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An invasive *Mimosa* in India does not adopt the symbionts of its native relatives

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• *Background and Aims* The large monophyletic genus *Mimosa* comprises approx. 500 species, most of which are native to the New World, with Central Brazil being the main centre of radiation. All Brazilian *Mimosa* spp. so far examined are nodulated by rhizobia in the betaproteobacterial genus *Burkholderia*. Approximately 10 Mya, trans-oceanic dispersal resulted in the Indian subcontinent hosting up to six endemic *Mimosa* spp. The nodulation ability and rhizobial symbionts of two of these, *M. hamata* and *M. himalayana*, both from north-west India, are here examined, and compared with those of *M. pudica*, an invasive species.
• *Methods* Nodules were collected from several locations, and examined by light and electron microscopy. Rhizobia isolated from them were characterized in terms of their abilities to nodulate the three *Mimosa* hosts. The molecular phylogenetic relationships of the rhizobia were determined by analysis of 16S rRNA, *nifH* and *nodA* gene sequences.
• *Key Results* Both native Indian *Mimosa* spp. nodulated effectively in their respective rhizosphere soils. Based on 16S rRNA, *nifH* and *nodA* sequences, their symbionts were identified as belonging to the alphaproteobacterial genus *Ensifer*, and were closest to the 'Old World' *Ensifer saheli*, *E. kostiensis* and *E. arboris*. In contrast, the invasive *M. pudica* was predominantly nodulated by Betaproteobacteria in the genera *Cupriavidus* and *Burkholderia*. All rhizobial strains tested effectively nodulated their original hosts, but the symbionts of the native species could not nodulate *M. pudica*.
• *Conclusions* The native *Mimosa* spp. in India are not nodulated by the *Burkholderia* symbionts of their South American relatives, but by a unique group of alpha-rhizobial microsymbionts that are closely related to the 'local' Old World *Ensifer* symbionts of other mimosoid legumes in north-west India. They appear not to share symbionts with the invasive *M. pudica*, symbionts of which are mostly beta-rhizobial.

Key words: *Mimosa hamata*, *Mimosa himalayana*, *Mimosa pudica*, Thar Desert, nodulation, *Cupriavidus*, *Burkholderia*, *Ensifer*, bacterial symbionts, rhizobia, Betaproteobacteria, nitrogen fixation, arid regions.

INTRODUCTION

The large monophyletic genus *Mimosa* (Mimosoideae; Fabaceae) consists of >500 species, mainly native to the New World (Barneby, 1991; Simon *et al.*, 2011). 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, including those that are low in nutrients and organic matter, low in pH and iron rich. *Mimosa* was considered by Barneby (1991) to have 'differentiated profusely in tropical and warm temperate savanna habitats', and it is particularly abundant and diverse in the cerrado and caatinga biomes of Brazil, where there are many endemics (Barneby, 1991; Simon and Proença, 2000; Simon *et al.*, 2011). Despite this high endemism, a few species have become pan-tropical invasive weeds, the most notorious of these being *M. diplotricha* (synonym *M. invisa*), *M. pigra* and *M. pudica* (Barneby, 1991; Chen *et al.*, 2005*a*; Parker *et al.*, 2007; Simon *et al.*, 2011). Nodulation by N_2-fixing bacteria (rhizobia) has been observed in almost all of approx. 100 *Mimosa* spp. that have been examined (dos Reis Junior *et al.*, 2010). Indeed, it is likely that their ability to nodulate profusely in alien environments has greatly assisted the spread of the invasive *Mimosa* spp. outside their predominantly native Americas (Chen *et al.*, 2005*a*; Parker *et al.*, 2007; Andrus *et al.*, 2012).

It is partly because of the seriousness of invasive *Mimosa* spp. as aggressive weeds that their bacterial symbionts have attracted a lot of interest in recent years, particularly as initial studies of invasive *M. diplotricha*, *M. pudica* and *M. pigra* in Taiwan showed that they were almost exclusively nodulated by strains of

Betaproteobacteria (Chen *et al.*, 2001, 2003*a*, *b*, 2005*a*). Legume nodulation by Betaproteobacteria ('beta-rhizobia') is a relatively recently described phenomenon; 'rhizobia' were formerly considered to consist exclusively of a limited number of genera in the order Rhizobiales in the Alphaproteobacteria (Graham, 2008; Sprent, 2009). Since their initial discovery, a considerable body of evidence has accumulated to show that legumes, particularly *Mimosa* spp. (Chen *et al.*, 2001, 2003*a*, *b*, 2005*a*, *b*, 2006; Barrett and Parker, 2005, 2006; Elliott *et al.*, 2007*a*; Andam *et al.*, 2007; Bontemps *et al.*, 2010; Mishra *et al.*, 2012), but also other mimosoids and some papilionoids, such as *Cyclopia* (Elliott *et al.*, 2007*b*), *Rhynchosia* (Garau *et al.*, 2009), common bean (*Phaseolus vulgaris*) (Talbi *et al.*, 2010) and *Lebeckia* spp. (Howieson *et al.*, 2013), may form effective nodules with bacteria in the genera *Burkholderia* and *Cupriavidus* (*Ralstonia*) (see review by Gyaneshwar *et al.*, 2011).

The consistent isolation of beta-rhizobia from *Mimosa* nodules worldwide suggested a special relationship between them and this legume genus, and this was investigated by a large study of symbionts of *Mimosa* spp. native to the cerrado and caatinga biomes of Central Brazil. These biomes are home to >250 *Mimosa* spp., most of them endemics to either the biomes as a whole or to specific (mainly highland) regions within them (Simon and Proença, 2000; Simon *et al.*, 2011). The surveys by Bontemps *et al.* (2010) and dos Reis Junior *et al.* (2010) showed that >95 % of the nodules from approx. 70 *Mimosa* spp. from the cerrado/caatinga contained *Burkholderia* spp. as their symbionts. These studies thus demonstrated that *Burkholderia* spp. are the predominant symbionts of *Mimosa* in its largest centre of radiation, i.e. Brazil. In addition, Bontemps *et al.* (2010) showed that there was high congruence between the core 'housekeeping' (16S rRNA, *recA*) and symbiosis-related (*nifH*, *nodC*) genes in the microsymbionts, and suggested that the symbiosis between *Mimosa* and *Burkholderia* spp. was 'ancient' (approx. 50 Myr old) and, therefore, unlikely to have been the result of recent transfer(s) of symbiosis-related genes from alpha-rhizobia.

In contrast to Brazil, in the second major centre of *Mimosa* radiation in the central highlands of Mexico, which has approx. 100 species (Barneby, 1991), it would appear that most of the endemic *Mimosa* spp. are nodulated not by Betaproteobacteria but by *Rhizobium* or *Ensifer* (synonym *Sinorhizobium*). This was first suggested by a study on just a single native Mexican species, *M. affinis* (Wang *et al.*, 1999), and then confirmed by a wider study on approx. 30 central Mexican species by C. Bontemps, Université de Lorraine, France and M. A. Rogel, Centro de Ciencias Genómicas, Mexico (unpubl. res.). This difference between Brazil and Mexico suggests that geographical separation/location (and possibly soil type) and host phylogenetic relationships (Simon *et al.*, 2011) have played a part in determining symbiont selection by *Mimosa* in the New World. In addition to the two major centres of *Mimosa* radiation in Brazil and Mexico there are two smaller ones in the Old World: Madagascar, with approx. 30 endemic species, and the Indian subcontinent with six (*M. angustisiliqua*, *M. barberi*, *M. hamata*, *M. himalayana*, *M. prainiana* and *M. rubicaulis*) (Gamble, 1920; Barneby, 1991; Simon *et al.*, 2011). These Old World species are phylogenetically nested in South American *Mimosa*, and it has been hypothesized that they arrived in Asia approx. 6 – 10 Mya via trans-Atlantic dispersal (Simon *et al.*, 2011).

Little is known about the symbionts of the Old World *Mimosa* spp., but given that they are closely related to South American species it might be expected that they would have retained their ability to nodulate with similar bacterial symbionts, i.e. with *Burkholderia* strains (Bontemps *et al.*, 2010). This appears to be the case with at least one species, *M. himalayana*, as it could nodulate effectively with the promiscuous *Mimosa* symbiont *B. phymatum* STM815^T, and ineffectively with *C. taiwanensis* LMG 19424^T (Elliott *et al.*, 2007*a*). The same symbiotic phenotype was evidenced by several South American species tested with these strains (Elliott *et al.*, 2007*a*; dos Reis Junior *et al.*, 2010). However, a recent study of legumes native to the Thar Desert in Rajasthan in western India showed that the symbionts of *M. hamata*, a species closely related to *M. himalayana*, include strains of *Ensifer* that are related to *E. saheli* (Gehlot *et al.*, 2012). The only other published study on *Mimosa* symbionts from India is that of Verma *et al.* (2004), who described two strains of *C. taiwanensis*, BHU1 and MS1, isolated from nodules on the non-native species, *M. pudica*, collected in the north (Uttar Pradesh) and south (Tamil Nadu) of India, respectively.

India thus represents a unique situation regarding *Mimosa* symbionts as, unlike other parts of sub-tropical and tropical Asia and Australasia, such as southern China (Liu *et al.*, 2011, 2012), Taiwan (Chen *et al.*, 2001, 2003*b*, 2005*a*), Australia (Parker *et al.*, 2007), New Guinea (Elliott *et al.*, 2009), the Philippines (Andrus *et al.*, 2012) and New Caledonia (Klonowska *et al.*, 2012) that harbour only invasive species (particularly *M. pudica*, which is common to them all), India also has native *Mimosa* spp. This raises the possibility of interaction(s) between the symbionts of the native/invasive species and their respective hosts. The present study, therefore, was aimed at: (1) examining in more detail the symbionts of native and invasive *Mimosa* spp. to determine their diversity and potential origins; and (2) determining if the native species share their environments and/or rhizobial symbionts with the invasive *M. pudica*.

MATERIALS AND METHODS

Collection of plant materials and soils for rhizobial 'trap' experiments and isolation of nodule symbionts

The sites in Rajasthan (RJ) from which the native Indian *Mimosa* species were sampled are characterized as semi-arid, whereas all the *M. pudica* sites are characterized as humid sub-tropical, with the exception of Bangalore (KA) which has a tropical wet/dry climate. Details are given in Table 1, where abbreviations for the locations can also be found in the footnote.

