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Burkholderia spp. are the most competitive symbionts of *Mimosa*, particularly under N-limited conditions

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Summary

Bacteria isolated from *Mimosa* nodules in Taiwan, Papua New Guinea, Mexico and Puerto Rico were identified as belonging to either the α- or β-proteobacteria. The β-proteobacterial *Burkholderia* and *Cupriavidus* strains formed effective symbioses with the common invasive species *Mimosa diplotricha*, *M. pigra* and *M. pudica*, but the α-proteobacterial *Rhizobium etli* and *R. tropici* strains produced a range of symbiotic phenotypes from no nodulation through ineffective to effective nodulation, depending on *Mimosa* species. Competition studies were performed between three of the α-proteobacteria (*R. etli* TJ167, *R. tropici* NGR181 and UPRM8021) and two of the β-rhizobial symbionts (*Burkholderia mimosarum* PAS44 and *Cupriavidus taiwanensis* LMG19424) for nodulation of these invasive *Mimosa* species. Under flooded conditions, *B. mimosarum* PAS44 out-competed LMG19424 and all three α-proteobacteria to the point of exclusion. This advantage was not explained by initial inoculum levels, rates of bacterial growth, rhizobia-rhizobia growth inhibition or individual nodulation rate. However, the competitive domination of PAS44 over LMG19424 was reduced in the presence of nitrate for all three plant hosts. The largest significant effect was for *M. pudica*, in which LMG19424 formed 57% of the nodules in the presence of 0.5 mM potassium nitrate. In this host, ammonium also had a similar, but lesser, effect. Comparable results were also found using an N-containing soil mixture, and environmental N levels are therefore suggested as a factor in the competitive success of the bacterial symbiont *in vivo*.

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Introduction

Nodulation in legumes occurs not only in symbiosis with members of the class α-*Proteobacteria* (including *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Devosia*), but also with several members of the β-*Proteobacteria* (Graham, 2008). This has been most conclusively established in the legume genus *Mimosa* (Mimosoideae) (Chen *et al*., 2003a,b; 2005a,b; Barrett and Parker, 2005; 2006; Andam *et al*., 2007; Parker *et al*., 2007). The β-*Proteobacteria* (β-rhizobia) known to nodulate *Mimosa* include several *Burkholderia* spp. as well as *Cupriavidus taiwanensis* (previously named *Ralstonia taiwanensis*: Vandamme and Coenye, 2004). Of the *Mimosa* symbionts so far identified, those in the genus *Burkholderia* appear to be more varied and more widespread, with several different species isolated from native and invasive *Mimosa* spp. across North, Central and South America [Brazil, Costa Rica, French Guiana, Mexico, Panama, Texas (USA), Venezuela] and from Taiwan (Chen *et al*., 2003a; 2005a,b; Barrett and Parker, 2005; 2006; Andam *et al*., 2007). *Cupriavidus taiwanensis* has also been isolated from invasive *Mimosa* species [*M. pudica*, *M. diplotricha* and *M. pigra* (syn. *M. pellita*)] in Taiwan (Chen *et al*., 2001, 2003a, 2005a) and in India (*M. pudica*; Verma *et al*., 2004). Further isolates of *C. taiwanensis*, and an undetermined *Cupriavidus* sp., have been found in the nodules of *M. pudica* and *M. pigra* in Costa Rica (Barrett and Parker, 2006), and *Cupriavidus* was also found to be the main effective symbiont of two native *Mimosa* spp. in Texas (USA) (Andam *et al*., 2007). Of the strains that have been tested on their original host species of *Mimosa*,

only four, *C. taiwanensis* LMG19424 (Chen *et al*., 2003b; Elliott *et al*., 2007a), *Burkholderia nodosa* Br3461 and *B. mimosarum* PAS44 and MAP3-5 (Chen *et al*., 2005a,b; 2006; 2007), have been confirmed to be effective symbionts using detailed microscopy. In addition, host-range and microscopical studies of *Burkholderia phymatum* STM815 (originally isolated from *Machaerium lunatum*, but not confirmed to nodulate this species) show that it too is a *Mimosa* symbiont (Elliott *et al*., 2007a).

Although these β-rhizobia appear to be the predominant symbionts of *Mimosa*, 'classic' α-proteobacterial rhizobia (α-rhizobia) are also capable of nodulating *Mimosa*. Chen and colleagues (2003a) found that although most isolates from *M. pudica* and *M. diplotricha* were *C. taiwanensis* variants, the remainder were mainly *Rhizobium* spp. Barrett and Parker (2005) also discovered *Rhizobium* spp. in nodules of *M. pudica* and *M. pigra* in Panama, and this was followed by their discovery of nodulating *Rhizobium*, *Burkholderia* and *Cupriavidus* spp. in similar numbers in *M. pudica*, in several cases within different nodules of the same plant (Barrett and Parker, 2006). Additional examples of α-rhizobia isolated from *Mimosa* have been reported earlier (Trinick, 1980; Oyaizu *et al*., 1993; Wang *et al*., 1999; Zurdo-Piñeiro *et al*., 2004). In contrast, surveys across Taiwan found no *Rhizobium* spp. to be present within *M. pigra* nodules, and unlike local studies on *M. pudica* and *M. diplotricha*, the overwhelmingly dominant genus isolated was found to be *Burkholderia* rather than *Cupriavidus* (Chen *et al*., 2005b).

Symbiosis-related genes, particularly those responsible for nitrogen fixation (*nif*) and nodulation (*nod*), are among the main genetical determinants of rhizobial compatibility with any potential host legumes (Graham, 2008), and yet comparisons between *Mimosa*-nodulating α- and β-rhizobia have shown that the sequences of these genes (e.g. *nifH* and *nodA*) are actually very distinct (Chen *et al*., 2003a). Why a single plant species selects one available bacterial symbiont over another is unknown, but given that *Mimosa* is known to nodulate with both α- and β-rhizobia, the present study was aimed at determining (i) if *Mimosa* had a preference for a particular type (or types) of symbiont (e.g. α- or β-) and (ii) what factors might underlie any preference. In the first part of the study we identified several strains belonging to either the α- or β-proteobacteria that were isolated from *Mimosa* nodules in several locations in the tropics (Taiwan, Papua New Guinea, Mexico and Puerto Rico), examined their phylogenetic relationships using their 16S rRNA and *nodA* sequences, and assessed their symbiotic effectiveness on invasive *Mimosa* species. Second, selected strains from these and from earlier studies, namely the β-rhizobia *B. mimosarum* PAS44, *B. phymatum* STM815 and *C. taiwanensis* LMG19424, and the α-rhizobia *Rhizobium etli* TJ167, *R. tropici* NGR181 and *R. tropici* UPRM8021, were then compared in pair-wise competition studies for nodulation of the common invasive species *M. diplotricha*, *M. pigra* and *M. pudica*. Finally, the possibility that other factors, including combined N, might affect this competition was also investigated.

Results

Identification and selection of strains

The phylogenetic positions of all the rhizobia used in this study were determined by comparisons of the sequences of their 16S rDNA and *nodA* genes, and Figs 1 and 2 were constructed using both Neighbour-Joining and Maximum-Likelihood analyses. As reported previously by Chen and colleagues (2003a), divergence between the α- and β-proteobacteria is clearly seen on analysis of 16S rDNA sequences, with a further divergence apparent within the β-rhizobia between *Burkholderia* and *Cupriavidus* (Fig. 1). The strains listed in Table 1 grouped into four clades: *Burkholderia*, *Cupriavidus*, *R. etli* and *R. tropici*. In the case of the two newly described β-rhizobial strains, NGR190 isolated from *M. diplotricha* nodules in Papua New Guinea was most likely a *B. mimosarum* strain, whereas the 16S rDNA sequence of NGR193A, which was originally isolated from *M. pudica* nodules in Papua New Guinea (Trinick, 1980), placed it in the genus *Cupriavidus*, although it was not sufficiently similar to any species (including *C. taiwanensis*) for it to be given a specific definition (Fig. 1). With regard to the α-rhizobia, the 16S rDNA sequence of NGR181, which was originally isolated from *M. diplotricha* nodules in Papua New Guinea (M.J. Trinick, unpublished), placed it within *R. tropici* along with UPRM8021 from *Mimosa ceratonia* nodules in Puerto Rico (Zurdo-Piñeiro *et al*., 2004). The 16S rDNA sequence of strain TJ172 from *M. diplotricha* nodules in Taiwan (Chen *et al*., 2003a) was not sufficiently close to the *R. tropici* clade to be included within this species, but was clearly closely related to it. The *R. etli* clade contained the two other Taiwanese strains, TJ167 and TJ173, and these were similar to three strains (Mim1, Mim2 and Mim7-4) isolated from the weedy herbaceous species, *Mimosa affinis*, in Mexico by Wang and colleagues (1999), which were placed by them in a new biovar, *R. etli* bv. *mimosae*.

