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# The geographical patterns of symbiont diversity in the invasive legume *Mimosa pudica* can be explained by the competitiveness of its symbionts and by the host genotype

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## Summary

**Variations in the patterns of diversity of symbionts have been described worldwide on *Mimosa pudica*, a pan-tropical invasive species that interacts with both α and β-rhizobia. In this study, we investigated if symbiont competitiveness can explain these variations and the apparent prevalence of β- over α-rhizobia. We developed an indirect method to measure the proportion of nodulation against a GFP reference strain and tested its reproducibility and efficiency. We estimated the competitiveness of 54 strains belonging to four species of β-rhizobia and four of α-rhizobia, and the influence of the host genotype on their competitiveness. Our results were compared with biogeographical patterns of symbionts and host varieties. We found: (i) a strong strain effect on competitiveness largely explained by the rhizobial species, with *Burkholderia phymatum* being the most competitive species, followed by *B. tuberum*, whereas all other species shared similar and reduced levels of competitiveness; (ii) plant genotype can increase the competitiveness of *Cupriavidus taiwanensis*. The latter data support the likelihood of the strong adaptation of *C. taiwanensis* with the *M. pudica* var. *unijuga* and help explain its prevalence as a symbiont of this variety over *Burkholderia* species in some environments, most notably in Taiwan.**

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## Introduction

Rhizobia are soil bacteria that form a symbiotic interaction with plants belonging to the family Fabaceae (commonly called legumes). Rhizobia induce the formation of nodules on the roots and, more rarely, on the stems of legumes, in which they differentiate into bacteroids and fix atmospheric dinitrogen into soluble compounds that are transferred to the plant in exchange for carbon compounds and a safer ecological niche (Gyaneshwar *et al.*, 2011). This plant-bacterial symbiosis is not compulsory for the two partners, as rhizobia compete with other microbes for their survival in soil in a free-living state before a compatible plant develops nearby. For its part, the legume plant is able to grow without the help of symbiotic bacteria when available forms of nitrogen are present in the soil. When a legume develops in a soil, its roots encounter diverse compatible rhizobia (i.e. those that are able to form nodules) with different symbiotic abilities (i.e. with variable rates of nitrogen fixation and capability to transfer the combined nitrogen to the host). If the compatibility with the plant relies mainly on a molecular dialogue involving flavonoids and Nod factors (for review, see Masson-Boivin *et al.*, 2009), the competition for nodulation (i.e. the competitiveness) among strains is also an important symbiotic trait for the success of nodulation by rhizobia. Indeed, compatible rhizobial populations will compete for the formation of nodules on the root system, as the plant limits the number of nodules formed through autoregulation by a shoot-derived signal (Ferguson *et al.*, 2010; Reid *et al.*, 2011). Competitiveness is often viewed as a trait that directly influences a strain's relative fitness in a population (for review, see Friesen, 2012). It can be

variable at both intraspecific and interspecific levels, as described in several plant-rhizobial model pairs (Amarger and Lobreau, 1982; Denton *et al*., 2003; Rangin *et al*., 2008). Several rhizobial properties have been identified as playing an important role in the competition for nodulation, including antibiosis (Robleto *et al*., 1998), motility and adhesion (Malek, 1992; Lodeiro *et al*., 2000), cell-surface characteristics (Lagares *et al*., 1992), or the catabolism of specific molecules (Triplett and Sadowsky, 1992; Murphy *et al*., 1995; Soedarjo and Borthakur, 1996; Toro, 1996; Fry *et al*., 2001; Wielbo *et al*., 2007).