Nodules were collected from some *M. hamata* plants growing naturally, e.g. near Jodhpur, Rajasthan (Gehlot *et al.*, 2012), but most *M. hamata* nodules were sampled from the roots of plants grown in pots using soil taken from the rhizosphere of *M. hamata* growing in its native range in various locations in the Thar Desert of Rajasthan (Table 1, Supplementary Data Fig. S1). Soil for 'trapping' *M. himalayana* rhizobia was sampled from the rhizosphere of this species growing in its native range in eastern Rajasthan (Bijoliya), which is characterized by a higher altitude and precipitation than that in the native

TABLE 1. *Sites from which* Mimosa *seeds and nodules were collected, their climatic types, soil characteristics (pH, %N) and nodulation of* Mimosa *spp. in rhizosphere soil used for 'trapping' of rhizobia*

Site (State)*	Coordinates	Altitude (m)	Site from which nodules and/or soil was sampled. Climate.	Soil pH	Soil %N	*Mimosa* spp. native to the soil	*Mimosa* spp. used to trap rhizobia†
Jodhpur (RJ)	26°14′49·85″N/73°1′18·65″E	230·61	Field near Bhagat ki Kothi (New Campus, JNVU) in the native range of *M. hamata*. Semi-arid (rainfall <300 mm p.a.).	8·2	0·0091	*M. hamata*‡	*M. hamata* (E), *M. himalayana* (E), *M. pudica* (E)
Deh (Nagaur) (RJ)	27°18′30·40″N/73°54′53·51″E	303·38	Soil from rhizosphere of *M. hamata* in the Thar Desert. Semi-arid.	8·3	0·0102	*M. hamata*	*M. hamata* (E), *M. himalayana* (E)
Fatehpur (Sikar) (RJ)	27°58′0·43″N/74°58′21·02″E	328·61	Soil from rhizosphere of *M. hamata* bordering the Thar Desert. Semi-arid.	8·5	0·0085	*M. hamata*	*M. hamata* (E)
Chhapar (Churu) (RJ)	27°45′43·57″N/74°27′12·25″E	329·8	Soil from rhizosphere of *M. hamata* bordering the Thar Desert. Semi-arid.	8·7	0·0097	*M. hamata*	*M. hamata* (E), *M. himalayana* (E)
Bikaner (RJ)	28°1′49·04″N/73°15′30·63″E	238·3	Soil from rhizosphere of *M. hamata* in the Thar Desert. Semi-arid.	8·4	0·0078	*M. hamata*	*M. hamata* (E)
Barmer (RJ)	25°39′54·66″N/72°0′54·03″E	227·1	Soil from rhizosphere of *M. hamata* bordering the Thar Desert. Semi-arid.	8·6	0·0071	*M. hamata*	*M. hamata* (E), *M. himalayana* (E)
Bijoliya (Bhilwara) (RJ)	25°7′25·78″N/75°16′24·28″E	508·79	Soil from rhizosphere of *M. himalayana* collected from field within its native range. Semi-arid with higher rainfall than the Thar Desert (rainfall = 600 mm p.a.)	7·8	0·0216	*M. himalayana*	*M. hamata* (–), *M. himalayana* (E), *M. pudica* (–)
Agra (UP)	27°16′60·00″N/77°58′0·00″E	324·85	Nursery seedlings collected from the field. Humid sub-tropical.	7·2	0·0352	*M. pudica*‡	ND
Bokaro (JH)	23°45′27·10″N/85°53′36·52″E	232·42	Konar, riverside near BTPS, Kothara. Humid sub-tropical.	6·9	0·0432	*M. pudica*‡	*M. hamata* (I), *M. himalayana* (E), *M. pudica* (E)
Bangalore (KA)	13°0′39·54″N/77°34′13·70″E	895·79	Nursery seedlings in the campus of Indian Wood Science Technology (IWST). Wet and dry tropical.	6·8	0·0352	*M. pudica*‡	ND
Haridwar (UT)	30°5′14·65″N/78°15′55·47″E	327·45	Plants on roadside near Rishikesh. Humid sub-tropical.	7·5	0·0322	*M. pudica*‡	ND
Jorhat (AS)	26°46′57·25″N/94°17′35·92″E	91	Field-grown plant in the grounds of the Rain Forest Research Institute (RFRI). Humid sub-tropical.	5·2	0·065	*M. pudica*‡	*M. hamata* (–), *M. himalayana* (–), *M. pudica* (E)
Shillong (ME)	25°39′18·83″N/91°53′52·85″E	3216	Plants on roadside in Barapani area near Shillong. Humid sub-tropical.	4·9	0·280	*M. pudica*‡	*M. hamata* (–), *M. himalayana* (–), *M. pudica* (E)

* Standard abbreviations used: AS, Assam; JH, Jharkhand; KA, Karnataka; ME, Meghalaya; RJ, Rajasthan; UP, Uttar Pradesh; UT, Uttarakhand.
† E, effective; I, ineffective; –, no nodules; ND, not determined.
‡ Nodules sampled directly from plants in the field.

range of *M. hamata* (Table 1). The *M. pudica* nodules/rhizospheric soils were sampled from plants growing in several parts of India, encompassing sites in the north-west (Haridwar, UT), centre (Agra, UP), west (Jodhpur, RJ), south (Bangalore, KA), east (Bokaro, JH) and north-east (Jorhat, AS; Shillong, ME) of the country (Table 1, Supplementary Data Fig. S1).

To trap symbionts of *M. hamata*, *M. himalayana* and *M. pudica* growing in the various rhizosphere soils, seeds of each species were germinated as previously described (Elliott *et al.*, 2007*a*), and the seedlings were then sown into soil in pots (8 kg soil per pot) and grown in a greenhouse for up to 12 weeks, at which time the plants were harvested and nodules were sampled from the roots. Bacteria were axenically isolated from single nodules, purified from single colonies and cultivated on yeast-mannitol (YM) medium (Vincent, 1970) essentially as described by Bontemps *et al.* (2010). Some of the nodules were also cut in half to determine if they were potentially effective, as judged by the appearance of a pink colouration due to the presence of leghaemoglobin (Lb). Pink nodules were then placed in vials containing 2·5 % glutaraldehyde in 50 mM phosphate buffer (pH 7·5) for microscopical analysis.

In addition to rhizobial trapping experiments in Indian soils, *M. hamata* and *M. himalayana* were also sown in soil taken from the rhizosphere of Brazilian *Mimosa* spp. at Embrapa-CENARGEN, Brasília, Brazil.

Microscopy and immunolabelling of Mimosa *nodules*

Nodules were embedded in resin and sectioned for light and transmission electron microscopy (TEM) coupled with *in situ* immunogold labelling with antibodies raised against *Burkholderia phymatum* STM815^T and *Cupriavidus taiwanensis* LMG 19424^T according to Elliott *et al.* (2007*a*). These antibodies have been shown previously to be specific, respectively, to the genus *Burkholderia* and to the species *C. taiwanensis* (Elliott *et al.*, 2007*a*; dos Reis Junior *et al.*, 2010). To confirm their symbiotic effectiveness, the nodule sections were also labelled with an antibody that was raised against the NifH protein of the nitrogenase enzyme (dos Reis Junior *et al.*, 2010). Non-immune serum was used as a negative control in all immunogold assays.

Genetic characterization of Mimosa*-nodulating rhizobia*

Potential rhizobial symbionts were isolated from nodules collected from the sites and/or trap plants described above (Table 1, Supplementary Data Fig. S1). Three nodules were sampled from each plant; in general, one symbiotic isolate per nodule was then obtained. All bacteria were grown in YM broth or on YM agar plates. The isolates were grouped based on their place of origin, and then further selected based upon their colony morphology on YM plates compared with known rhizobial type strains, and finally on their ability to nodulate their host species of *Mimosa*. Confirmed nodulating strains from each group from each location were then further characterized by PCR amplification and sequencing of their 16S rRNA genes, and representative strains from each 16S rRNA cluster were selected for sequencing of their *nifH* and *nodA* genes (Table 2). PCR amplifications were performed with genomic DNA that was extracted as described in Moulin *et al.* (2004). For all strains, the nearly full-length 16S rRNA gene was amplified and sequenced with primers AGAGTTTGATCCTGGCTCAG and AAGGAGGTGATCCAGCC (Weisburg *et al.*, 1991). Partial *nifH* fragments from the isolates were amplified with primers CGTTTTACGGCAAGGGCGGTATCGGCA and TCCTCCAGCTCCTCCATGGTGATCGG (Perret and Broughton, 1998) for Alphaproteobacteria or with primers CGCIWTYTACGGIAARGGIGG and GGIKCRTAYTSGATIACIGTCAT for Betaproteobacteria (Chen *et al.*, 2003*b*). Partial *nodA* fragments were amplified with primers TGCRGTGGAARNTRNNCTGGGAAA and GGNCCGTCRTCRAAWGTCARGTA (Haukka *et al.*, 1998) for Alphaproteobacteria, with primers NodAF, AGTTGGGCCGGMGCNAGGCCTGA, and NodAR1, CAACGAACTGTTAATTGGCA, for *Burkholderia* strains, and with primers nodA F, 5′TGCRGTGGARDCTRYGCTGGGAAA 3′, and nodA R, 5′TCACARCTCKGGCCCGTTCCG-3′, for *Cupriavidus* strains (Mishra *et al.*, 2012). The PCR conditions for amplification were essentially as described earlier (Bontemps *et al.*, 2010; Gehlot *et al.*, 2012). The amplified gene products were purified using the QIAquick^{TM} PCR purification kit. Sequencing was performed at Xcelris Genomics, Ahmedabad, India, using a ABI SOLiD V4·0 System, at the University of Wisconsin Madison DNA Sequencing Facility, and at the National Kaohsiung Marine University using an Applied Biosystems ABI Prism 3730 sequencer.

Phylogenetic and taxonomic analysis

For molecular phylogenetic analyses, sequences of type strains and/or NCBI reference (NR) sequences were downloaded from NCBI. The GenBank accession numbers are listed in parentheses for the 16S rRNA, *nifH* and *nodA* genes used in this analysis. All the sequences were aligned using CLUSTAL W (Thompson *et al.*, 1997) and the alignment was exported to molecular evolutionary genetics analysis (MEGA) format in MEGA5 software (Tamura *et al.*, 2011). The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). Evolutionary distances were computed using the Kimura two-parameter method in units of the number of base substitutions per site (Kimura, 1980). To obtain confidence values, the original data set was resampled 1000 times using the bootstrap analysis method (Felsenstein, 1985). The MEGA5 software (Tamura *et al.*, 2011) was used for construction of phylogenetic trees, inferring distances and percentage similarity.