Phylogenetic analyses of *nodA* gene sequences (Fig. 2) appeared to reinforce 16S rRNA gene sequence data, showing clear differences between the *Mimosa*-nodulating α- and β-proteobacteria, but with the divergence between the two β-proteobacterial genera less apparent. Again, the *nodA* sequence of *B. mimosarum* NGR190 placed it firmly within the *B. mimosarum* clade, and *Cupriavidus* sp. NGR193A was in a clade close to *C. taiwanensis* LMG19424 and other *Cupriavidus* strains

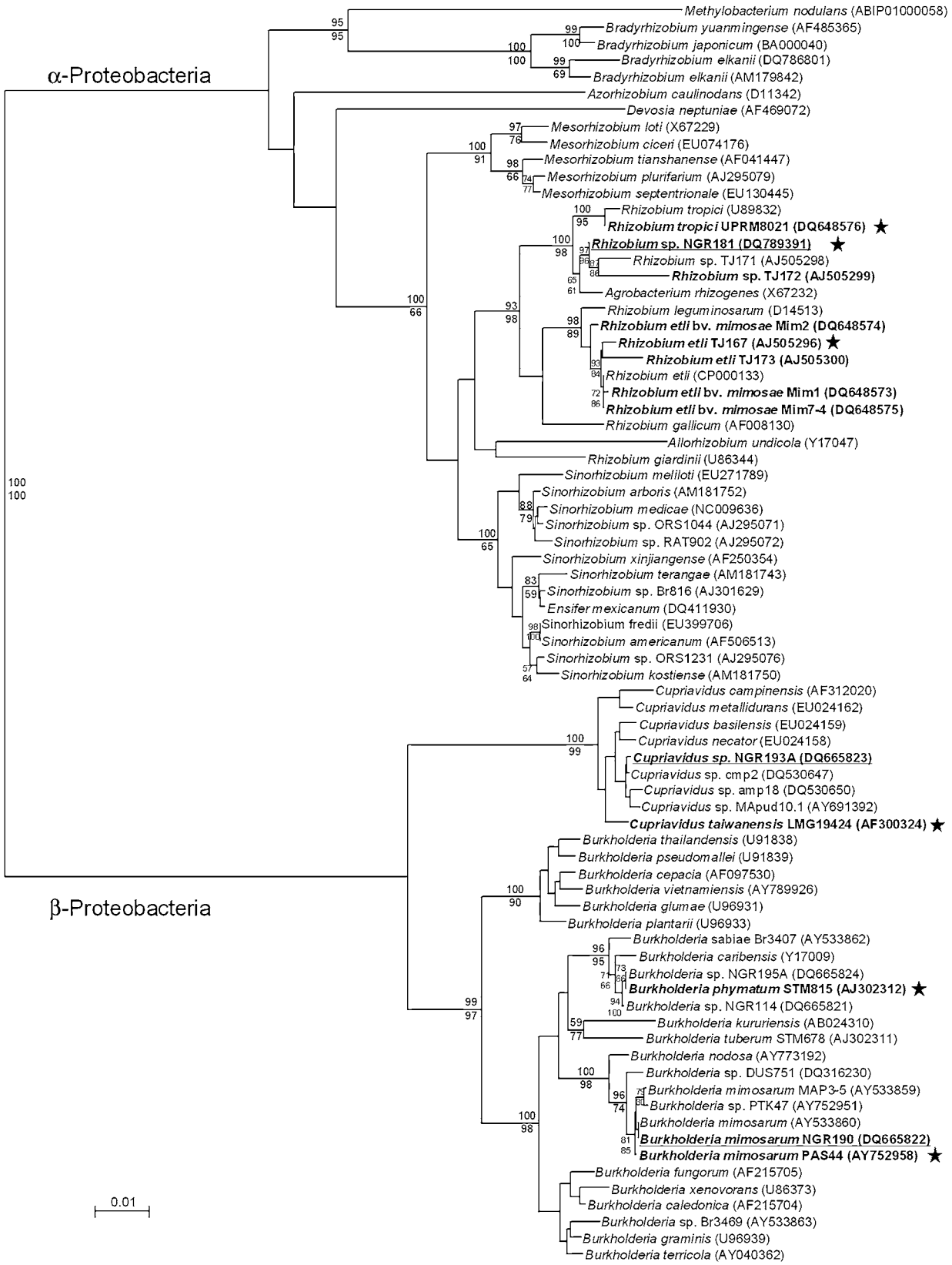

Fig. 1. Phylogenetic relationships of selected α- and β-proteobacteria based on 16S rRNA genes (1240 nucleotides). Strains listed in bold type are those used in this study (Table 1). Strains underlined are those for which sequences were derived in this study. Strains annotated with a star are those used in competition experiments in this study. The tree was built using Neighbour-Joining and bootstrap percentages over 50% from Neighbour-Joining and Maximum-Likelihood are listed above and below the branches respectively.

(and also *Burkholderia caribensis* TJ182). The *nodA* sequences of the two Taiwanense *R. etli* strains (TJ167, TJ173) were in a clade with the Mexican *R. etli* bv. *mimosae* strains, and those of the *Mimosa*-nodulating *R. tropici* strains (NGR181 and UPRM8021) were grouped together within the *R. tropici* clade along with *Rhizobium* sp. TJ172 (Fig. 2).

Following phylogenetic analysis, all strains (Table 1) not already shown to be functional symbionts were tested for their ability to nodulate the three invasive *Mimosa* species, *M. diplotricha*, *M. pigra*, *M. pudica*, as well as their original hosts (except UPRM8021, as no seeds of *M. ceratonia* were available) (Table 2). This resulted in effective (i.e. with significant acetylene reduction activity) nodulation of *M. diplotricha* by all strains (*B. mimosarum* NGR190, *Cupriavidus* sp. NGR193A, *R. etli* strains TJ167 and TJ173, and *R. tropici* strains NGR181 and UPRM8021), except for *Rhizobium* sp. TJ172 and the three Mexican *R. etli* Mim strains which produced ineffective nodules without any nitrogenase activity. In contrast, *M. pigra* was nodulated effectively only by the two β-rhizobia (NGR190 and NGR193A) and by *R. tropici* UPRM8021, although it was also nodulated ineffectively by all the other strains. *Mimosa pudica* was nodulated effectively by NGR190, NGR193A, and by the Taiwanense *R. etli* strains, TJ167 and TJ173, but was only ineffectively nodulated by the Mexican *R. etli* bv. *mimosae* strains (Mim1, Mim2, Mim7-4), *R. tropici* UPRM8021 and *Rhizobium* sp. TJ172, with NGR181 not nodulating it at all (Table 2). All the *R. etli* strains, including strains TJ167 and TJ173, nodulated *M. affinis* effectively (Table 2). The nodules formed on *Mimosa* spp. by the α-rhizobia were examined further by light and transmission electron microscopy (TEM). Sections of these confirmed the symbiotic phenotypes described in Table 2; for example, *R. etli* TJ67 formed effective nodules on *M. diplotricha* (Fig. 3A) similar to those previously seen on this species effectively nodulated with a β-rhizobial symbiont (e.g. *C. taiwanensis* LMG19424; Chen *et al.*, 2003b), and formed ineffective nodules on *M. pigra* (Fig. 3B). Moreover, effective nodules, such as those on *M. diplotricha* infected with TJ167 (not shown), *R. tropici* UPRM 8021 (Fig. 3C) and *R. tropici* NGR181 (Fig. 3D), contained bacteroids that expressed nitrogenase *nifH* (Fe-)protein. Sections from ineffective nodules and negative control sections with the antibody against the *nifH* protein substituted with non-immune serum gave no significant immunogold reaction (not shown).

Selection and transformation of strains for competition studies

Representative α- and β-proteobacteria that nodulated their original hosts effectively were then chosen from the strains listed in Tables 1 and 2 for competition studies using the three invasive *Mimosa* species (*M. diplotricha*, *M. pigra* and *M. pudica*). These were *B. mimosarum* PAS44 (original host *M. pigra*; Chen *et al*., 2005b), *C. taiwanensis* LMG19424 (*M. pudica*; Chen *et al*., 2003b), *R. etli* TJ167 (*M. diplotricha*; this study) and *R. tropici* NGR181 (*M. diplotricha*; this study). A further *R. tropici* strain, UPRM8021 (*M. ceratonia*; Zurdo-Piñeiro *et al*.,

Table 1. Strains used in this study including genetic identifications, provenance and source.

Strain	16S rRNA identity	Host plant	Provenance	Source
LMG19424[a]	*Cupriavidus taiwanensis*	*Mimosa pudica*	Taiwan	Chen *et al*. (2001)
Mim1	*Rhizobium etli* bv. *mimosae*	*Mimosa affinis*	Mexico	Wang *et al*. (1999)
Mim2	*Rhizobium etli* bv. *mimosae*	*Mimosa affinis*	Mexico	Wang *et al*. (1999)
Mim7-4	*Rhizobium etli* bv. *mimosae*	*Mimosa affinis*	Mexico	Wang *et al*. (1999)
NGR181[a]	*Rhizobium tropici*	*Mimosa diplotricha*[b]	Papua New Guinea	This study[c]
NGR190	*Burkholderia mimosarum*	*Mimosa diplotricha*[b]	Papua New Guinea	This study[c]
NGR193A	*Cupriavidus* sp.	*Mimosa pudica*	Papua New Guinea	Trinick (1980)
PAS44[a]	*Burkholderia mimosarum*	*Mimosa pigra*	Taiwan	Chen *et al*. (2005b)
STM815	*Burkholderia phymatum*	*Machaerium lunatum*[d]	French Guiana	Vandamme *et al*. (2002)
TJ167[a]	*Rhizobium etli*	*Mimosa diplotricha*	Taiwan	Chen *et al*. (2003a)
TJ172	*Rhizobium* sp.	*Mimosa diplotricha*	Taiwan	Chen *et al*. (2003a)
TJ173	*Rhizobium etli*	*Mimosa diplotricha*	Taiwan	Chen *et al*. (2003a)
UPRM8021[a]	*Rhizobium tropici*	*Mimosa ceratonia*	Puerto Rico	Zurdo-Piñeiro *et al*. (2004)

a. Selected for use in competition study.
b. Originally identified as *Mimosa invisa*, but now thought most likely to be *M. diplotricha*.
c. Originally isolated by Trinick in 1964, but previously unidentified.
d. Postulated as an error, with the actual host suggested as a *Mimosa* sp. (Elliott *et al*., 2007a).