Rhizobia are a polyphyletic group within the α and β classes of the Proteobacteria, and the terms α- and β-rhizobia have been proposed for convenience (Chen *et al*., 2003; Gyaneshwar *et al*., 2011). Most legume species are known to nodulate with α-rhizobia, while β-rhizobia are so far mostly restricted to legume families within the tribe Mimoseae and in some Papilionoids (Elliott *et al*., 2007a; Garau *et al*., 2009; dos Reis *et al*., 2010; Gyaneshwar *et al*., 2011; da Silva *et al*., 2012; Taulé *et al*., 2012; Bournaud *et al*., 2013; Howieson *et al*., 2013). β-rhizobia were described only 12 years ago, and their occurrence seemed to be uncommon at that time, but it is now well established that they are the dominant symbionts of many legume species within the genus *Mimosa*, although α-rhizobia are also found in *Mimosa* nodules but less frequently (Chen *et al*., 2003; Barrett and Parker, 2005; 2006; Parker *et al*., 2007; Bontemps *et al*., 2010; Liu *et al*., 2012; Mishra *et al*., 2012; Gehlot *et al*., 2013). The β-rhizobia described so far belong to two genera (*Burkholderia* and *Cupriavidus*), and to 10 species, namely *B. phymatum*, *B. tuberum*, *B. mimosarum*, *B. nodosa*, *B. sabiae*, *B. symbiotica*, *B. diazotrophica*, *B. phenoliruptrix* (Gyaneshwar *et al*., 2011; Sheu *et al*., 2012; 2013; Bournaud *et al*., 2013), *C. taiwanensis* and *C. necator* (Chen *et al*., 2001; da Silva *et al*., 2012). Phylogenetic analyses of house-keeping and nodulation genes have shown that *Burkholderia* species are ancient symbionts of *Mimosa* (Bontemps *et al*., 2010), while *Cupriavidus* species have probably acquired symbiotic genes from a *Burkholderia* symbiont more recently (da Silva *et al*., 2012).

Several diversity studies on *Mimosa* species symbionts have been conducted across tropical regions, with a particular attention on three *Mimosa* species because of their invasive status (*M. pudica*, *M. pigra* and *M. diplotricha*). For these latter species, structured biogeographical diversity patterns are clearly apparent in both their native and invasive territories.

In South America, the major centre of diversification of *Mimosa*, with more than 300 native species (Simon *et al*., 2011), *Mimosa* symbionts are dominated by *Burkholderia* species (Chen *et al*., 2005a; Bontemps *et al*., 2010; Mishra *et al*., 2012). In Costa Rica (Central America), almost even frequencies of *Cupriavidus*, *Rhizobium* and *Burkholderia* were isolated from *M. pudica* nodules, whereas *Burkholderia* dominated in *M. pigra* nodules (Barrett and Parker, 2006). In Taiwan, *C. taiwanensis* is the major symbiont of *M. diplotricha* and *M. pudica* (80% and 85.7% of described symbionts, Chen *et al*., 2003), while *B. mimosarum* dominates in *M. pigra* nodules (Chen *et al*., 2005a). In Southern China, *Burkholderia* and *Cupriavidus* occupy almost evenly the nodules of *M. pudica* and *M. diplotricha* (Liu *et al*., 2012). In New Caledonia and the Philippines (South Pacific), *Cupriavidus* dominates in *M. pudica* nodules (Andrus *et al*., 2012; Klonowska *et al*., 2012). Finally, endemic *Mimosa* species in India are nodulated by *Sinorhizobium* spp., while the invasive species *M. pudica* remains associated with β-rhizobial symbionts, with neither *Burkholderia* nor *Cupriavidus* being dominant as a whole, although there are locations in India where one type is more prevalent than the other (Gehlot *et al*., 2013).

These diversity studies have revealed the uneven frequencies of *Mimosa* symbionts in various geographical areas and have underlined the prevalence of β-rhizobia in the nodules of several *Mimosa* species. Alpha-rhizobia are also often found in *Mimosa* nodules, although at a lower frequency than β-rhizobia. These α-rhizobia belong to several *Rhizobium* species, namely *R. mesoamericanum*, *R. tropici* and *R. etli* bv. *mimosae* (Wang *et al*., 1999; Chen *et al*., 2003; Elliott *et al*., 2009; López-López *et al*., 2012; Mishra *et al*., 2012).

The unequal frequencies of each bacterial species might result from several factors, among which competition for nodulation might play a central role. Elliott and colleagues (2009) compared competitiveness among 3 α-rhizobia and 3 β-rhizobia isolates. They concluded that *B. mimosarum* PAS44 strain out-competed *C. taiwanensis* LMG19424, *R. etli* and *R. tropici* strains on *M. pudica*, *M. pigra* and *M. diplotricha* but that the dominance of PAS44 over LMG19424 was reduced when several abiotic conditions were altered (e.g. presence of nitrate or ammonium, and the density of the soil medium). The authors also compared PAS44 against *B. phymatum* STM815 and found them to be equally competitive on all hosts, except for *M. pudica* where STM815 was more competitive.