Nodulation tests with wild-type and GUS-marked strains

Representative strains from all three species were tested for nodulation of their original hosts (*M. hamata*, *M. himalayana*, *M. pudica*), and some strains were also selected for cross-inoculation tests on the same three hosts and on *M. affinis*, a Mexican species that is known to prefer to nodulate with alpha-rhizobia (Wang *et al.*, 1999; Elliott *et al.*, 2009). More detailed nodulation tests combined with microscopy were performed with selected strains that were marked with a pCAM121 transposon containing constitutively expressed glucuronidase (GUS) (Wilson *et al.*, 1995). Briefly, *Escherichia coli* strain β2155 (Dehio and Meyer, 1997), which requires diaminopimelic acid, was transformed with the plasmid, pCAM121,

TABLE 2. *Rhizobial strains isolated from native and invasive* Mimosa *spp. in India and Brazil and their putative identification via matching of their 16S rRNA gene sequences with those in the databases; data are also shown for nodulation tests of selected strains with* M. hamata *(*Mha*),* M. himalayana *(*Mhi*) and* M. pudica *(*Mp*)*

Strain no.	Plant host (no. of isolates obtained)	Geographical origin (State)	16S rRNA GenBank accession no.	Closest 16S rRNA BLASTN match (% similarity)	*nifH* GenBank accession no.	*nodA* GenBank accession no.	*Mha*	*Mhi*	*Mp*
MH1b	*M. hamata* (2)	Nagaur (Rajasthan)	GQ355314	*E. saheli* LMG 7837^T (99 %)	JQ951757	JQ951758	E	ND	
MH3	*M. hamata* (2)	Sikar (Rajasthan)	GQ355315	*E. saheli* LMG 7837^T (99 %)	JQ951759	JQ951760	E	–	–
MH3a*	*M. hamata*	Sikar (Rajasthan)	JN867012	*E. saheli* LMG 7837^T (99 %)		JQ951761	E	–	–
MH8*	*M. hamata* (7)	Jodhpur (Rajasthan)	JN867013	*E. saheli* LMG 7837^T (99 %)	KC478282	JQ951762	E	–	–
MH9	*M. hamata*	Jodhpur (Rajasthan)	GQ355316	*E. saheli* LMG 7837^T (99 %)		JQ951763	E	ND	–
MH32	*M. hamata* (5)	Chhapar (Rajasthan)	JX843749	*E. saheli* LMG 7837^T (99 %)	JX843757	JX843746	E	ND	–
MH37	*M. hamata* (3)	Bikaner (Rajasthan)	JX843750	*E. saheli* LMG 7837^T (99 %)	JX843758	JX843747	E	E	–
MH40	*M. hamata* (3)	Barmer (Rajasthan)	JX843751	*E. saheli* LMG 7837^T (99 %)	JX843759	JX843748	E	E	–
MHM1	*M. himalayana* (7)	Bijoliya (Rajasthan)	JQ951764	*E. saheli* LMG 7837^T (99 %)	JX843760	JX843744	–	E	–
MHM2	*M. himalayana*	Bijoliya (Rajasthan)	JQ951766	*E. saheli* LMG 7837^T (99 %)			–	E	–
MHM3	*M. himalayana*	Bijoliya (Rajasthan)	JQ951768	*E. saheli* LMG 7837^T (99 %)			–	E	–
MHM4	*M. himalayana*	Bijoliya (Rajasthan)	JQ951770	*E. saheli* LMG 7837^T (99 %)			–	E	–
MHM12	*M. himalayana*	Bijoliya (Rajasthan)	JQ951772	*E. saheli* LMG 7837^T (99 %)	JQ951773	JQ951774	–	E	–
MHM22	*M. himalayana* (4)	Jodhpur (Rajasthan)	JQ951776	*E. saheli* LMG 7837^T (99 %)	JX112774	JQ951777	–	E	–
MHM24	*M. himalayana* (3)	Nagaur (Rajasthan)	JX843752	*E. saheli* LMG 7837^T (99 %)	JX843761	JX112778	–	E	–
MHM32	*M. himalayana* (3)	Chhapar (Rajasthan)	JQ951778	*E. saheli* LMG 7837^T (99 %)	JX112775	JQ951779	–	E	–
MHM40	*M. himalayana* (3)	Barmer (Rajasthan)	JX843753	*E. saheli* LMG 7837^T (99 %)	JX843762	JX843745	–	E	–
MP3	*M. pudica* (2)	Bangalore (Karnataka)	JQ951791	*C. oxalaticus* DSM 1105^T (97 %)	JX843754	JQ951780	I	I	E
MP6	*M. pudica* (2)	Haridwar (Uttarakhand)	GQ355321	*C. taiwanensis* LMG 19424^T (99 %)	JX843755	JX843742	ND	ND	E
MP7	*M. pudica* (4)	Jodhpur (Rajasthan)	GQ355322	*C. taiwanensis* LMG 19424^T (99 %)	JQ951781	JQ951782	ND	ND	E
MP10	*M. pudica* (3)	Agra (Uttar Pradesh)	GQ355325	*R. vallis* CCBAU 65647^T (100 %)	JQ951784	JQ951783	I	ND	E
MP15	*M. pudica* (3)	Agra (Uttar Pradesh)	GQ355324	*C. taiwanensis* LMG 19424^T (99 %)	JX843756	JX843743	–	–	E
MP20	*M. pudica* (5)	Bokaro (Jharkhand)	GQ355318	*B. phymatum* STM815^T (99 %)	JQ951785	JQ951786	I	E	E
MPB1	*M. pudica* (10)	Barapani (Meghalaya)	KC287136	*B. mimosarum* PAS44^T (99 %)	KC440177	KC478283	ND	ND	E
MPB6	*M. pudica*	Barapani (Meghalaya)	KC287137	*B. mimosarum* PAS44^T (99 %)	KC440178		ND	ND	E
MPB8	*M. pudica*	Barapani (Meghalaya)	KC287138	*B. mimosarum* PAS44^T (99 %)	KC440179	KC478284	ND	ND	E
MPB11	*M. pudica*	Barapani (Meghalaya)	KC287139	*B. mimosarum* PAS44^T (99 %)	KC440180		ND	ND	E
MPJ1	*M. pudica* (4)	Jorhat (Assam)	JQ951792	*B. phymatum* STM815^T (99 %)	JQ951788	JX843740	ND	ND	E
MPJ4	*M. pudica* (4)	Jorhat (Assam)	JQ951793	*B. mimosarum* PAS44^T (99 %)	JQ951789	JX843739	ND	ND	E
MPJ11	*M. pudica* (4)	Jorhat (Assam)	JQ951794	*C. taiwanensis* LMG 19424^T (98 %)	JQ951790	JX843741	ND	ND	E
STM815^T	*M. pudica*†	French Guiana	NR_027555	*B. phymatum* STM815^T (100 %)	AJ505319	AJ505318	I	E†	E†
LMG19424^T	*M. pudica*	Taiwan	NR_028800	*C. taiwanensis* LMG 19424^T (100 %)	NC_010529	AJ505311	I	I†	E†
Mim-1	*M. affinis*	Mexico	DQ648573	*R. etli* bv. *mimosae* Mim-1 (100 %)			–	E	I‡
MHM (B) 2 III	*M. himalayana* (7)	Brazil	KC791149	*E. mexicanum* ITTG-R7^T (99 %)			ND	ND	ND
MHM (B) 5	*M. himalayana*	Brazil	KC791150	*E. mexicanum* ITTG-R7^T (99 %)			ND	ND	ND
MHM (B) 8	*M. himalayana*	Brazil	KC791151	*E. mexicanum* ITTG-R7^T (99 %)			ND	ND	ND

* Previously reported by Gehlot *et al.* (2012).
† See Elliott *et al.* (2007*a*) for details.
‡ See Elliott *et al.* (2009) for details.

and the transposon was then mobilized into the *M. pudica* isolates *Cupriavidus* sp. MP3 and *B. phymatum* MP20 by conjugation. The transconjugants were selected on YM agar containing 100 μg ml^{-1} spectinomycin and screened for GUS activity on YM agar containing 20 μg ml^{-1} X-gluc. One colony showing GUS activity and no apparent growth defect was selected for nodulation studies. Seeds of *M. hamata*, *M. himalayana*, *M. pudica* and *M. affinis* were scarified with concentrated sulphuric acid for 5 min, washed with sterile distilled water five times and germinated on water agar (1 %) plates. Seven-day-old seedlings were transferred to 150-mL glass tubes containing sterile vermiculite and inoculated with 10^9–10^{10} cells of various bacterial strains grown on YM medium. The inoculated seedlings were then incubated in a growth chamber at 25 °C either under a 16/8-h light/dark cycle or under a natural day/night cycle. Un-inoculated seedlings served as controls. The number of nodules, their appearance (e.g. if they were expressing Lb) and the health of the host plants was determined at 30 d after inoculation (dai) for *M. pudica* and *M. affinis* and at 40 dai for *M. hamata* and *M. himalayana*. Representative nodules from all species/strain combinations were also prepared for light microscopy and TEM as described above.

RESULTS

Nodulation of native and invasive Mimosa *spp. in India*

Mimosa hamata (Fig. 1A, B) is native to the Thar Desert and to surrounding semi-arid regions of Rajasthan and north-west India (Gehlot *et al.*, 2012). The other native Indian species in this study, *M. himalayana* (Fig. 1C, D), is much more widespread (Ali, 1973; Shetty and Singh, 1987; Bora and Kumar, 2003), and generally prefers higher altitude (non-desert) regions in Rajasthan and in other parts of northern India that have significantly higher rainfall than the Thar Desert. In this study, the two native species were not found to inhabit the same environments. Nodulation of *M. hamata* growing near Jodhpur has previously been reported by Gehlot *et al.* (2012), and the ability of this species to nodulate in this semi-arid environment was confirmed in the present study via trap experiments using soil from several other locations in the Thar Desert (Table 1, Fig. 1E, Supplementary Data Fig. S1). In the case of *M. himalayana*, soil was obtained from the rhizosphere of natural stands of plants growing near Bijoliya in the east of Rajasthan (Fig. S1). This soil, which was more fertile than the *M. hamata* rhizospheric soils from the Thar Desert (Table 1), was used to trap rhizobia with *M. himalayana* seedlings that had been sown into it. Mature nodules had formed on *M. himalayana* by 2 months after seeds had been sown into the soil, similar to the time taken for *M. hamata* nodules to form when grown in pots of soil under the conditions used in the present study. Nodules on both species were branched and appeared to be indeterminate (Fig. 1E, F). This was confirmed by light microscopy of longitudinal sections, which demonstrated that *M. hamata* nodules were similar to those on other *Mimosa* spp. from semi-arid environments (dos Reis Junior *et al.*, 2010), i.e. indeterminate with a pronounced meristem and invasion zone, and with an outer cortex with a 'corky' hypodermis layer (Fig. 2A, C), with cells containing phenolic compounds and/or tannins. The structure of nodules on *M. himalayana* was similar to that of *M. hamata* nodules, and has been described previously by Elliott *et al.* (2007*a*). TEM coupled with immunogold labelling with an antibody against the NifH protein of nitrogenase confirmed that bacteroids in field-grown or trap soil-grown nodules expressed this enzyme (Fig. 2B, D), strongly suggesting that both species form symbiotic N_2-fixing nodules in the field and/or in their native soils.