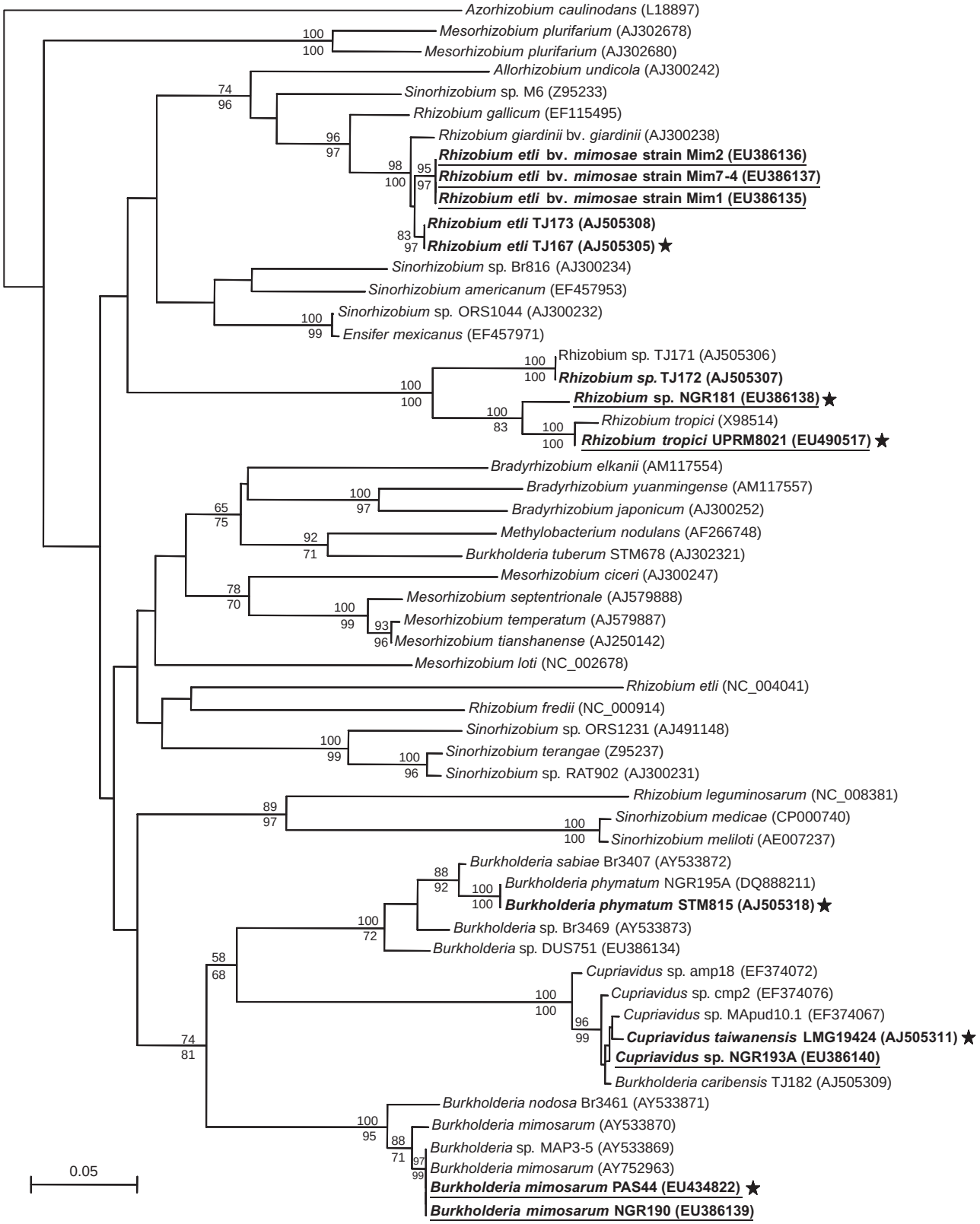

Fig. 2. Phylogenetic relationships of selected α- and β-proteobacteria based on a 227 nt *nodA* sequence alignment. Strains listed in bold type are those used in this study (Table 1). Strains underlined are those for which sequences were derived in this study. Strains annotated with a star are those used in competition experiments in this study. The tree was built using Neighbour-Joining and bootstrap percentages over 50% from Neighbour-Joining and Maximum-Likelihood are listed above and below the branches respectively.

2004), was included as a strain not originally isolated from one of these three invasive host plant species, but which could effectively nodulate at least one of them (this study), thus giving five test strains. *Burkholderia phymatum* STM815 was used for comparison with *B. mimosarum* PAS44 only.

Strains tagged with GUS or fluorescent proteins showed no significant difference in growth or, nodulation in comparison with their wild-type ancestors (data not shown). Further confirmation that the three *Rhizobium* strains and their XFP-tagged derivatives could form effective symbioses with *M. diplotricha* was obtained by confocal laser scanning microscopy (CLSM) (Fig. 4). This showed that *R. etli* TJ167rfp (Fig. 4A and B), *R. tropici* NGR181ecfp (Fig. 4C and D) and *R. tropici* UPRM8021eyfp (Fig. 4E and F) were, indeed, the symbionts within their *M. diplotricha* hosts. Similarly, *B. mimosarum* PAS44*nodD*GUS had identical symbiotic performance to the parent wild-type strain and formed effective nodules on all three invasive species, e.g. *M. pigra* (Fig. 4G and H).

Growth of a mixed culture of *C. taiwanensis* LMG19424 and *B. mimosarum* PAS44 over 36 h showed no growth inhibition of either strain in YEM broth (data not shown). Inoculum survival in the presence of plant roots was checked by cell counts 21 days after inoculation of single strains. All inocula maintained or increased their cell density during this critical period for nodule formation (Fig. 5). Nodulation rates of the three *Mimosa* species were monitored after inoculation with single bacterial strains. *Mimosa diplotricha* formed nodules more quickly with *R. tropici* NGR181, *R. etli* TJ167 and *B. mimosarum*

Table 2. Nodulation and nitrogenase activity of strains tested on *Mimosa* spp.

Strain	*Mimosa* species	Nodule No.	ARA (nmol C_2H_4 $plant^{-1}$ h^{-1})
Burkholderia mimosarum NGR190	*M. diplotricha*	22 ± 7	1992 ± 12
	M. pigra	39 ± 7	3206 ± 454
	M. pudica	21 ± 3	1299 ± 81
Cupriavidus sp. NGR193A	*M. diplotricha*	24 ± 6	794 ± 39
	M. pigra	43 ± 10	369 ± 133
	M. pudica	25 ± 5	2046 ± 354
Rhizobium etli TJ167	*M. affinis*	15 ± 2	1191 ± 204
	M. diplotricha	33 ± 1	551 ± 251
	M. pigra	30 ± 6	0
	M. pudica	9 ± 3	166 ± 13
Rhizobium etli TJ173	*M. affinis*	18 ± 2	941 ± 317
	M. diplotricha	20 ± 12	473 ± 238
	M. pigra	34 ± 8	0
	M. pudica	6 ± 2	194 ± 65
Rhizobium etli bv. *mimosae* Mim-1	*M. affinis*	17 ± 3	1035 ± 144
	M. diplotricha	15 ± 5	0
	M. pigra	16 ± 6	0
	M. pudica	11 ± 3	0
Rhizobium etli bv. *mimosae* Mim-2	*M. affinis*	21 ± 5	695 ± 260
	M. diplotricha	16 ± 7	0
	M. pigra	17 ± 3	0
	M. pudica	14 ± 3	0
Rhizobium etli bv. *mimosae* Mim-7-4	*M. affinis*	14 ± 3	900 ± 11
	M. diplotricha	10 ± 5	0
	M. pigra	15 ± 4	0
	M. pudica	14 ± 3	0
Rhizobium tropici NGR181	*M. diplotricha*	12 ± 4	958 ± 210
	M. pigra	29 ± 6	0
	M. pudica	0	0
Rhizobium sp. TJ172	*M. diplotricha*	20 ± 11	0
	M. pigra	10 ± 4	0
	M. pudica	16 ± 7	0
Rhizobium tropici UPRM 8021	*M. diplotricha*	10 ± 7	400 ± 346
	M. pigra	19 ± 9	96 ± 83
	M. pudica	4 ± 4	0

All plants were assessed 42 days after inoculation. Six replicate plants were used, and standard deviations are shown.

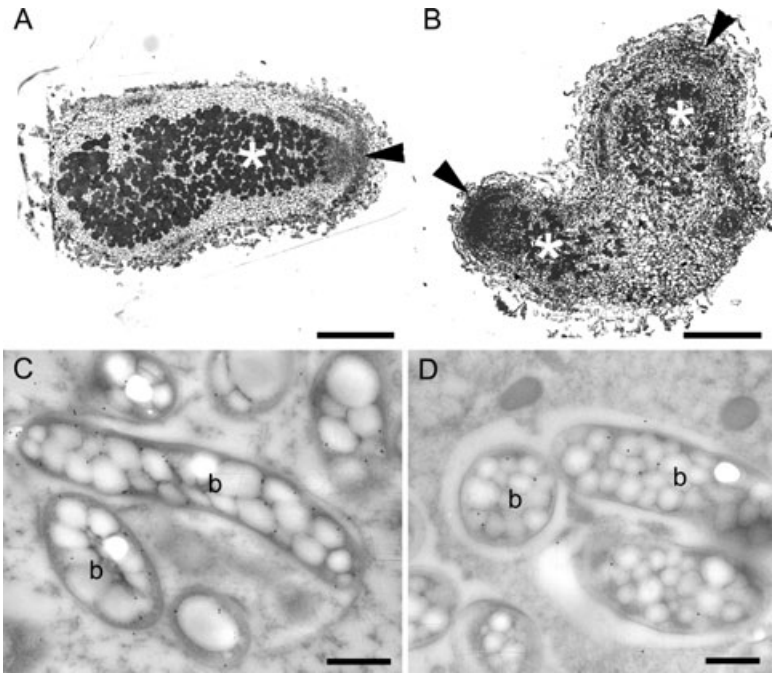

Fig. 3. Light microscopy (A and B) and transmission electron microscopy (TEM) (C and D) of resin-embedded sections of *Mimosa* nodules harvested at 42 days after inoculation with strains of *Rhizobium*.
A and B. (A) Effective nodule from *Mimosa diplotricha* and (B) an ineffective nodule from *M. pigra* inoculated with *Rhizobium etli* TJ167. The sections of both nodules were stained with 1% toluidine blue, and the meristems (arrowheads) and infected zone (*) are clearly shown in both cases, but note that the infected zones in the *M. pigra* nodule are less dense compared with those in the *M. diplotricha* nodule.
C and D. The TEM sections of bacteroids in *M. diplotricha* nodules in (C) and (D) were immunogold labelled with an antibody raised against the *nifH* (nitrogenase) protein. The bacteroids of *R. tropici* UPRM 8021 (C) are abundantly marked with gold particles, but those of *R. tropici* NGR181 (D) are less so, although the labelling still indicates the presence of the enzyme.
Bars, 500 μm (A and B), 200 nm (C), 500 nm (D).

