N

North and Central American spp.	
Widespread and Brazilian spp.	
Effective nodulation	E
Ineffective nodulation	N
No nodulation	–
Not tested	nt

Plants (two to four replicates per species/strain combination) were grown in sterile glass tubes and rooted in sterile vermiculite/perlite (1 : 1) for 6–8 wk after inoculation.
[a]Results obtained by Elliott *et al.* (2007) or dos Reis Junior *et al.* (2010).
[b]Results obtained by Elliott *et al.* (2009).

so uncommon at the sample locations. A dedicated search, particularly of nonendemic *Mimosa* species in the *M. occidentalis*/*M. orthocarpa* clade M of Simon *et al.* (2011), would no doubt reveal greater diversity.

Many species of *Rhizobium* and *Ensifer* are symbionts of endemic Mexican *Mimosa* species

The *Mimosa*-nodulating alphaproteobacteria were divided into five distinct *16S* rRNA-*recA* clusters: one *Ensifer* and four *Rhizobium*. Some individual clusters encompassed several reference species, so they can be regarded as species complexes. There was substantial sequence diversity among the MMS within each cluster, indicating their affiliation to more than one species. *Ensifer* (Cluster 2) has not previously been reported as a *Mimosa* symbiont in the Neotropics, but has been isolated from other mimosoid legumes in central Mexico, such as *E. americanum* from *Acacia* (*s.l.*) spp. (Toledo *et al.*, 2003) and *E. mexicanum* and *E. chiapanecum* from *Acaciella angustissima* (Lloret *et al.*, 2007; Rincón-Rosales *et al.*, 2009). Three *Ensifer* (*Sinorhizobium*) isolates were reported from the USA–Mexican native *M. strigillosa* in Texas (Andam *et al.*, 2007). In addition, *E. mexicanum* strains were isolated from nodules on *M. himalayana*, an Indian species that was used for trap experiments in Brazilian Cerrado soils by Gehlot *et al.* (2013). The *Ensifer* symbionts isolated in the present study fall into at least five species-level clades (Fig. 1), including some that are not closely related to any described species. The three main *Rhizobium* clusters (4, 5 and 6) contained *R. mesoamericanum*, *R. etli*/*R. phaseoli* and *R. tropici*/*R. leucaenae*/*R. calliandrae*, respectively, plus strains not yet given a formal species designation; these species all contain strains already known to nodulate *Mimosa* spp. and/or other mimosoids, such as *Leucaena* and *Calliandra*, but also to nodulate the promiscuous papilionoid legume *P. vulgaris* (Wang *et al.*, 1999; Zurdo-Piñeiro *et al.*, 2004; Elliott *et al.*, 2009; Klonowska *et al.*, 2012; López-López *et al.*, 2012; Mishra *et al.*, 2012; Ribeiro *et al.*, 2012; Rincón-Rosales *et al.*, 2013; Melkonian *et al.*, 2014). The present study has greatly extended the sampling of these clusters to include other closely related strains and possibly new *Mimosa*-nodulating species. Indeed, the type strains of *R. etli*, *R. tropici* and *R. leucaenae*, which are efficient nodulators of *P. vulgaris*, are also capable of nodulating *M. affinis* (albeit ineffectively), and *Mimosa* strains from each of the five Alphaproteobacterial clusters (2–6) can nodulate *P. vulgaris* effectively (this study).

The *nodA* and *nodC* phylogenies of the *Ensifer* and *Rhizobium* symbionts are largely congruent with those of the *16S* rRNA and *recA* genes, indicating that horizontal gene transfer (HGT) has not been common. The depth of the branches in the trees indicates that the common ancestor of these sets of symbiosis genes was ancient. The Mexican *Mimosa* rhizobia situation is, therefore, quite similar to that observed with the *Burkholderia* symbionts of the Brazilian *Mimosa* endemics, as the latter also exhibited very