Nodules from the invasive *M. pudica* that were sampled from several parts of India were also examined by microscopy, and the structure of these was as reported previously (Chen *et al.*, 2003*a*). Sections of nodules of all three species were also probed with antibodies specific to the common beta-rhizobial *Mimosa* symbionts, *B. phymatum* and *C. taiwanensis* (Elliott *et al.*, 2007*a*; dos Reis Junior *et al.*, 2010). None of the nodules examined from either of the native species was recognized by these antibodies (a section of an *M. hamata* nodule that was probed with the *C. taiwanensis* antibody is shown in Supplementary Data Fig. S2A), but nodules of *M. himalayana* that had been nodulated by *B. phymatum* $STM815^T$ from the study of Elliott *et al.* (2007*a*) reacted strongly with the *B. phymatum* antibody (Fig. S2B). *Mimosa pudica* nodules obtained from trap plants grown in soil from the rhizosphere of *M. hamata* near Jodhpur (RJ) (Table 1) were strongly labelled with the *C. taiwanensis* antibody (Fig. S2C), but not the *B. phymatum* antibody (Fig. S2D), and this was also the case with *M. pudica* nodules sampled directly from plants at three other locations at Agra (UP) (Fig. S2E), Bangalore (KA) and Haridwar (UT) (data not shown). On the other hand, nodule samples from another location, Bokaro (JH), in eastern India, were strongly labelled with the *B. phymatum* antibody (Fig. S2F).

The native and invasive *Mimosa* spp. were also tested for nodulation in some of the rhizospheric soils (Table 1). *Mimosa himalayana* nodulated in several of the *M. hamata* rhizospheric soils from the Thar Desert (Table 2), but *M. hamata* failed to nodulate in the more fertile *M. himalayana* rhizosphere soil from Bijoliya (RJ). Neither of the native species was able to nodulate in any of the *M. pudica* rhizospheric soils, with the exception of the Bokaro (JH) soil, in which *M. himalayana* (but not *M. hamata*) nodulated. *Mimosa pudica* was able to nodulate readily in the *M. hamata* rhizospheric soil from Jodhpur (Table 2), but not in the *M. himalayana* rhizosphere soil from Bijoliya (data not shown).

Mimosa hamata grew poorly and only formed the occasional ineffective nodule in Brazilian cerrado soil (Fig. 3A, B), whereas *M. himalayana* grew well and nodulated profusely and effectively (Fig. 3C, D). Sections of the nodules from neither species reacted with the *B. phymatum* and *C. taiwanensis* antibodies (data not shown), which strongly suggests that neither of these beta-rhizobial types is present in the nodules (dos Reis Junior *et al.*, 2010).

Molecular characterization of symbionts of native and invasive Mimosa *spp. in India*

Rhizobia were isolated from nodules of native and invasive *Mimosa* spp. under axenic conditions and their phylogenetic relationships were determined by analysis of 16S rRNA gene sequences (Figs 4 and 5). In addition to strains MH3a and MH8 that were directly isolated, respectively, from *M. hamata* nodules sampled near Sikar and Jodhpur (RJ) by Gehlot *et al.*

FIG. 1. Native Indian *Mimosa* spp. in the wild. (A) *Mimosa hamata* is a shrub that grows in the Thar Desert of Rajasthan. It grows to approx. 3 m maximum height, and the plant in this photograph is approx. 2 m. (B) Detail of the foliage and flowers of *M. hamata*; the spiny stems and the spherical pink inflorescences are very typical of the genus *Mimosa*. (C) *Mimosa himalayana* has a similar growth habit to *M. hamata* and it grows to a similar size, but it prefers wetter environments, in which it grows among other lush vegetation. (D) Detail of the foliage and flowers of *M. himalayana*; note that the stems, foliage and flowers are very similar to the closely related *M. hamata*. (E) Large branched nodules (arrow) on an *M. hamata* plant grown in soil taken from the rhizosphere of a plant growing in the Thar Desert of Rajasthan. (F) Nodules (*) on an *M. himalayana* plant grown in soil taken from the rhizosphere of a plant growing in the Bijoliya region of Rajasthan. Scale bars: (E) = 1 cm; (F) = 500 μm.

(2012), six further defined strains were isolated from *M. hamata* nodules obtained from soil trapping experiments using soil from four more sites in the Thar Desert of Rajasthan (Tables 1 and 2). Five defined rhizobial strains were isolated from *M. himalayana* nodules obtained from trapping experiments using soil from the rhizosphere of *M. himalayana* sampled from Bijoliya in eastern Rajasthan, and four additional strains were isolated from *M. himalayana* nodules on seedlings grown in *M. hamata*

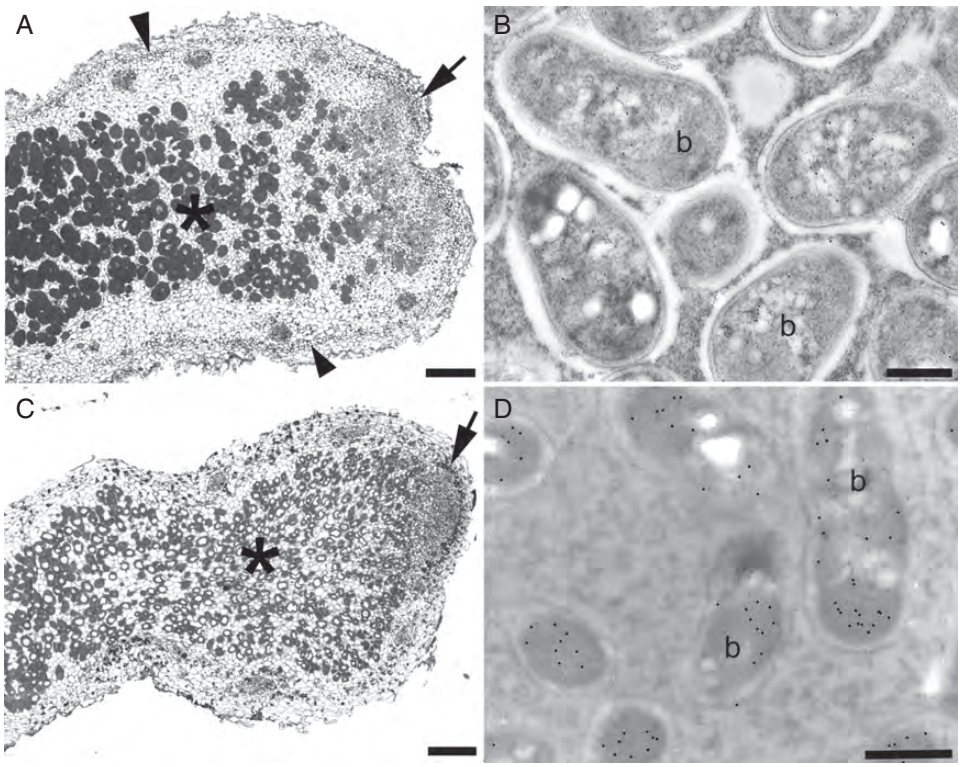

FIG. 2. Light microscopy (A, C) and transmission electron microscopy (TEM) combined with immunogold labelling with an antibody against the NifH (Fe-)protein of nitrogenase (B, D) of *M. hamata* (A, B) and *M. himalayana* (C, D) nodules. Longitudinal sections of the nodules (A, C) show them to be broadly similar to those on other *Mimosa* spp., i.e. typically indeterminate with a persistent meristem (arrows) and an infected zone of N_2-fixing cells (*), but in the case of *M. hamata* they also have a pronounced hypodermis (arrowheads in A). The bacteroids (b) in nodules from both species strongly express the NifH protein (B, D). Scale bars: (A, C) = 200 μm; (B, D) = 500 nm.

rhizospheric soil from four sites in the Thar Desert (Table 1). As shown in Fig. 4, all the strains from *M. hamata* and *M. himalayana* grouped together and showed highest 16S rRNA gene sequence similarity to sequences from *Ensifer saheli* in the Alphaproteobacteria. The 16S rRNA sequences of the rhizobia isolated from *M. himalayana* plants grown and nodulated in Brazilian cerrado soil also placed these in *Ensifer*, but in this case they were more closely related to *E. mexicanum* (Fig. 4). The identities of the strains nodulating the native species contrast with those isolated from the invasive species *M. pudica* as, with the exception of MP10 from Agra (UP) which was related to *Rhizobium vallis* (Table 2, Fig. 4), all of the symbiotically effective *M. pudica* isolates belonged to genera/species in the Betaproteobacteria (Fig. 5).

All the betaproteobacterial isolates from *M. pudica* nodules sampled in Bangalore (KA), Agra (UP) and Haridwar (UT) and one isolate from Jorhat (AS) were related to *C. taiwanensis*, as were the isolates 'trapped' by *M. pudica* seedlings that were grown in *M. hamata* rhizosphere soil from Jodhpur (Fig. 5). These strains all clustered with the *C. taiwanensis* type strain, LMG 19424^T, but strain MP3 from Bangalore (KA) was closer to the South Indian *Cupriavidus* sp. strain from Tamil Nadu (MS1) than to the north Indian one from Uttar Pradesh (BHU1), both of which had been isolated from *M. pudica* nodules by Verma *et al.* (2004). In contrast to *C. taiwanensis* being the apparently predominant symbiont of *M. pudica* in north-western, central and southern India, mostly bacteria showing maximum 16S rRNA sequence similarity to the common *M. pudica*-nodulating *Burkholderia* spp., *B. mimosarum* and *B. phymatum*, were isolated from *M. pudica* growing in eastern (Bokaro, JH) and north-eastern (Jorhat, AS; Shillong, ME) parts of India (Fig. 5).