PAS44 than with *C. taiwanensis* LMG19424 and *R. tropici* UPRM8021, and the final numbers of nodules formed were the highest with *R. etli* TJ167 (Fig. 6). In the case of *M. pigra*, however, *B. mimosarum* PAS44 and *C. taiwanensis* LMG19424 were quicker to form nodules than *R. etli* TJ167 and *R. tropici* UPRM8021, with *C. taiwanensis* LMG19424 giving the highest number of nodules (Fig. 6). Similarly, *C. taiwanensis* LMG19424 and *B. mimosarum* PAS44 both formed more nodules on *M. pudica* than did *R. etli* TJ167, with *C. taiwanensis* LMG19424 showing a faster nodulation rate than *B. mimosarum* PAS44, although final numbers of nodules produced by these two were identical (Fig. 6).

Competition between β-rhizobia

Comparisons between *B. mimosarum* PAS44, originally isolated from *M. pigra*, and *C. taiwanensis* LMG19424, originally isolated from *M. pudica*, showed that when plants were grown in an N-free liquid medium, *B. mimosarum* PAS44 completely out-competed *C. taiwanensis* LMG19424 in all three invasive *Mimosa* species (Table 3). Even when initial inoculation cell densities were altered to favour *C. taiwanensis* LMG19424 by a factor of 10^4, no nodules were formed by this strain. A reduction in initial pH of the medium from pH 6.8 to 5.8 also had no effect (results not shown). Addition of N in the form of 0.5 mM ammonium or nitrate ions had no significant effect on nodule number, but 2.5 mM of either ion caused a reduction in total nodule numbers that was statistically significant in some cases (Fig. 7). Overall, the results with ammonium and nitrate suggested that *M. pigra* nodulation was the least sensitive to applied nitrogen of both types, with *M. diplotricha* being the most sensitive to ammonium and *M. pudica* the most sensitive to nitrate (Fig. 7). The addition of nitrogen shifted the balance of competition and allowed *C. taiwanensis* LMG19424 to form some nodules in competition with *B. mimosarum* PAS44 on each host species. This effect was the greatest on *M. pudica* and least on *M. pigra* (Fig. 8).

On plants grown in N-free solid rooting medium, nodule occupancy of *M. pigra* was unaltered, but there were slight yet significant increases in competitiveness of *C. taiwanensis* LMG19424 on *M. diplotricha* and *M. pudica* (Fig. 8, Table 3). Similar tests in an autoclaved soil/sand mixture (with a concentration of 0.1% total N) showed a statistically insignificant shift towards occupancy by *C. taiwanensis* LMG19424 in *M. pigra* nodules, but with much larger highly significant swings towards LMG19424 on *M. diplotricha* and *M. pudica* (Fig. 8). Competition experiments between *B. mimosarum* PAS44 and another *Burkholderia* strain, *B. phymatum* STM815, showed no significant difference between the two strains for nodulation of *M. diplotricha* or *M. pigra* under either liquid or solid N-free rooting conditions, but *B. phymatum* STM815 formed significantly more nodules on *M. pudica* than *B. mimosarum* PAS44 in both cases (Table 3).

Competition between α- and β-rhizobia

Comparisons between the two β-rhizobial strains (*B. mimosarum* PAS44 and *C. taiwanensis* LMG19424) and

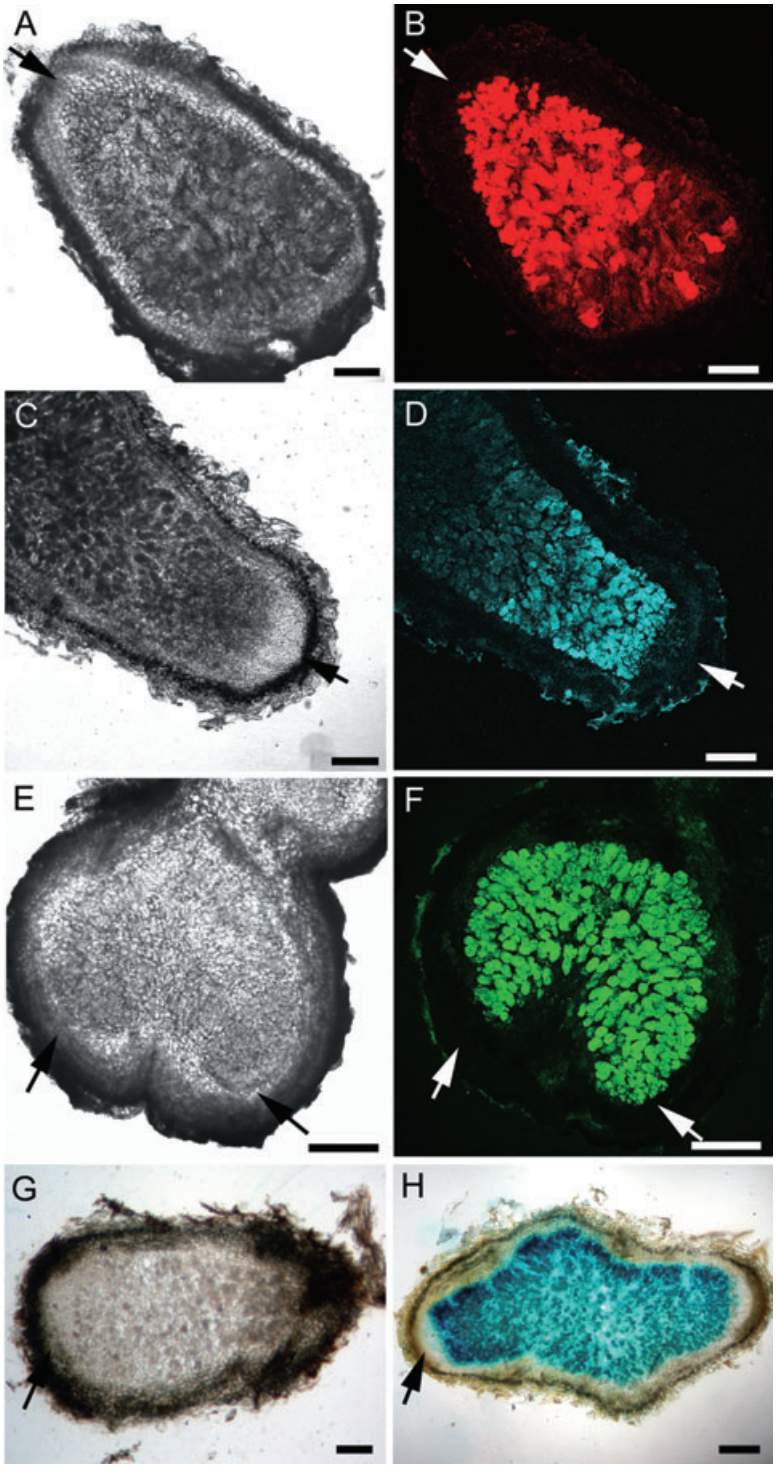

Fig. 4. A–F. *Mimosa diplotricha* nodules infected with *R. etli* TJ167rfp (A and B), *R. tropici* NGR181ecfp (C and D) and *R. tropici* UPRM 8021eyfp (E and F). Images (A), (C) and (E) viewed under transmitted light, with corresponding (B), (D) and (F) images viewed under confocal fluorescence microscopy.
G and H. (G) and (H) are, respectively, of *M. pigra* nodules infected with *Burkholderia mimosarum* PAS44 wild type and PAS44nodDGUS stained with X-gluc.
Bars, 200 μm.

three α-rhizobia (*R. etli* TJ167, *R. tropici* UPRM 8021 and *R. tropici* NGR181) were performed. *Rhizobium etli* TJ167 was compared on all three host plant species with both *B. mimosarum* PAS44 and *C. taiwanensis* LMG19424. Overall, *B. mimosarum* PAS44 again showed an advantage over its competitor strain, but there were differences depending on the strain and the host plant (Table 3). For example, *R. etli* TJ167 formed 40% and 15% of the nodules on *M. diplotricha* when it was grown, respectively, in liquid and solid rooting medium, but with *M. pigra* it formed only 2% and 3% of nodules under these conditions, while it failed to form any nodules on *M. pudica* in a

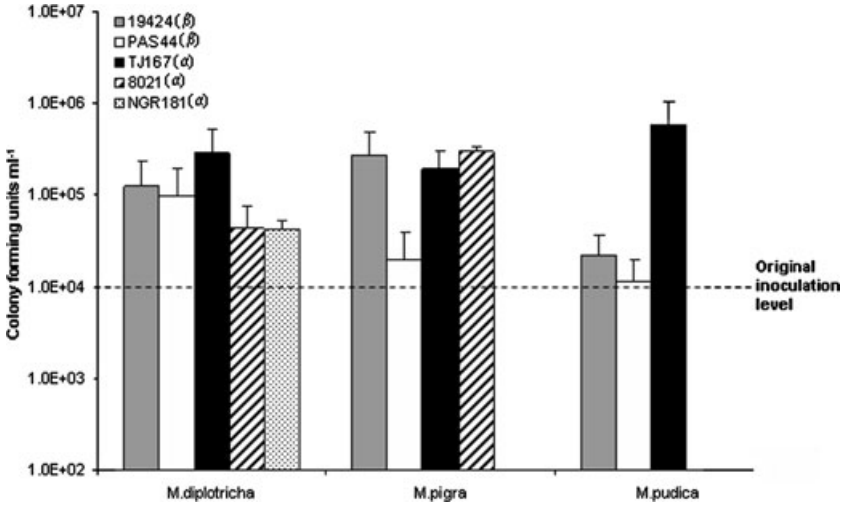

Fig. 5. Numbers of bacteria (colony-forming units ml^{-1}) in the liquid rooting medium of three *Mimosa* spp. at 42 days after inoculation. The original inoculum level (1×10^4 colony-forming units ml^{-1}) is indicated by a line. Bars indicate ± standard deviation of at least six replicates.