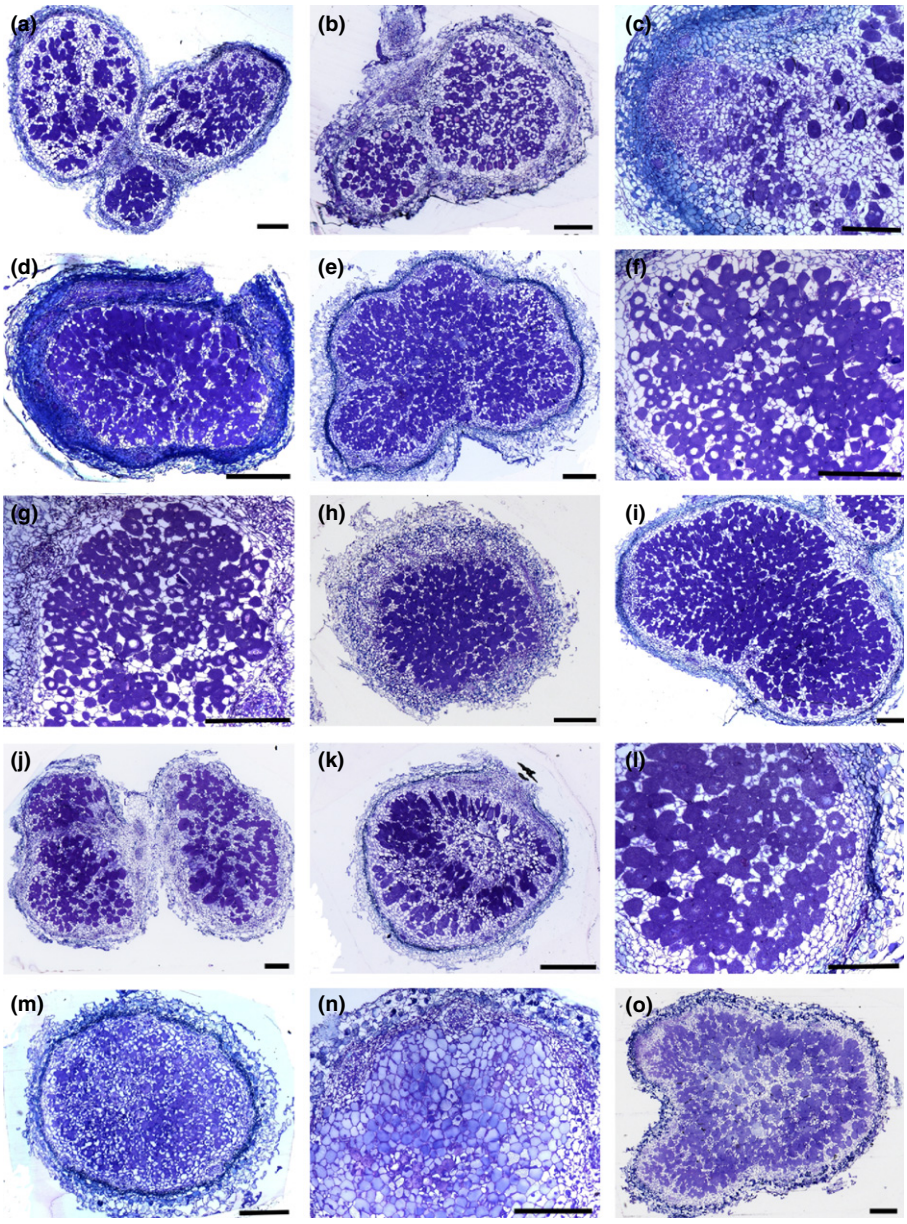


Fig. 5 Light microscopy of semi-thin (1-μm-thick) sections of Mexican *Mimosa* nodules from cross-inoculation experiments. All the nodules were effective, except for those indicated. *Ensifer* sp. JPY1220+ (a) *M. borealis*, (b) *M. luisana* and (c) *M. albida*; *Rhizobium* sp. JPY934+ (d) *M. albida* (ineffective); *Rhizobium* sp. JPY940+ (e) *M. borealis*, (f) *M. dysocarpa*, (g) *M. polyantha* and (h) *M. tequilana*; *Rhizobium* sp. JPY1198+ (i) *M. borealis* and (j) *M. luisana*; *R. etli* sv mimosae Mim-1+ (k) *M. borealis* (ineffective) and (l) *M. aculeaticarpa*; *Burkholderia* sp. CCGE1002+ (m) *M. borealis* (ineffective) and (*n*) *M. polyantha* (ineffective); (o) *B. phymatum* STM815+ *M. orthocarpa*. Bars, 200 μm.