To determine the relatedness of the rhizobia of the invasive and native *Mimosa* spp. further, the DNA sequences of genes that are essential for N_2 fixation (*nifH*) and symbiosis (*nodA*) were analysed (Figs 6 and 7). The *nifH* gene encodes the iron (Fe-) protein component of the nitrogenase enzyme complex and is essential for mutualistic N_2-fixing symbioses, although it is not specific to rhizobia and is present in all free-living diazotrophs (Young, 2005). A phylogenetic analysis of the *nifH* sequences of the strains that nodulated the native Indian *M. hamata* and *M. himalayana* showed them to be clustered together and that they were closest to the *E. kostiensis* type strain HAMBI 1489^T, which was isolated from *Acacia senegal* in Sudan (Nick *et al.*, 1999), with the next closest sequence being that of the *E. saheli* type strain, ORS609^T, from *Sesbania cannabina* (de Lajudie *et al.*, 1994). In the case of the *M. pudica* isolates, the *nifH* sequence from *Rhizobium* sp. MP10 clustered with that of *R. etli* bv. *mimosae* Mim7-4, a symbiont of *M. affinis* from Mexico (Wang *et al.*, 1999) and with *R. etli* TJ173 from *M. pudica* nodules in Taiwan (Elliott *et al.*, 2009),

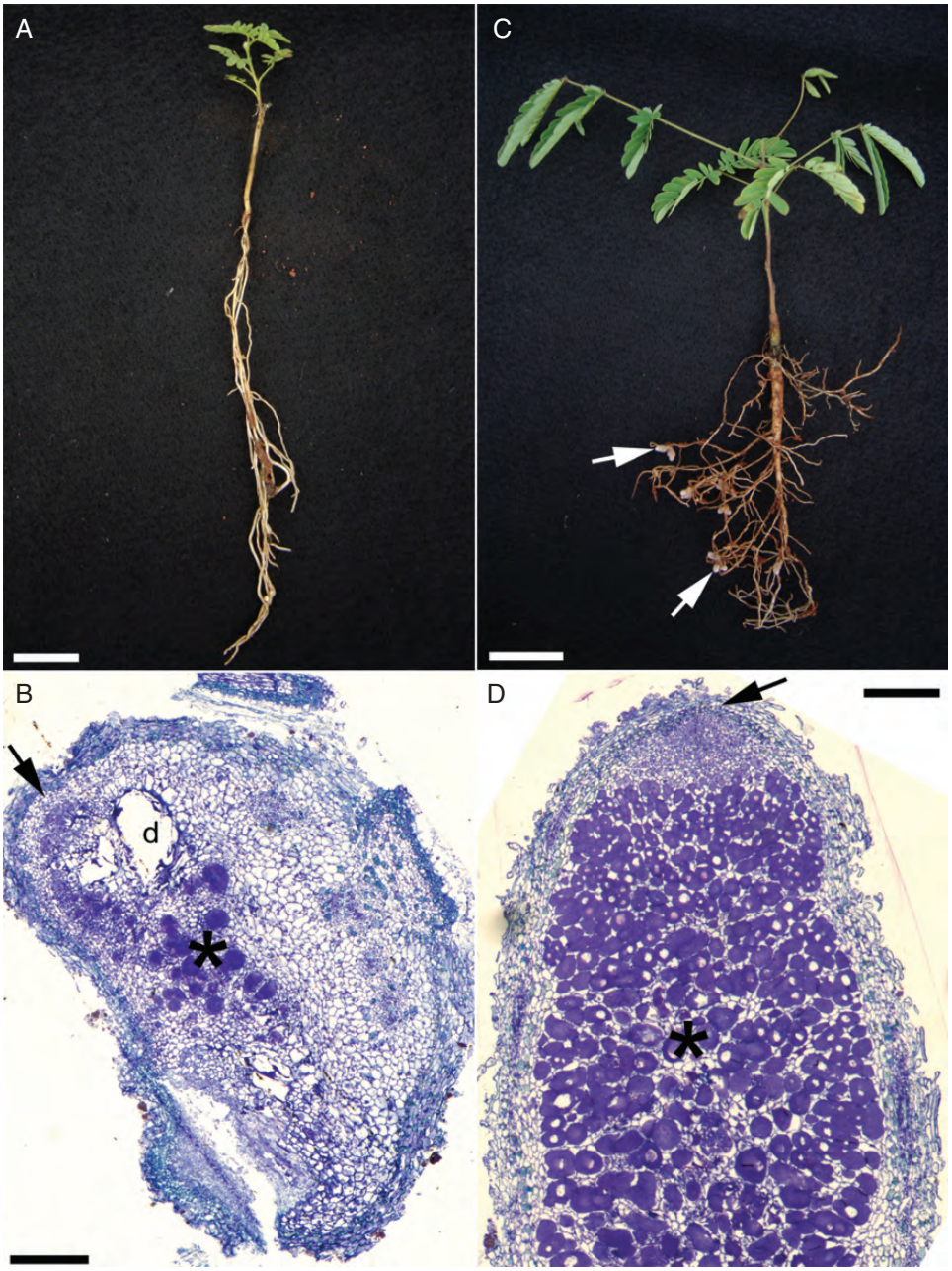

FIG. 3. *Mimosa hamata* (A, B) and *M. himalayana* (C, D) grown in Brazilian cerrado soil for 3 months. Note that there are no (or few) nodules on *M. hamata* (A) and that the plant is small and unhealthy. This is reflected in the structure of the single nodule taken from an *M. hamata* plant (B); it is clearly ineffective and contains areas of degraded tissue (d). In contrast, *M. himalayana* is green and healthy and well nodulated (arrows in C), and the nodules are effective in appearance (D). An arrow indicates the nodule meristem in (B) and (D), and the infected, N_2-fixing zone is indicated by an asterisk (*) in each case. Scale bars: (A) = 1 cm; (B) = 2 cm; (C, D) = 200 μm.

whereas the *M. pudica*-nodulating *C. taiwanensis* and *B. phymatum* strains showed maximum similarity to the *nifH* sequences of their respective type strains, *C. taiwanensis* LMG 19424^T and *B. phymatum* STM815^T, but the *Cupriavidus nifH* sequences were different from those of the previously isolated Indian *Cupriavidus* strains BHU1 and MS1 (Verma *et al.*, 2004). Finally, the *nifH* sequences of *B. mimosarum* MPJ4 and MPB1, MPB6, MPB8 and MPB11 showed highest similarity to that of the *B. mimosarum* type strain, PAS44^T (Fig. 6).

In contrast to *nifH* genes, the *nod* genes are present only in legume-nodulating rhizobia, in which they are involved in the synthesis of Nod factors. In most legume–rhizobial symbioses studied to date, these are essential components of the signal exchange between the soil-dwelling rhizobia and the roots of their potential legume host, an exchange which will ultimately lead to the formation of functional N_2-fixing nodules (Sprent, 2009). Slight alterations ('decorations') on the chemical structure of the lipo-chito-oligosaccharide backbone of the Nod

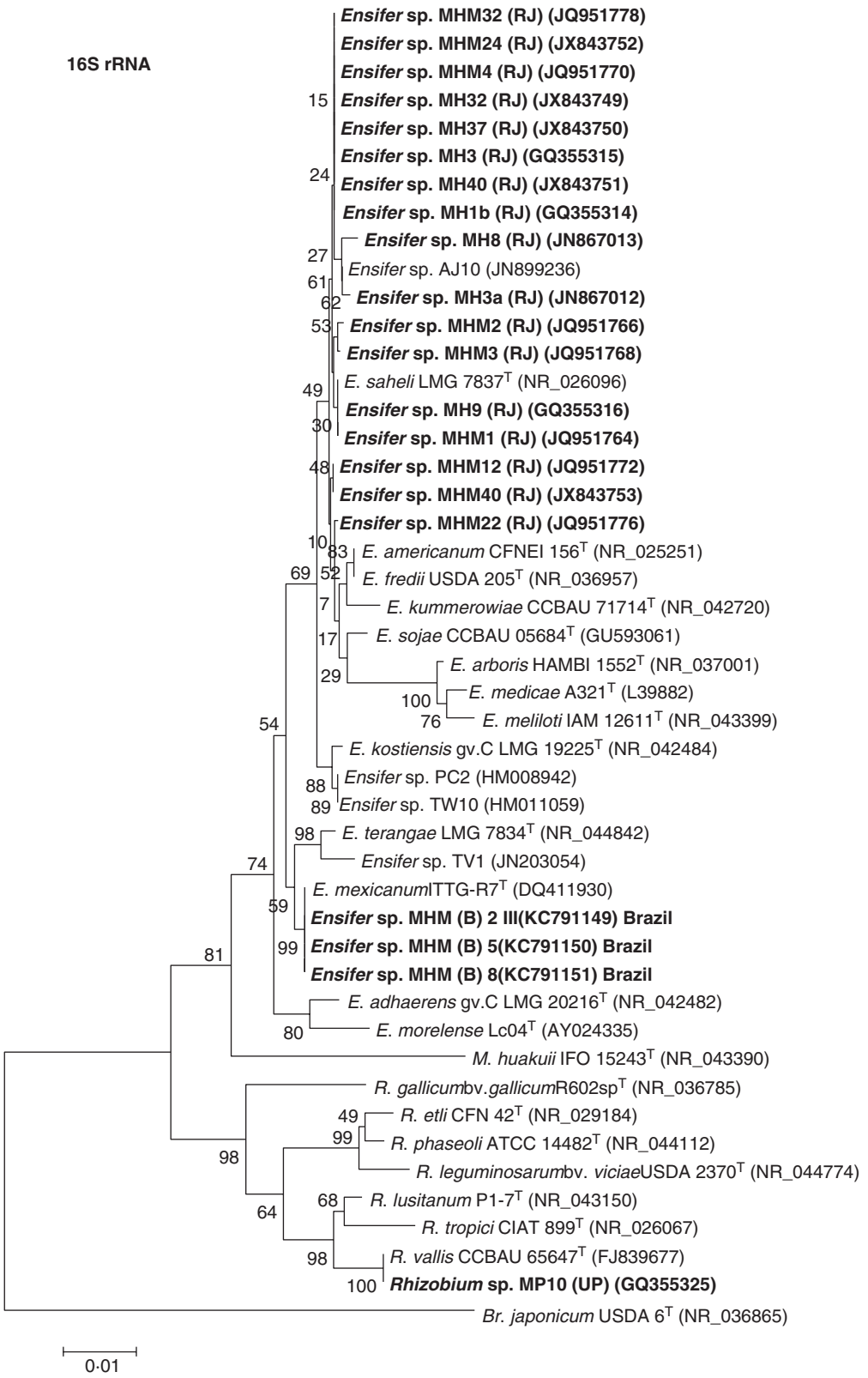

FIG. 4. Neighbour-joining phylogenetic tree for 16S rRNA gene sequences of *Ensifer* and *Rhizobium* strains isolated from native Indian *Mimosa* species with type/reference strains and close relatives. Bootstrap values calculated for 1000 replications are indicated at the internodes. The scale bar indicates 1 % substitutions per site. GenBank accession numbers are given in parentheses. Abbreviations: *Br.*, *Bradyrhizobium*; *E.*, *Ensifer*; *M.*, *Mesorhizobium*; *R.*, *Rhizobium*; T, type strain; (NR), NCBI reference sequence. Strains with the prefixes MH and MHM were isolated from *M. hamata* and *M. himalayana*, respectively. Strains isolated in the present study are marked in bold.