Table 3. Nodule occupancy of strains on *Mimosa* test species following competitive co-inoculation and subsequent plant growth.

Mimosa test species	PAS44 (β)	LMG19424 (β)	STM815 (β)	TJ167 (α)	UPRM8021 (α)	NGR181 (α)	*n*	*P*-value
Liquid root medium								
Mimosa diplotricha	63	0					63	< 0.0005
	11		12				23	ns
	24			16			40	ns
		6		36			42	< 0.0005
	65				1		66	< 0.0005
		17			7		24	< 0.05
	23					0	23	< 0.0005
		5				43	48	< 0.0005
Mimosa pigra	49	0					49	< 0.0005
	19		25				44	ns
	64			1			65	< 0.0005
		5		15			20	< 0.05
	38				0		38	< 0.0005
		5			19		24	< 0.005
Mimosa pudica	39	0					39	< 0.0005
	7		32				39	< 0.0005
	31			0			31	< 0.0005
		9		16			25	ns
Solid root medium								
Mimosa diplotricha	13	1					14	< 0.0005
	14		6				20	ns
	23			4			27	< 0.0005
		5		17			22	< 0.05
	16				0		16	< 0.0005
		18			0		18	< 0.0005
	15					1	16	< 0.0005
		8				5	13	ns
Mimosa pigra	26	0					26	< 0.0005
	14		7				21	ns
	28			1			29	< 0.0005
		5		12			17	ns
	24				0		24	< 0.0005
		7			0		7	< 0.05
Mimosa pudica	26	5					31	< 0.0005
	0		25				25	< 0.0005
	18			2			20	< 0.0005
		11		7			18	ns

Pairs of strains were co-inoculated in equal numbers (1×10^4 bacterial cells ml^{-1} media) into liquid or solid media in which the roots of 5-day-old *Mimosa* seedlings were placed. Plants were harvested 42 days after inoculation to yield *n* mature nodules in each test case, with test strains re-isolated and identified morphologically. Variations from equal distribution of nodules between the two strains tested in each case were considered as non-significant (ns) when χ^2 tests showed a *P*-value of > 0.05.

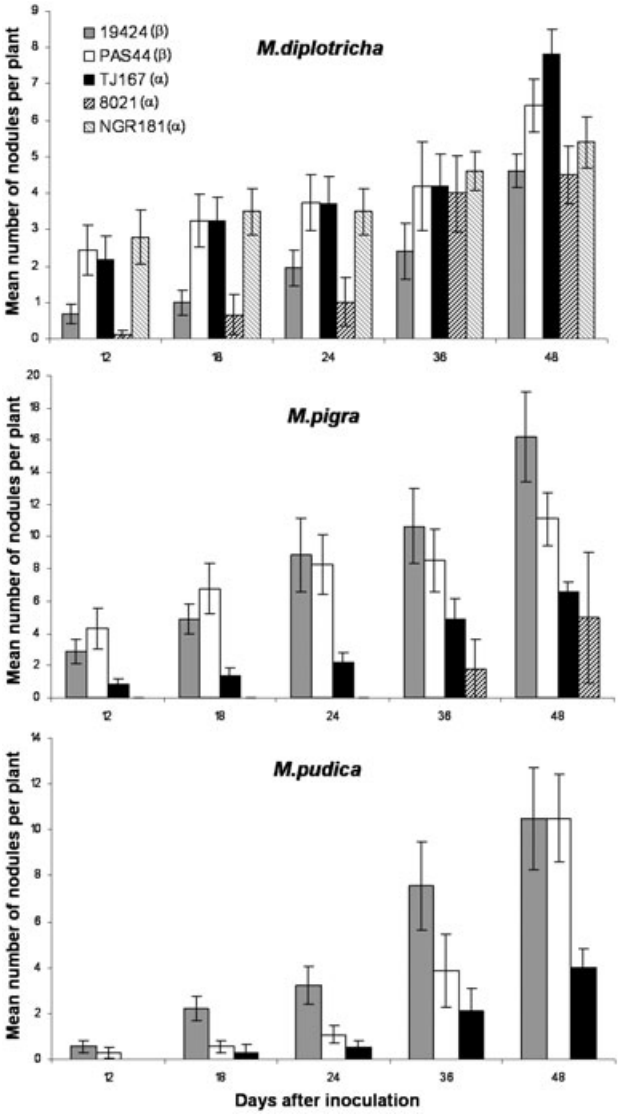

Fig. 6. Nodulation rate of all tested strains on three invasive *Mimosa* species. Nodule counts were made at 12, 18, 24, 36 and 48 days after inoculation of test plants with equal cell numbers of individual strains. All plants were grown under identical conditions in modified Jensen's N-free liquid medium. Bars indicate ±1 standard error based on at least six replicates.

liquid medium, and only 10% of the nodules in a solid medium (Table 3). However, *R. etli* TJ167 was more competitive with *C. taiwanensis* LMG19424, and it formed the majority of nodules on all plants in both growth media, except on *M. pudica* grown in a solid medium, where only 39% of the nodules contained *R. etli* TJ167. *Rhizobium tropici* UPRM 8021 was compared with *B. mimosarum* PAS44 and *C. taiwanensis* LMG19424 on *M. diplotricha* and *M. pigra* only, as it failed to nodulate *M. pudica* effectively (Table 2). Again, *B. mimosarum* PAS44 was extremely competitive and formed virtually all the nodules (145 out of 146) on both plants grown in both liquid and solid conditions (Table 3). Competition between *R. tropici* UPRM 8021 and *C. taiwanensis* LMG19424 showed a mixed response depending on plant rooting medium, with UPRM 8021 yielding 30% and 79% nodulation on *M. diplotricha* and *M. pigra*, respectively, in liquid medium, while on solid substrates both plants only produced nodules containing *C. taiwanensis* LMG19424 (Table 3). *Rhizobium tropici* NGR181 was only compared using *M. diplotricha* as a host and, again, *B. mimosarum* PAS44 out-competed the α-rhizobial strain, giving 100% nodulation in liquid and 94% in solid rooting media. Comparison of *R. tropici* NGR181 with *C. taiwanensis* LMG19424 showed the former out-competing the latter in liquid (90% *R. tropici* NGR181 nodulation), with roles somewhat reversed in solid medium (62% *C. taiwanensis* LMG19424 nodulation) (Table 3).

To reduce symbiotic motility/chemoattraction requirements, direct inoculation was performed (i.e. immersing roots in a concentrated bacterial suspension prior to 'planting' rather than adding the inoculant to the medium in which the seedling is already planted), comparing *B. mimosarum* PAS44 under both liquid and solid growth conditions with *R. etli* TJ167 in *M. pudica*, with *R. tropici* NGR181 in *M. diplotricha* and with *C. taiwanensis* LMG19424 in *M. pigra*. Only *R. tropici* NGR181 with *B. mimosarum* PAS44 in *M. diplotricha* in liquid medium showed a different and significant response from that under 'standard' inoculation conditions, with an increase from 0 out of 23 nodules containing *R. tropici* NGR181 to 4 out of 17 nodules (23%) with direct inoculation ($P < 0.05$).

Discussion

Why study competition in Mimosa *symbionts?*

β-Rhizobia have now been found to nodulate a range of legumes from all three subfamilies, with particularly good evidence for *Mimosa* in the Mimosoideae (Elliott *et al*., 2007a), *Cyclopia* and *Macroptilium* in the Papilionoideae (Elliott *et al*., 2007b), and some evidence for *Chamaecrista* in the Caesalpinioideae (E.K. James, P. Whitty, G.N. Elliott and J.I. Sprent, unpublished). Many previous reports have described symbionts, both α- and β-rhizobia, from the large, economically and ecologically important genus *Mimosa*, making it ideal for a study on competitive nodulation by these two very different groups of rhizobia. Our discovery that β-rhizobia are so dominant as symbionts in this genus, and particularly that this dominance can be reduced by soil N-content, raises the possibility that they may also be dominant in other genera in other environments, and hence 'traditional' concepts about rhizobial taxonomy/phylogeny *vis-à-vis* host plants across the nodulating members of the Leguminosae family may have to be thought anew.