little HGT, and the phylogenies of their housekeeping genes were closely aligned with those of the symbiotic loci (Bontemps *et al.*, 2010). Nevertheless, our phylogenetic studies also showed that some *P. vulgaris* symbionts have genes closely related to those of the MMS, supporting a possible symbiotic overlap which we have confirmed for selected MMS by nodulation tests. It is now clear that the diversity of rhizobia able to nodulate *Mimosa* is much greater than previously thought, but that this diversity is only found in certain rhizobial species and nodulation gene clades, indicating that *Mimosa* nodulation requires some degree of specificity, the basis of which is still unknown.

Mexican *Mimosa* species prefer Alphaproteobacterial symbionts

The widespread Mesoamerican species, *M. affinis*, was found to be a good 'common' host for nodulation tests, as it could nodulate with most of the MMS. Interestingly, very few MMS strains could form nodules on the widespread and pan-tropical species *M. pudica*, whereas this has been a useful common host for testing the symbionts of South American *Mimosa* spp., which are mainly betarhizobia (Bontemps *et al.*, 2010; Mishra *et al.*, 2012). This is not so surprising, in fact, as *M. pudica* has shown only a slight ability to nodulate with alpharhizobia, and the nodules formed are often ineffective or partially effective (Barrett & Parker, 2006; Elliott *et al.*, 2009; Gehlot *et al.*, 2013; Melkonian *et al.*, 2014). Cross-inoculation tests have confirmed that the native southern USA and Mexican *Mimosa* spp. prefer alpha- to betarhizobia, but also that the degree of preference depends on the clade. None of the closely related species in the southern USA–Mexican Clade B could nodulate effectively with promiscuous *Burkholderia* strains (Elliott *et al.*, 2007; this study), but those in the 'mixed' Clade T could. The latter includes *M. albida*, which is a very common species in Central America and Mexico, and *M. tequilana*, which

is endemic to the Tequila municipality in Jalisco; both could nodulate effectively with *B. phymatum* STM815, but in the field they appear to be nodulated exclusively by alpharhizobia. The other species in this clade, such as *M. debilis* and *M. velloziana* are nodulated by *Burkholderia* in Brazil (Bontemps *et al.*, 2010; dos Reis Junior *et al.*, 2010), and cannot nodulate with the promiscuous *Mimosa*-nodulating *Rhizobium* strain JPY940 (this study); in this respect they differ from their cousins *M. albida* and *M. tequilana* in Mexico, which have adapted to nodulate with the local alpharhizobial MMS. A similar situation was recently reported for a native Indian *Mimosa* species (*M. himalayana*), which is related to Brazilian species (Simon *et al.*, 2011); although it nodulates with 'local' *Ensifer* spp. in the field, it can still nodulate with *Burkholderia* (Gehlot *et al.*, 2013).