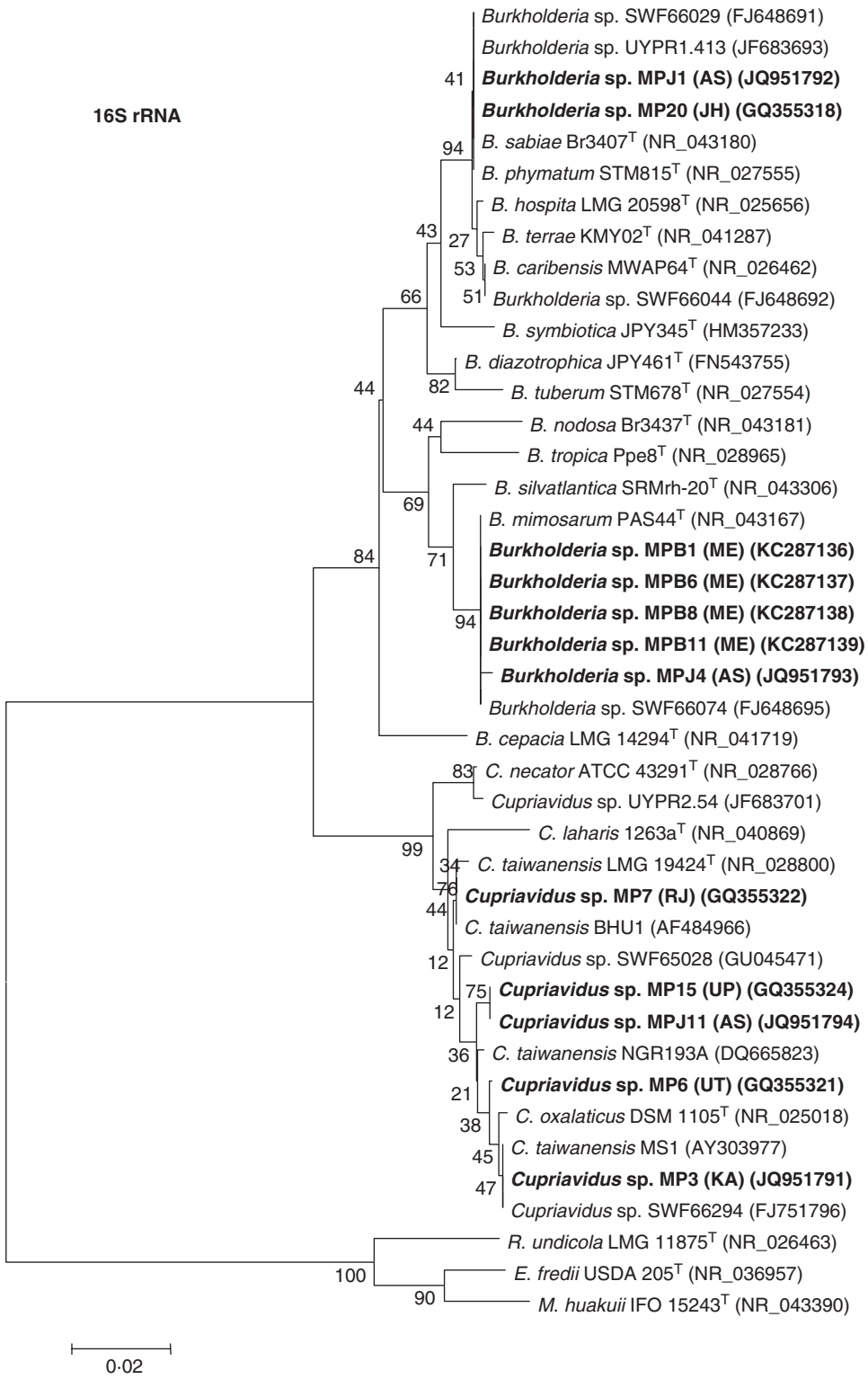

FIG. 5. Neighbour-joining phylogenetic tree for 16S rRNA gene sequences of *Burkholderia* and *Cupriavidus* strains isolated from the invasive species *Mimosa pudica* with type/reference strains and close relatives. Bootstrap values calculated for 1000 replications are indicated at the internodes. The scale bar indicates 2 % substitutions per site. GenBank accession numbers are given in parentheses. Abbreviations: *B*., *Burkholderia*; *C*., *Cupriavidus*; *E*., *Ensifer*; *M*., *Mesorhizobium*; *R*., *Rhizobium*; T, type strain; (NR), NCBI reference sequence. Strains isolated in the present study are marked in bold.

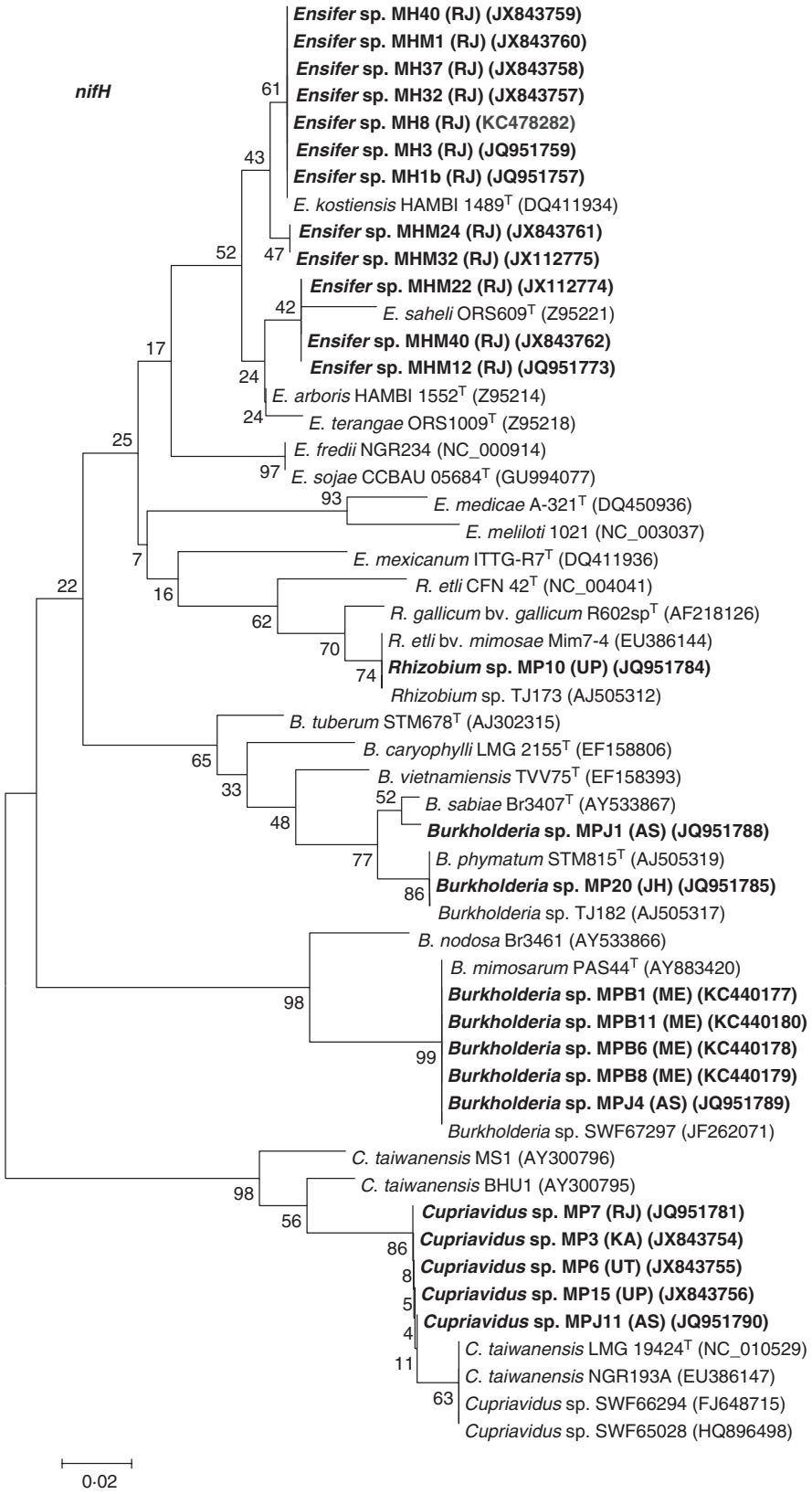

FIG. 6. Neighbour-joining tree of *nifH* gene sequences showing the phylogenetic relationships of the root nodule bacteria isolated from *Mimosa* spp. Bootstrap values calculated for 1000 replications are indicated at the internodes. GenBank accession numbers are given in parentheses. The scale bar represents 2 % nucleotide substitutions per site. Abbreviations: *B.*, *Burkholderia*; *C.*, *Cupriavidus*; *E.*, *Ensifer*; *R.*, *Rhizobium*; T, type strain. Strains with the prefixes MH, MHM and MP were isolated from *M. hamata*, *M. himalayana* and *M. pudica*, respectively. Strains isolated in the present study are marked in bold.