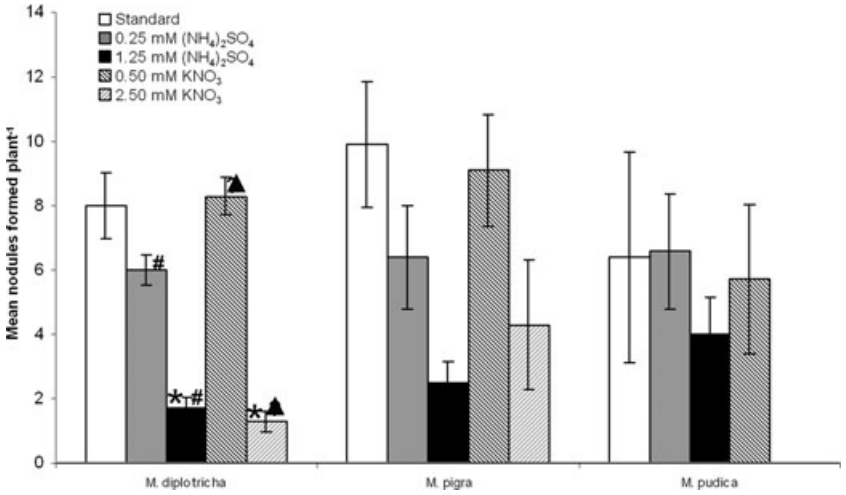

Fig. 7. Effect of N concentration on nodulation for three invasive *Mimosa* spp. co-inoculated with β-proteobacterial strains *Burkholderia mimosarum* PAS44 and *Cupriavidus taiwanensis* LMG19424. Total nodule counts were made 48 days after inoculation of test plants, with all performed with five or more replicates. Bars indicate ± 1 standard error. Data were tested for significance using minimum significant difference generation for multiple pair-wise comparisons (Tukey–Kramer method at $P < 0.05$). The hash (#) indicates a significant difference between $(NH_4)_2SO_4$ treatments, the triangle (▲) indicates a significant difference between KNO3 treatments and the asterisk (*) indicates a significant difference between standard treatment and the treatment indicated. No nodules were formed by *M. pudica* in the presence of 2.5 mM KNO_3.

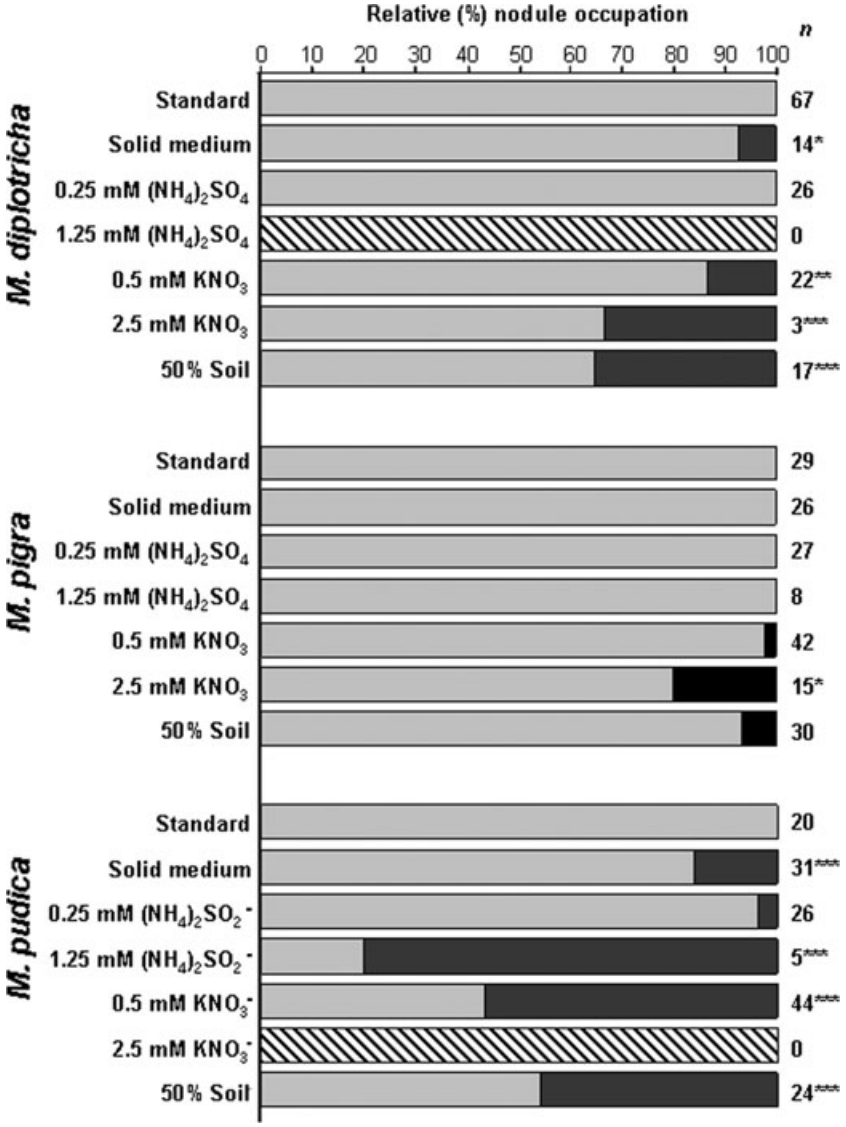

Fig. 8. Effect of N concentration on competition success in terms of nodule occupancy for β-proteobacterial strains *Burkholderia mimosarum* PAS44 and *Cupriavidus taiwanensis* LMG19424. Strains were inoculated in equal numbers and identified morphologically after re-isolation from nodules 42 days later. Light bars indicate *B. mimosarum* PAS44-containing and dark bars indicate *C. taiwanensis* LMG19424-containing nodules as a percentage of total nodules tested (*n*). Striped bars indicate treatments from which no nodules were successfully isolated from. Asterisks indicate significant differences from response in relevant standard medium (i.e. 100% *B. mimosarum* PAS44 occupation), with *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All plants, apart from those in N-free vermiculite-perlite (VP) or in 50% soil (described below), were grown in modified Jensen's N-free liquid medium with one of the following treatments: (i) no additions; (ii) addition of $(NH_4)_2SO_4$ to either 0.25 or 1.25 mM final concentration; (iii) addition of KNO_3 to either 0.5 or 2.5 mM final concentration. Nitrogen-free VP consisted of a 1:1 mixture of vermiculite and perlite soaked in liquid medium and then drained of excess, whereas 50% soil was a 1:1 mixture of a Taiwanese soil with sand (final total N concentration 0.1%).

Molecular phylogeny and symbiotic effectiveness of α- and β-rhizobia from invasive Mimosa *species*

This study has shown that 10 rhizobial strains isolated largely from invasive *Mimosa* species from various parts of the tropics can be grouped according to their 16S rDNA and *nodA* gene sequences with already known legume symbionts. Two β-rhizobial strains, NGR190 and NGR193A, originally isolated over 40 years ago from *M. diplotricha* and *M. pudica* nodules in Papua New Guinea by Trinick (M.J. Trinick unpublished; Trinick, 1980), were shown to belong, respectively, to the species *B. mimosarum* and the genus *Cupriavidus*. This is the first report of a *B. mimosarum* strain isolated from *M. diplotricha*, as all previously described strains have come either from another invasive species, *M. pigra*, or from *Mimosa scabrella*, a tree native to South East Brazil (Chen *et al*., 2005a,b; 2006). The exact identity of *Cupriavidus* sp. NGR193A awaits more detailed examination, although it is most probably another strain of *C. taiwanensis*, a species that has been isolated from *M. pudica* on many occasions in other parts of South-East Asia (Chen *et al*., 2001; 2003a; Verma *et al*., 2004). The 16S rDNA and *nodA* sequences of the α-rhizobia examined in the present study showed that NGR181, which was also originally isolated by M.J. Trinick (unpublished) from *M. diplotricha* nodules in Papua New Guinea, belongs to *R. tropici* along with already described *Mimosa* isolates, such as UPRM 8021 from *M. ceratonia* in Puerto Rico (Zurdo-Piñeiro *et al*., 2004), whereas the *R. etli* strains from *M. diplotricha* nodules in Taiwan, TJ167 and TJ173, grouped closely with the three *R. etli* bv. *mimosae* strains from *M. affinis* in Mexico (Mim1, Mim2 and Mim7-4). It is possible that TJ167 and TJ173 could also be included in this biovar as suggested by Silva and colleagues (2005). Our results confirm the suggestion of Chen and colleagues (2003a) that the α-rhizobia and β-rhizobia have very separate lineages of *nodA*. What is also clear from the present study is that the lineages of the *nodA* sequences of the α-rhizobia from *Mimosa* nodules can be further subdivided into two clades, one based on the *R. tropici* and one on the *R. etli* strains.

It is now well established that nodulation in some species of the legume genus *Mimosa* is largely dominated by symbioses with β-rhizobia (see *Introduction* for references). However, 'classical' α-rhizobia have also been isolated many times from *Mimosa* nodules over the last 15 years (Oyaizu *et al*., 1993; Wang *et al*., 1999; Chen *et al*., 2003a; Barrett and Parker, 2006; Andam *et al*., 2007; this study), although these strains, which include members of the genera *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium*, have been largely uncharacterized in symbiotic terms until now. This study is the first detailed report of α-rhizobia effectively nodulating *Mimosa*. Although previous studies have suggested that this could be the case, either no symbiotic data were presented (e.g. for *R. etli* strains TJ167 and TJ173; Chen *et al.*, 2003a) or the strains appeared to be symbiotically ineffective (e.g. *Rhizobium* sp. strains MApud2.5 and Hpud7.2; Barrett and Parker, 2006), or not to nodulate at all (e.g. *Sinorhizobium* sp. strain TJ170; Chen *et al.*, 2003a). In other cases, strains isolated from *Mimosa* spp. have only been tested on promiscuous non-host species, such as *Macroptilium atropurpureum* (e.g. *R. tropici* strain UPRM 8021; Zurdo-Piñeiro *et al*., 2004), and *Leucaena leucocephala*/*Phaseolus vulgaris* (e.g. *R. etli* bv. *mimosae* strains Mim1, 2 and 7-4; Wang *et al*., 1999). Interestingly, the present study has shown that not only can the Mexican *R. etli* bv. *mimosae* strains form N-fixing symbioses with their original host, *M. affinis*, but also that this native Mexican plant can be nodulated effectively by Taiwanense *R. etli* strains (TJ167 and TJ173) that were originally isolated from the invasive *M. diplotricha*. This lends support to the possibility that TJ167 and TJ173 may be *R. etli* bv. *mimosae* strains (Silva *et al*., 2005; this study), although they differ from the three Mexican *R. etli* strains in that the latter appear not to be capable of effectively nodulating other *Mimosa* spp., whereas TJ167 and TJ173 can nodulate both *M. diplotricha* and *M. pudica* effectively (Table 2). It should be noted, however, that *M. affinis* is a relatively non-selective host for nodulation, as it can also be nodulated effectively by a broad host range *Burkholderia* strain (*B. phymatum* STM815) and ineffectively by *C. taiwanensis* LMG19424 (Elliott *et al*., 2007a).