Alpharhizobia have been isolated from *Mimosa* in previous studies, but have been only a minor part of the symbiont diversity, and they are often ineffective or non-nodulating (Barrett & Parker, 2006; Elliott *et al.*, 2009; Mishra *et al.*, 2012; Melkonian *et al.*, 2014). In central Mexico, however, they are clearly the major part of the rhizobial diversity associated with the genus, at least as it is represented by the 25 species in the present study, and this apparent preference of Mexican species for alpharhizobial symbionts is in almost complete contrast to Brazilian species, where all but two of the 143 symbionts isolated from 47 *Mimosa* spp. were *Burkholderia* (Bontemps *et al.*, 2010). Mexico is second only to Brazil as a centre of diversity of the large and important genus *Mimosa*, and geographical separation of these two diversification centres has most likely affected the type of symbiont selected by the *Mimosa* spp. in each. Differences in soils, such as fertility (e.g. N content) and pH, are important factors in governing how and why *Mimosa* spp. select particular symbionts (Elliott *et al.*, 2009; Liu *et al.*, 2012; Mishra *et al.*, 2012; Gehlot *et al.*, 2013). For example, under N-limited conditions, invasive *Mimosa* spp. overwhelmingly prefer to nodulate with *Burkholderia* rather than *Cupriavidus* or *Rhizobium*, (Elliott *et al.*, 2009; Melkonian *et al.*, 2014), but the predominance of *Burkholderia* can be overcome at higher N concentrations, which demonstrates that soil N-content is an important factor in *Mimosa* symbiont selection (Elliott *et al.*, 2009). Furthermore, soils in central Brazil are generally acid (many are less than pH 5.0; dos Reis Junior *et al.*, 2010), which would favour the acid-tolerant genus *Burkholderia* (Garau *et al.*, 2009; Stopnisek *et al.*, 2014), whereas those from central Mexico are either weakly acidic, neutral or slightly alkaline (Camargo-Ricalde *et al.*, 2010; this study), which would favour most species of *Rhizobium*, and also *Cupriavidus* (Klonowska *et al.*, 2012; Liu *et al.*, 2012; Mishra *et al.*, 2012; Gehlot *et al.*, 2013). Further studies using soils and seeds from both Brazil and Mexico could help to determine if (and what) soil characteristics are factors in the selection of symbionts by *Mimosa* spp. endemic to Brazil and Mexico.

Concluding remarks

Independent evolution following geographic separation of *Mimosa* lineages may help to explain symbiont preferences in the various clades. A possible scenario is that after the ancestors of the main Mexican and Brazilian lineages became separated, their descendants coevolved with the 'local' rhizobial microflora inhabiting the mainly highland soils in which the genus speciated: *Burkholderia* in the case of Brazil and *Rhizobium*/*Ensifer* in the case of Mexico. These old plant lineages, particularly those rich in endemic habitat-specific species, have had time to become increasingly specialized to a particular group of symbionts, and so have eventually lost (or possibly never had) an ability to associate with other types of bacteria. For example, *Burkholderia* is not a symbiont of Clade B, which diverged *c.* 20 Myr ago (Ma), soon after the emergence of the genus *c.* 28 Ma (Simon *et al.*, 2011). However, there have been several subsequent divergences between Mexican and Brazilian *Mimosa* lineages, and many are quite recent (1–6 Ma; Simon *et al.*, 2011). These younger lineages, such as those in the 'mixed' Clade T which diverged 2–6 Ma, have also adopted alpharhizobial symbionts in Mexico, but have retained their ability to nodulate with the *Burkholderia* symbionts of their South American cousins. If the closely related Indian species *M. hamata* and *M. himalayana* are an appropriate example (Gehlot *et al.*, 2013), it might be expected that the ability to nodulate with multiple symbiont types will eventually be lost in the more endemic Mexican species in this clade, such as *M. tequilana*, but retained in the widespread species *M. albida*. Another example is *M. affinis*, the Mesoamerican 'sister' to the widespread *M. pudica*: in the short time since they diverged (*c.* 2 Ma) *M. affinis* has developed a much stronger affinity for Alphaproteobacteria than its Betaprotobacteria-loving sister (Elliott *et al.*, 2009; Melkonian *et al.*, 2014; this study). In summary, although plant symbiotic preference can evolve relatively rapidly following the colonization of a new area/continent (e.g. a shift from beta to alpha preference in Mexican mimosas that recently diverged from South American lineages), there appears to be a trend towards symbiotic specialization, particularly in endemic plant lineages confined to specific habitats.

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Supporting Information

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

Notes S1 Sampling locations with *Mimosa* herbarium voucher numbers in KML format for visualization in Google Earth.

Fig. S1 Mexican *Mimosa* species growing *in situ*.

Fig. S2 Light microscopy of semi-thin sections of Mexican *Mimosa* nodules collected in the field.

Fig. S3 Phylogenetic relationships of strains isolated from *Mimosa* nodules in this study and reference strains based on 737 bp 16S rRNA and 337 bp *recA* sequences.

Table S1 Plant voucher and bacterial accession numbers, together with location details of Mexican *Mimosa* spp.

Table S2 Characteristics of rhizosphere soil from *Mimosa* species collected in Mexico

Table S3 Primers (Oligonucleotides) which were used for PCR and sequencing

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