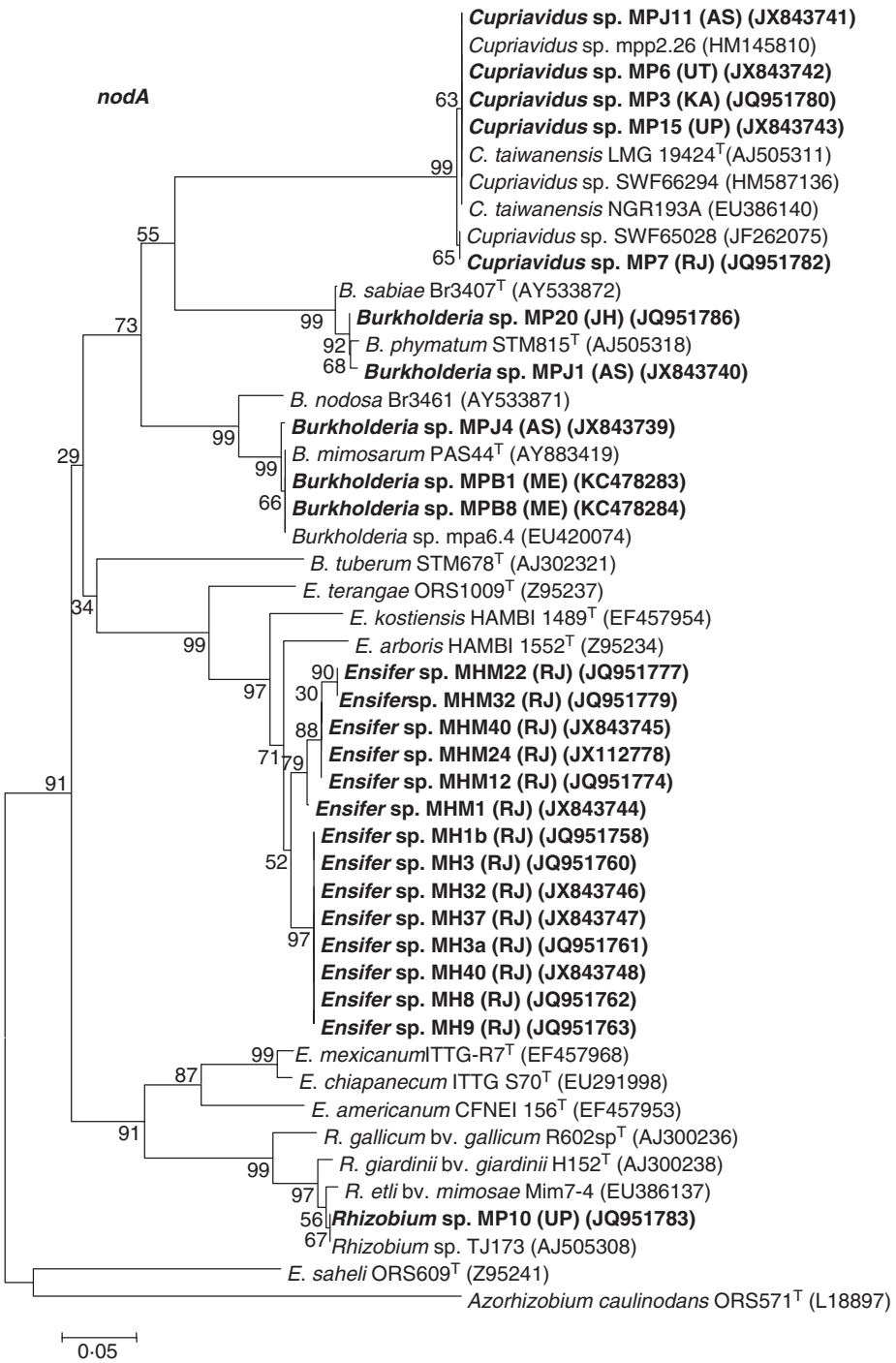

FIG. 7. Neighbour-joining phylogenetic tree for *nodA* gene sequences of nodulating strains isolated from *Mimosa* spp. with close relatives and type strains. Bootstrap values calculated for 1000 replications are indicated at the internodes. The scale bar indicates 5 % nucleotide substitutions per site. GenBank accession numbers are given in parentheses. Abbreviations: *B.*, *Burkholderia*; *C.*, *Cupriavidus*; *E.*, *Ensifer*; *R.*, *Rhizobium*; T, type strain. Strains with the prefixes MH, MHM and MP were isolated from *M. hamata*, *M. himalayana* and *M. pudica*, respectively. Strains isolated in the present study are marked in bold.

factors can greatly affect the host range of a particular rhizobial strain (Pueppke and Broughton, 1999; Kobayashi and Broughton, 2008). Analysis of the sequences of the *nodA* genes of the various native and invasive *Mimosa* isolates (Fig. 7) showed that the *Ensifer* strains from *M. hamata* and *M. himalayana* clustered together, but they were not closely related to any type strains, with their sequences being closest to the African strains *E. arboris* HAMBI 1552^T from *Prosopis chilensis*, *E. kostiensis* HAMBI 1489^T from *Acacia senegal* and *E. terangae* ORS1009^T from *A. laeta* (de Lajudie *et al.*, 1994;

Haukka *et al.*, 1998; Nick *et al.*, 1999). For the *M. pudica* rhizobial strain, as with its *nifH* sequence (Fig. 6), the *nodA* sequence from *Rhizobium* sp. MP10 showed highest similarity to those of the *Mimosa*-nodulating strains *R. etli* biovar *mimosae* Mim7-4 and *R. etli* TJ173 (Fig. 7). The *nodA* sequences of the *Cupriavidus* strains were all similar to the *C. taiwanensis* type strain, LMG 19424^T from Taiwan, whereas those of the *Burkholderia* strains MP20 and MPJ1 were closest to *B. phymatum* STM815^T, and those of *Burkholderia* strains MPJ4, MPB1 and MPB8 were closest to *B. mimosarum* PAS44^T (Fig. 7).

Cross inoculation studies using wild-type and GUS-marked strains

All of the strains isolated from the Indian native and invasive *Mimosa* spp. featured in Figs 4–7 were tested positive for nodulation on their original hosts (Table 2). Some of these strains were also tested for nodulation of the other species in this study. None of the *M. hamata* and *M. himalayana* strains was capable of nodulating *M. pudica* (Table 2), but *M. hamata* strains, such as *Ensifer* sp. MH37 and MH40, could effectively nodulate both *M. hamata* and *M. himalayana* (Supplementary Data Fig. S3A, B), whereas the opposite was not true, i.e. no *M. himalayana* strains could nodulate *M. hamata* (Fig. S3A, Table 2). A Mexican *Mimosa* strain, *R. etli* bv. *mimosae* Mim-1, which was isolated from *M. affinis* by Wang *et al.* (1999) and which can effectively nodulate this species (Elliott *et al.*, 2009), was also capable of nodulating *M. himalayana* (Fig. S3C) but not *M. hamata* (data not shown). *Mimosa affinis* could be nodulated by the *M. hamata Ensifer* sp. strains MH37 and MH40, but the nodules were small, white and ineffective (Fig. S3D). *Mimosa affinis* could not be nodulated by the *M. himalayana Ensifer* sp. strain MHM12 (data not shown).

It was previously shown that *M. himalayana* can be effectively nodulated by the *M. pudica*-nodulating strain *B. phymatum* STM815^T and ineffectively nodulated by *C. taiwanensis* LMG 19424^T (Elliott *et al.*, 2007*a*). This has been confirmed in the present study (data not shown), and we have also found that *M. hamata* is ineffectively nodulated by both these strains (Table 2). However, neither of these beta-rhizobial strains was isolated in India, and so *Cupriavidus* sp. strain MP3 (from Karnataka), *B. phymatum* strain MP20 (from Jharkhand) and *Rhizobium* sp. MP10, all of which were capable of nodulating *M. pudica* effectively (Table 2), were tested on *M. hamata* and *M. himalayana*. As with the type strain of *C. taiwanensis*, LMG 19424^T, MP3 nodulated both the native species ineffectively, whereas MP20 followed the same pattern as the *B. phymatum* type strain, STM815^T, and nodulated *M. himalayana* effectively but *M. hamata* ineffectively (Table 2). *Rhizobium* sp. MP10, like many *Rhizobium* strains isolated from *Mimosa* nodules (Barrett and Parker, 2006; Elliott *et al.*, 2009; Mishra *et al.*, 2012), is not completely effective on its original host, *M. pudica*, producing plants with prematurely senescing nodules and yellow–green leaves, and it was also ineffective at nodulating *M. hamata* (Table 2). Additional studies were performed using variants of strains MP3 and MP20 that were marked with a transposon-based constitutively expressed *gusA* gene (Wilson *et al.*, 1995), and these confirmed the nodulation phenotypes of the wild-type strains (e.g. on *M. hamata*; Supplementary Data Fig. S3E, F).

DISCUSSION

The native Indian species *Mimosa hamata* and *M. himalayana* are nodulated by *Ensifer (Sinorhizobium)* spp.

Mimosa hamata, a species which is native to the Thar Desert of Rajasthan and to semi-arid parts of neighbouring Pakistan (Barneby, 1991; Kumar and Sane, 2003), was shown in a study by Gehlot *et al.* (2012) to be nodulated near the city of Jodhpur and in Sikar district by rhizobial strains in *Ensifer*. This was confirmed in the present study by the isolation and characterization of strains from trap experiments using soils from the rhizosphere of *M. hamata* from several other locations in the Thar Desert. These strains were shown to form effective nodules on their host, and as no other symbiotic bacterial types were isolated from *M. hamata*, it is reasonable to state that it is preferentially nodulated by *Ensifer* spp. in its native range. This is reinforced by the demonstration that *M. hamata* cannot be nodulated effectively (or at all) by other *Mimosa*-nodulating strains; this includes alpha- and betaproteobacterial strains known to be promiscuous nodulators of several *Mimosa* spp. (Elliott *et al.*, 2007*a*, 2009; dos Reis Junior *et al.*, 2010; Gyaneshwar *et al.*, 2011) and strains isolated from other *Mimosa* spp. in India (this study).

Mimosa himalayana is another native Indian species, but it prefers wetter and more fertile environments than its close relative *M. hamata*. It is also more widespread than *M. hamata*, and is native to several states in northern India, as well as neighbouring countries, such as Afghanistan and Nepal, where, as its name suggests, it is often found growing in highland regions bordering the Himalayas (Ali, 1973; Shetty and Singh, 1987; Barneby, 1991; Bora and Kumar, 2003). As with *M. hamata*, *M. himalayana* was also nodulated in the present study by *Ensifer* strains when it was sown into soil sampled from the rhizosphere of mature plants in its native range in eastern Rajasthan. The strains that were isolated from both the native Indian *Mimosa* spp. were closely related to each other on the basis of their 16S rRNA sequences, and were also somewhat related to *E. saheli*, a species known to nodulate *Acacia* spp. (de Lajudie *et al.*, 1994) and is the most commonly isolated symbiont from several other native legumes in the Thar Desert, including all the mimosoids examined (Gehlot *et al.*, 2012). Indeed, *Ensifer* spp. are often the preferred symbionts of mimosoid legumes, such as those in the genera *Acacia* (*sensu lato*), *Acaciella*, *Calliandra*, *Leucaena* and *Prosopis*, that are native and/or introduced to tropical and sub-tropical ecosystems in both the Old World (de Lajudie *et al.*, 1994; McInroy *et al.*, 1999; Nick *et al.*, 1999; Räsänen *et al.*, 2001; Bala and Giller, 2001; Bala *et al.*, 2003; Wolde-Meskel *et al.*, 2005; Ben Romdhane *et al.*, 2006; Benata *et al.*, 2008; Xu *et al.*, 2013) and the New World (Moreira *et al.*, 1998; Toledo *et al.*, 2003; Lloret *et al.*, 2007; Rincón-Rosales *et al.*, 2009). Generally speaking, the *Ensifer* strains nodulating Old World mimosoids are in (or related to) the species *E. arboris*, *E. kostiensis*, *E. saheli* and *E. terangae*, whereas those from the New World belong to a group represented by *E. americanum*, *E. chiapenecum* and *E. mexicanum* (Rincón-Rosales *et al.*, 2009).

Although their association with mimosoid legumes is well established by these examples, *Ensifer* spp. have not previously been reported as symbionts of *Mimosa* spp. in their native ranges (Barrett and Parker, 2005, 2006; Andam *et al.*, 2007; Bontemps *et al.*, 2010; Mishra *et al.*, 2012). Moreover, although an *Ensifer*

strain (TJ170) was isolated from nodules on invasive *M. pudica* in Taiwan by Chen *et al.* (2003*b*), this strain was not capable of nodulation, and so the present study is the first published demonstration that *Ensifer* strains can effectively nodulate *Mimosa* spp.