Both *R. tropici* strains, NGR181 and UPRM 8021, effectively nodulated the invasive *Mimosa* spp., but NGR181 could only nodulate its original host, *M. diplotricha*, whereas UPRM 8021 nodulated both *M. diplotricha* and *M. pigra*. Interestingly, UPRM 8021, which was isolated from *M. ceratonia*, but not yet tested on this plant (Zurdo-Piñeiro *et al*., 2004), was the only α-rhizobial strain in the present study to effectively nodulate *M. pigra*. Indeed, all of the non-Mexican α-rhizobia seemed to show a distinct preference for nodulating *M. diplotricha* (Table 2), which was also the *Mimosa* species in the study of Chen and colleagues (2003a) whose nodules were most likely to yield an effective α-rhizobial symbiont. This lends support to the suggestion of Barrett and Parker (2006) that, compared with other invasive species, *M. pigra* may be a relatively selective host. Indeed, studies with *M. pigra* symbionts from various parts of the world have shown that this plant has a distinct preference for *Burkholderia* (Chen *et al*., 2005a,b; Parker *et al*., 2007), whereas nodules on the other invasive species, *M. diplotricha* and *M. pudica*, are more likely to contain a wider variety of bacteria. Nodulation studies have also demonstrated that, in comparison with *M. pigra*, these two host species more readily enter into symbioses with bacteria other than

Burkholderia (Chen *et al*., 2003a; Barrett and Parker, 2005; 2006; Elliott *et al*., 2007a). In conclusion, the present study suggests that the order of preference of α-rhizobial nodulation of the three invasive *Mimosa* spp. is *M. diplotricha* first, followed by *M. pudica* and *M. pigra*, but with all three, including *M. diplotricha*, still showing a general ability to form more highly effective symbioses with β-rhizobia (this study, but also see Chen *et al*., 2005b; Barrett and Parker, 2006).

Competition between α- and β-rhizobia in nodulation of invasive Mimosa *species*

Competition experiments for nodule formation have been extensively performed between α-rhizobia strains on several legume crops, such as soybean (*Glycine max*), alfalfa (*Medicago sativa*), bean (*P. vulgaris*) and clover (*Trifolium* spp.), and these studies have allowed the identification of strains better adapted to hosts and of genes involved in competitiveness (Graham, 2008). Similar experiments between α- and β-rhizobia have not been performed until now because (i) β-rhizobia were unknown until recently and (ii) there was no suitable host that was compatible with both rhizobial types. However, we demonstrate here that *Mimosa* spp. can nodulate effectively with both α- and β-rhizobia, allowing us to compare natural symbionts from both the α- and β-proteobacteria on original host plant species under identical *in vitro* conditions. Initial comparisons between β-rhizobial genera were of interest as two earlier studies in Taiwan showed differences in the species profiles of the strains. The first study by Chen and colleagues (2003a) showed that of 190 isolates from *M. pudica* and *M. diplotricha*, 93% were *C. taiwanensis*, only 1% were *Burkholderia* and the remainder were α-proteobacteria. However, in the second study, Chen and colleagues (2005b) found the opposite trend in *M. pigra*, with 96% of 191 isolates identified as *Burkholderia* and the remaining 4% as *C. taiwanensis* (with no α-proteobacteria being isolated). The results of the present study, showing the exclusion of *C. taiwanensis* LMG19424 in the presence of *B. mimosarum* PAS44 in all three plant species under N-free conditions, were particularly surprising in view of the observations of Chen and colleagues (2003a). Comparisons between PAS44 and the well-studied *Mimosa*-nodulating strain *B. phymatum* STM815 (Elliott *et al*., 2007a) showed that PAS44 does not retain this competitive superiority in the presence of another *Burkholderia* strain, and so the high level of dominance exhibited by PAS44 over all other strains in the competition studies under N-free conditions may be a generic characteristic of *Mimosa*-nodulating *Burkholderia* rather than a specific trait of strain PAS44.

The dominance of nodulation of *Mimosa* spp. by *B. mimosarum* strain PAS44 in N-free liquid rooting media could not be explained through such factors as competitive strain inhibition during initial culture (none was detected), strain survival during experimentation (PAS44 was not favoured, Fig. 5) or bacterial migration to the host roots (the effect persisted with direct inoculation). However, experiments were not performed in terms of competition strain inhibition past the initial growth phase (30 h in liquid culture), and so the present study cannot be directly related to studies such as that of Robleto and colleagues (1997) who examined the relative competitiveness of *R. etli* strains in the rhizosphere of bean plants over a longer period (4 days). Also, the direct inoculation methods used in the present study would not have removed the advantage that a bacterium superior in chemoattractive motility might have in travelling along the root to a transient nodulation zone (Bhuvaneswari *et al*., 1980; 1981; Vande Broek and Vanderleyden, 1995; López-García *et al*., 2002). However, as nodulation rates of *B. mimosarum* PAS44 when inoculated in isolation were inferior to those of *C. taiwanensis* LMG19424 (Fig. 5), the assertion that nodulation rate alone largely determines competitive nodulation success (Lupwayi *et al*., 1996) is not supported by the present study on *Mimosa* nodulation.

The near universal dominance of *Burkholderia* over all α-rhizobia tested under N-free hydroponic conditions was only challenged by *R. etli* TJ167 on *M. diplotricha*. Indeed, the original host of both *R. etli* TJ167 and *R. tropici* NGR181 is *M. diplotricha*, and although *R. tropici* NGR181 did not compete as well against the *Burkholderia* strain as did *R. etli* TJ167, a similar dominance of *R. tropici* NGR181 over *C. taiwanensis* LMG19424 in liquid culture demonstrated that α-rhizobia can still be competitive against some β-rhizobia in nodulating *M. diplotricha*, a species that appears to have a greater compatibility with α-rhizobia compared with the other invasive species (see earlier). Nevertheless, the third α-rhizobial strain, *R. tropici* UPRM8021 which, unlike the two former α-rhizobia, was not isolated from *M. diplotricha*, fared poorly in competition against *C. taiwanensis* LMG19424 for nodulation of this species.

Substrate composition affects the competitiveness of β-rhizobial nodulation of Mimosa

The different natural growth conditions to which the three host species (and their symbionts) are adapted to are likely to be factors when explaining the differences found between the present and previous studies of *Mimosa* nodulation. For example, under water-logged conditions, the *Mimosa* species used here grow and nodulate most successfully in the order of *M. pigra*, *M. pudica*, *M. diplotricha*, both in the field and in the laboratory (James *et al*.,

2001; Chen *et al*., 2005a,b). Nevertheless, we used a liquid rooting medium extensively in the competition experiments with all three *Mimosa* species, regardless of differences in their respective abilities to tolerate waterlogging. This was done in order to minimize any potential problems that could have occurred with a solid rooting medium, such as variations in bacterial density, N-content and pH (Vlassak and Vanderleyden, 1997). As a consequence of this, *in vitro* data from plants grown in the liquid medium may more closely reflect the natural growth and nodulation conditions of the flooding-tolerant *M. pigra* than those of the less tolerant *M. pudica* and *M. diplotricha*. Indeed, changes in the dominance of *Burkholderia* over *C. taiwanensis* were found to occur in all three plant species when grown in a mixture of soil and sand, in that *M. pudica* and *M. diplotricha* were much more likely to be nodulated by *C. taiwanensis* than was *M. pigra*. Of all the data presented here, these findings are the most similar to those found in studies of rhizobial strain composition in *Mimosa* nodules from the field (Chen *et al*., 2003a; 2005b), thus indicating that substrate conditions (i.e. flooding versus non-flooding, and mineral N-levels) are important in competition for nodule development by these bacteria.

Indications that it is the N-content of the plant growth substrate that most affects host-symbiont competitive specificity at the generic level are very interesting, as it is well known that increased nitrate or ammonium levels can adversely affect nodulation by 'conventional' rhizobia (for reviews, see Streeter, 1988; Patriarca *et al*., 2002). The literature has examples of N-content having no effect on competitive nodule occupancy (Kosslak and Bohlool, 1985; Sheoran *et al*., 1997), while others show measurable effects (McNeil, 1982; Somasegaran and Bohlool, 1990), although it should be noted that these studies compared closely related strains of the same species for nodulation, whereas in the present study the strains compared on their natural hosts were not only from different genera, but actually from different classes of the proteobacterial phylum. With regard to the effect of different sources of mineral N on nodulation of *Mimosa* spp., the only previously published study, to our knowledge, is that of Goi and colleagues (1997), who showed that nitrate had a negative effect on nodule number and plant growth of *Mimosa caesalpiniifolia* (a tree native to Brazil that also nodulates with *Burkholderia*; Chen *et al.*, 2005a; 2007), but ammonium increased nodulation and plant growth. Although in the present study the presence of N in the form of ammonium sulfate affected competition in *M. pudica*, only the higher level of N from ammonium (1.25 mM) gave levels of *C. taiwanensis* nodule occupancy similar to both those found with 50% soil (Fig. 8), and these were also commensurate with the *in situ* findings in Taiwanese soils by Chen and colleagues (2005b). On the other hand, when mineral N was added in the form of nitrate (Fig. 8), much lower mineral N concentrations (< 0.5 mM), similar to those more likely to be encountered in Taiwanese soils (mineral N generally being between 1% and 2% of total N soil content, which translates to between 0.12% and 0.14% total N; W.-M. Chen, unpubl. data), were sufficient to result in the nodule occupancy data found previously for all three *Mimosa* spp. in Taiwan (Chen *et al*., 2003a; 2005b). Further studies are needed to determine the exact mechanisms that cause the differential effects of nitrate and ammonium on the competitive ability of *Burkholderia* and *C. taiwanensis* in their nodulation of the three invasive *Mimosa* spp., but one possibility is that they could be due to the different modes of action of the ammonium- and the nitrate-dependent inhibition of N-fixing nodule formation (Patriarca *et al*., 2002).