When considering relationships between legumes and their symbionts, core 'housekeeping' genes, such as *rrs* (16S rRNA) and *recA*, can only tell part of the story, as the ability of rhizobia to nodulate and fix N_2 with particular legume hosts depends on their symbiosis-related genes (*nod* and *nif*), which in many rhizobial genera, including *Ensifer*, are borne on mobile *Sym* plasmids (Martinez-Romero, 2009; Sprent, 2009; Rogel *et al.*, 2011). In spite of their potential to be transferred between bacterial types via horizontal gene transfer (HGT), the phylogenetic trees for symbiosis-related genes, such as *nifH* and *nodA*, are often similar to each other and to the core genomes of both alpha- and betaproteobacterial legume symbionts (Rincón-Rosales *et al.*, 2009; Bontemps *et al.*, 2010; Mishra *et al.*, 2012). *Ensifer* strains that nodulate mimosoids are a case in point, as the phylogenetic trees for their *nifH* and *nodA* genes, for example, generally follow those for their housekeeping genes (such as 16S rRNA), and accordingly they also show a clear separation between the *Ensifer* strains isolated from the Old and New Worlds (Haukka *et al.*, 1998; Rincón-Rosales *et al.*, 2009). However, when the *nifH* and *nodA* genes from the native Indian *Mimosa* symbionts were examined in the present study, they were shown to have different phylogenetic relationships. Although the *nifH* genes of strains from both the native Indian species clustered together in two clades that were relatively close to each other and to other Old World *Ensifer* mimosoid symbionts, particularly *E. kostiensis* (Haukka *et al.*, 1998), their *nodA* genes were different from any described rhizobial strains, being closely grouped together in a distinct clade that was distant from the nearest described rhizobial species, *E. kostiensis* and *E. arboris*. This was particularly true of the *nodA* sequences of strains from *M. hamata*, which were in a subclade that was distinct from those of the *M. himalayana* strains.

Given that the *nod* genes, including *nodA*, are those that confer host selectivity upon rhizobia (Kobayashi and Broughton, 2008; Martinez-Romero, 2009; Cummings *et al.*, 2009; Rogel *et al.*, 2011), the different phylogenetic patterns of their *nodA* genes suggested that the host ranges of the *M. hamata* and *M. himalayana* symbionts were different, and so the ability of representative strains from the *M. hamata* and *M. himalayana* symbionts to nodulate various *Mimosa* hosts was examined. These experiments showed that *M. hamata* strains nodulated *M. himalayana*, but that the reverse was not true, i.e. *M. himalayana* strains could not nodulate *M. hamata*.

Taken together, these data demonstrate that the symbiosis-related genes of native Indian *Mimosa* spp. are more closely related to Old World *Ensifer* mimosoid symbionts than to New World ones, but that the *nodA* genes are in separate groups from each other and from other mimosoid *Ensifer* strains. In the case of *M. hamata*, this has resulted in the species being highly dependent on being nodulated by symbionts with very specific *nodA* sequences, and so it might be appropriate to consider that the *M. hamata Ensifer* symbionts described in the present study belong to a new 'symbiovar' (Rogel *et al.*, 2011). *Mimosa himalayana* is a slightly different case, as although it appears to nodulate preferentially in its native soil with *Ensifer* strains that are closely related to *M. hamata* symbionts (with which it can also nodulate), it differs from *M. hamata* in that it is more promiscuous and can nodulate with other rhizobial types, including *Burkholderia* (Elliott *et al.*, 2007*a*).

The invasive Mimosa *species in India* M. pudica *is mainly nodulated by* Cupriavidus *and* Burkholderia

Mimosa pudica is a widespread invasive plant in India, and is present in most (if not all) states, where it is found as a weed growing on roadsides, wasteground and pastures. It generally prefers wetter and more fertile environments, and so has not been recorded in (for example) arid and/or semi-arid regions, such as the Thar Desert (Shetty and Singh, 1987; Kumar and Sane, 2003). As with many sub-tropical and tropical South East Asian countries in which it has been introduced (Chen *et al.*, 2003*b*; Elliott *et al.*, 2009; Liu *et al.*, 2011, 2012; Klonowska *et al.*, 2012; Andrus *et al.*, 2012), *M. pudica* in India is mainly nodulated by Betaproteobacteria in the genera *Burkholderia* and *Cupriavidus* (Verma *et al.*, 2004; this study). The degree to which *M. pudica* is nodulated by each beta-rhizobial genus, *Burkholderia* or *Cupriavidus*, appears to depend upon the location; in Taiwan and New Caledonia it is almost exclusively nodulated by *Cupriavidus* (Chen *et al.*, 2003*b*; Klonowska *et al.*, 2012), whereas in southern China and the Philippines it is nodulated by a relatively equal proportion of both genera. In India, it was previously shown by Verma *et al.* (2004) that *M. pudica* was nodulated by *Cupriavidus* in two locations, one in the north (Uttar Pradesh) and the other in the south (Tamil Nadu). The present study has confirmed that *Cupriavidus* strains similar to *C. taiwanensis* are common symbionts of *M. pudica* in several other locations in India, but has gone further and shown for the first time that strains in the species *B. mimosarum* and *B. phymatum* are also common *M. pudica* symbionts, and even that some *Rhizobium* strains (e.g. MP10, which is similar to *R. vallis*; Wang *et al.*, 2011) can be symbiotic with this species in India. Our study of *M. pudica* symbionts in India has some parallels with that of Liu *et al.* (2012) from southern China, in which the same three species, *C. taiwanensis*, *B. mimosarum* and *B. phymatum*, were always found to nodulate *M. pudica* in varying proportions depending upon location, but the present study differs from Liu *et al.* (2012) in that some sites in India were dominated by one symbiont type, e.g. Haridwar (UT), Agra (UP) and Bangalore (KA) by *C. taiwanensis*, Bokaro (JH) by *B. phymatum*, and Shillong (ME) by *B. mimosarum*, whereas others, such as Jorhat (AS), had *M. pudica* plants that were nodulated with all three symbiont types.

Are soil characteristics and/or plant taxonomy and geographical isolation responsible for the selection of symbionts by native and invasive Indian Mimosa *spp.?*

The results from this study have shown clearly that the rhizobial symbionts of native and invasive *Mimosa* spp. in India are distinct and host-specific, and are most likely not shared between the two types. In the case of the native species, *M. hamata* and *M. himalayana* are both nodulated by *Ensifer*, but the fact that these symbionts are more closely related to those of other Mimosoideae in the same region suggests that their geographical isolation of approx. 10 Myr from the main

centres of *Mimosa* diversity in the New World (Simon *et al.*, 2011) has resulted in these *Mimosa* spp. evolving a relationship with variants of the 'local' mimosoid symbionts rather than with the *Burkholderia* symbionts (Bontemps *et al.*, 2010) of their closest New World relatives in Brazil (Simon *et al.*, 2011). Indeed, in the case of *M. hamata*, it has become so adapted to its particular environment in the Thar Desert that it appears no longer to be capable of nodulating effectively with other *Mimosa*-nodulating rhizobia of any type, and this could be related to the high pH and low fertility of the soils in this region (Sprent and Gehlot, 2010; Gehlot *et al.*, 2012). *Mimosa himalayana*, by contrast, which is a more widespread species than *M. hamata*, has retained the ability of its South American ancestors to nodulate with *Burkholderia* (Elliott *et al.*, 2007*a*), and thus it also appears to be adaptable to several soil types. It can nodulate in low-fertility Thar Desert soils and in more fertile soils in its native range and in Bokaro (JH). Of potentially even more significance is the fact that, unlike the closely related *M. hamata*, *M. himalayana* can nodulate so effectively in Brazilian cerrado *Mimosa* rhizospheric soils. However, given its ability to nodulate with *Burkholderia* and the preponderance of native and endemic *Mimosa* spp. nodulated by *Burkholderia* in cerrado soils (Bontemps *et al.*, 2010; dos Reis Junior *et al.*, 2010), it is surprising that the symbionts isolated from the *M. himalayana* trap plants were all closely related to *E. mexicanum*, a species originally isolated from nodules on *Acaciella* spp. in Mexico (Toledo *et al.*, 2003; Rincón-Rosales *et al.*, 2009). Further studies are currently being undertaken to determine the origin (i.e. the original hosts) of these symbionts in Brazil, and to characterize them in terms of their symbiosis-related genes.

In contrast to the native species, no *M. pudica* plants from any of the sites/soils were nodulated with *Ensifer* spp., even when they were nodulated after being sown into *M. hamata* rhizospheric soils from Jodhpur. It thus appears that *M. pudica* in India is nodulated by the same types of symbionts as in other Asian locations, including neighbouring China (Liu *et al.*, 2011, 2012), and that as with other invasive legumes (e.g. *Acacia saligna*; Crisóstomo *et al.*, 2013) it most likely brings its symbionts with it as it invades new territories, including those that are already occupied by native *Mimosa* spp. These symbionts are (mainly) a combination of beta-rhizobial types, and the type (or combination of types) depends on the location, but what is it about each location that might be involved in their selection? Soil characteristics are considered to be important for the selection of symbionts by invasive *Mimosa* spp., especially soil pH (Bontemps *et al.*, 2010; dos Reis Junior *et al.*, 2010; Mishra *et al.*, 2012; Liu *et al.*, 2012) and fertility (Elliott *et al.*, 2009). Low fertility (i.e. low soil N-concentration) generally favours the selection of *Burkholderia* as symbionts by *Mimosa* spp., almost to the complete exclusion of other rhizobial types, but this dominance is broken in favour of *C. taiwanensis* as soil N-concentrations increase (Elliott *et al.*, 2009). In the case of pH, it has been noted from studies on *M. pudica* symbionts in French Guiana (Mishra *et al.*, 2012) and southern China (Liu *et al.*, 2012) that soils with values below pH 7·0 harbour plants that are generally nodulated by *Burkholderia* spp., whereas plants growing in soils with higher pH values are likely to have *C. taiwanensis* as their symbionts. With the exception of the low-fertility alkaline soils in Jodhpur (RJ) (pH 8·2) that resulted in *M. pudica* trap plants selecting *C. taiwanensis*, and the acidic soils in Shillong (ME) (pH. 4·9) that resulted in *M. pudica* selecting *B. mimosarum*, there are no clear reasons as to why the soils in many of the locations in the present study produced the particular *M. pudica* symbionts that they did based upon pH and fertility alone. However, sampling from *M. pudica* was very low for each site, as the study was designed only to get a wider picture of the variety of symbionts nodulating this invasive species in India, and more in-depth sampling will almost certainly show that the diversity of *M. pudica* symbionts at each site is much more complex than has been demonstrated here.

SUPPLEMENTARY DATA

Supplementary data are available online at www.oab.oxfordjournals.org and consist of the following. Figure S1: map of India showing locations where *Mimosa* nodules and/or rhizosphere soil were collected, and from which species. Figure S2: immunogold-labelled sections of nodules of *Mimosa* spp. sampled from plants collected in the field from various locations in India. Figure S3: cross-inoculation tests with Indian and Mexican rhizobial strains on various *Mimosa* species.

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