Concluding remarks

Although several legumes (e.g. *Leucaena*, *Macroptilium*, *Phaseolus*) are known to nodulate with a very wide range of α-rhizobial genera (Graham, 2008), none of these has yet been reported to nodulate effectively with either or both of the described genera of β-rhizobia (i.e. *Burkholderia* and *Cupriavidus*). Hence the range of effectively symbiotic strains from invasive *Mimosa* spp., confirmed in the present and earlier studies, is the most diverse found to date in any legume genus. Indeed, although the 'discovery' of symbiotic β-rhizobia dates only from 2001, it is clear from the recent molecular characterization of selected strains from the 1962–64 Papua New Guinea ('NGR') collection of Trinick, including *R. tropici* NGR181, *B. mimosarum* NGR190, *Cupriavidus* sp. NGR193A and *B. phymatum* NGR195A (Trinick, 1980; Elliott *et al*., 2007a; this study), that symbionts from both the α- and β-rhizobia have routinely been isolated from *Mimosa* nodules in the past 50 years or so. However, because of the difficulties in distinguishing the different types of bacteria before the advent of molecular techniques, the profound genotypic differences between α- and β-rhizobia would not necessarily have been apparent, and it is possible that in many cases the β-rhizobia, with their fast growth rates compared with 'normal' rhizobia, were discarded as 'contaminants' (Vincent, 1970), and hence Trinick should be credited with unique foresight in the conservation of these unusual NGR isolates.

The present study has also shown that, although *Rhizobium* strains can nodulate certain *Mimosa* species, particularly *M. diplotricha*, they are not as promiscuous or as competitive as other *Mimosa* symbionts, especially *Burkholderia* strains, such as the French Guianian *B. phymatum* STM815, which have a particular affinity for nodulating *Mimosa* spp. (Elliott *et al*., 2007a). This may explain why *Rhizobium* spp. are not so commonly isolated as the

Burkholderia symbionts, at least in the context of native species in South and Central America (see *Introduction* for references). However, given that the dominant symbiont of *M. affinis* in Mexico appears to be *R. etli* bv. *mimosae* (Wang *et al*., 1999), and that *Cupriavidus* spp. are the dominant symbionts of two invasive species (*M. diplotricha*, *M. pudica*) outside their native habitats (e.g. in Taiwan and India), and possibly also of native *Mimosa* species from Texas (Andam *et al*., 2007), this may not be the case in all localities. Indeed, although other factors, such as host genotype and geographical isolation, will be important, the present study suggests that the concentration and type of N in the soils from these different regions will almost certainly have also played a large part in the selection of symbionts by both native and invasive *Mimosa* spp.

Experimental procedures

Bacterial strains

All rhizobial strains (Table 1) were obtained from published sources, except for most of the NGR strains, originally isolated from *Mimosa* nodules between 1962 and 1964 (Trinick, 1980; Elliott *et al*., 2007a), which were kindly provided by Professor Ivan Kennedy from the Papua New Guinea collection of Mike Trinick housed in the University of Sydney. All strains were grown in YEM medium (Vincent, 1970) at 28°C.

Amplification, sequencing and phylogenetic analysis

Nearly full-length 16S rDNA from NGR strains 181, 190 and 193A was amplified and sequenced using primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) (Weisburg *et al*., 1991). For all strains listed in Table 1 (apart from LMG19424, STM815, TJ167, TJ172 and TJ173 which were all sequenced previously), *nodA* gene fragments were amplified with a touchdown PCR programme with annealing temperature varying from 60°C to 50°C for 20 cycles and followed by 25 additional cycles at 50°C. A 576 bp *nodA* product was amplified with primers 5′-TGGARVBTNYSYTGGGAAA-3′ and 5′-TCACARCTCGGCCCGTTCCG-3′ for strains Mim1, Mim2 and Mim7-4 and a 478 bp *nodA* product with primers 5′-ACSTAYGGDCCDACMGG-3′ and ATGRCGGYKCCGTYNGGCCA for strains PAS44, NGR190, NGR193 and DUS751. Nucleotide alignments were constructed with CLUSTALX 1.83, imported into BioEdit 4.8.4 (Hall, 1999) and manually corrected. Phylogenetic tree constructions were based on a 1240 nt rDNA 16S or a 227 nt *nodA* alignment. Neighbour-Joining analyses were performed using Kimura two-parameter distance correction with phylo_win 2.0 (Galtier *et al*., 2006) and Maximum-Likelihood analyses with a fully estimated GTR model with the PhyML software (Guindon *et al*., 2005). For Neighbour-Joining, robustness of the tree branches was estimated with 1000 bootstrap replications, and with 100 for Maximum-Likelihood.

Tests for symbiotic effectiveness

Seeds from *M. diplotricha* and *M. pigra* were obtained from wild plants in Taiwan, *M. affinis* seeds from wild plants in Mexico (Wang *et al*., 1999), and *M. pudica* seeds were from Thompson and Morgan (UK) (Ipswich, UK). All were surface-sterilized with concentrated sulfuric acid for 10 min followed by several washes with sterile distilled water. Seeds were subsequently placed onto 1% water agar for germination at 20°C in darkness. Following germination, nodulation tests with all the strains listed in Table 1 were carried out using a variation of the semi-enclosed tube method of Gibson (1963) by suspending seedlings through Parafilm into 30 ml tubes filled with sterile modified Jensen's N-free medium in liquid form (agar-free) (Somasegaran and Hoben, 1994). At 42 days after inoculation, acetylene reduction assays (ARAs) were carried out on the plants according to Chen and colleagues (2003b). After the ARAs, nodules were fixed and embedded for light microscopy, and some were also prepared for TEM coupled with immunogold labelling using antibodies raised against the nitrogenase iron protein NifH (Chen *et al*., 2003b; 2005a,b).

Competition studies

Five strains (*B. mimosarum* PAS44, *C. taiwanensis* LMG19425, *R. etli* TJ167, *R. tropici* NGR181 and *R. tropici* UPRM8021) were selected for competition studies. The three *Rhizobium* strains were directly transformed with plasmids pMP4641, pMP4658 and pMP4662 (Bloemberg *et al*., 2000) using a modified freeze–thaw method of Vincze and Bowra (2006) to create, respectively, *R. etli* NGR181ecfp, *R. tropici* UPRM 8021eyfp and *R. etli* TJ167rfp. *Burkholderia mimosarum* PAS44 was transformed with plasmid pRG960sd-32 (Van den Eede *et al*., 1992) through triparental mating according to Chen and colleagues (2003b) and Elliott and colleagues (2007b). These transformed strains, as well as *C. taiwanensis* LMG19424gfp (Elliott *et al*., 2007a), were then used to inoculate seedlings of *M. diplotricha*, *M. pigra* and *M. pudica* growing either hydroponically using the semi-enclosed tube method described above or in a solid medium. Plants were grown in two types of solid medium: sterile vermiculite/perlite (1:1), with modified Jensens N-free medium added and any excess drained, and autoclaved soil/sand (1:1) moistened with sterile water. The soil used consisted of 27% sand, 53% silt and 20% clay; organic matter, 2%; total N, 0.2%, and was derived from the Pingtung region of Taiwan. All plants were grown at 26°C under Triplus T5 triphosphor plant growth lamps at 339.5 ($\pm$ 28.2) $\mu E \cdot m^{-2}\ s^{-1}$ with a 12 h day length and all plants were grown for 42 days before harvesting, unless stated otherwise.

Seedlings were placed into growth media and inoculated, 5 days after germination, with the equivalent of 1×10^4 bacterial cells ml^{-1} medium unless stated otherwise. Where stated, a direct inoculation method was used, where plant roots were directly immersed in high densities of cells ($> 10^7\ ml^{-1}$) for 2 min. Seedlings were then replaced into the appropriate medium for growth. Nitrogen levels were altered by adding either KNO_3 (to 0.5 or 2.5 mM) or $(NH_4)_2SO_4$ (to 0.25 or 1.25 mM) to give final (starting) concentrations of 3.5 and 17.5 mg N l^{-1} medium for both nitrogen sources. Levels of acidity were also raised in some liquid-medium experi-

ments by lowering the pH to 5.8 (starting level) by the addition of 20 µl of 1 M HCl to 30 ml tubes immediately prior to inoculation. All studies were performed using at least six individual plants in separate tubes as replicates.

For isolation of bacteria, including assessment of nodule occupancy by marked strains, root nodules were soaked in sterile water overnight if dry or carefully picked from the root if fresh, then surface-sterilized in 95% ethanol for 30 s, followed by 3 min in 3% sodium hypochlorite, then washed six times with sterile distilled water. Individual nodules were crushed, spread onto YEM agar containing Congo red dye and incubated at 28°C. Identification in competition studies was performed using gross morphological traits, with microscopy for confirmation. Fluorescence activity within colonies was observed under UV light and/or epifluorescence microscopy. Nodules harvested for microscopy were sectioned at approximately 50 µm thickness using a Vibratome 1500 (Agar Scientific, Stansted, UK), with nodules to be assessed for GUS activity being exposed to 1 mM X-Gluc before viewing using dark-field microscopy. Nodule sections were also analysed using standard light microscopy, epifluorescence miscroscopy and CLSM according to Elliott and colleagues (2007a).

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