In the present study, we took advantage of a large collection (54) of α- and β-rhizobial symbionts of *M. pudica* that were isolated from geographical areas throughout its native and invasive ranges worldwide in order to investigate if competitiveness could explain the overall dominance of β-rhizobia on *Mimosa* species, as well as the different patterns of diversity of rhizobial species in *M. pudica*. More precisely, we tested: (i) if β-rhizobia are, as a whole, more competitive than α-rhizobia on *Mimosa* species, (ii) how the different

species of β-rhizobia behave against each other and thus explain (or not) the frequency of particular symbiont species in the previously mentioned diversity studies, and (iii) if the competitiveness of rhizobial species was influenced by the *M. pudica* host genotype used in the tests.

## Results

### *Genetic screening of the bacterial collection*

The 54 rhizobial strains were chosen to maximize within species genetic diversity (initially based on DNA fingerprinting of a larger collection of 522 strains, by repetitive extragenic palindromic element (REP)-polymerase chain reaction (PCR) (Table 1), and their taxonomic position was further confirmed by phylogenetic analyses based on two loci (16S RNA and *recA*). Our sampling included from 9 to 15 strains per rhizobial species, except for the α-rhizobia for which sampling was smaller because they were less frequently identified as *Mimosa* symbionts. Most of the strains were isolated from *M. pudica* nodules, but some originated from other *Mimosa* species (Table 1). All strains were proven to form a nitrogen-fixing symbiosis on *M. pudica* (data not shown).

The 54 strains used in the present study were sampled from various locations in the tropics, and some had been previously identified via molecular typing (Table 1 and references therein). The phylogenetic tree based upon a 16S rRNA-*recA* concatenated data set is presented in Fig. 1A. Forty-three strains could be assigned to one of the four known β-rhizobial species: *B. tuberum* (9 isolates), *B. mimosarum* (9), *B. phymatum* (10) and *C. taiwanensis* (15). One strain (STM3675) was considered as *B. phymatum* despite an unresolved position between *B. sabiae* and *B. phymatum* in maximum likelihood (ML) trees but with a clear clustering with other *B. phymatum* strains in its *nodC* phylogeny (Fig. 1B). The other strains belonged to *Rhizobium* species, with six strains isolated from *M. pudica* in Guinea that might form a new species of the genus *Rhizobium* (*Rhizobium* sp. 1), two *R. mesoamericanum*, two *R. etli* and one *R. tropici*. The *nodC* and *nifH* trees (Fig. 1B and Supplementary Fig. S1) also grouped the strains according to their species, except for strains of *R. mesoamericanum* and of *Rhizobium* sp. 1 that share similar *nodC* and *nifH* genes.

### *Development and validation of a high-throughput methodology for the estimation of competitiveness*

As we were studying a large collection of strains, a method to compare them with a reference strain was chosen, and this involved their co-inoculation in equal proportion of each strain against a common competitor, the *B. phymatum* strain STM815-GFP. This GFP strain was chosen among others based on an experiment with a small subset of strains from different species showing that it was moderately competitive and hence suitable for measuring the proportion of nodulation by each strain (Experiment I in Table 2), and also sufficiently fluorescent within the nodules (supplementary Fig. S2) and in subcultures after nodules were crushed. This strain is altered in its competitiveness compared with the wild-type strain (Table 2), and we considered that this altered competitiveness is stable in the competition experiments whatever the pairs thus not modifying the final rank order. Several strains were tested two or three times following the same protocol (Table 2). In general, the strain and species rankings were retained in the different experiments despite variations in % of nodule occupancy, thereby validating our approach.

### *Competitiveness for nodulation of the rhizobial collection*

Using commercial seeds of *M. pudica* as a host plant, the nodulation competitiveness of the 54 strains included in the study was estimated by competition experiments against a common competitor, the *B. phymatum* strain STM815-GFP.

The percentage of nodule occupancy ranged from 0% to 92% (Table 1), depending on the strain, and the strain effect was highly significant ($P \leq 0.0001$). This variation was largely explained by the rhizobial species [$P \leq 0.001$; $R^2$ coefficient of determination in analysis of variance (ANOVA) of 0.70]. The average competitiveness per species is presented in Fig. 2. The rank of competitiveness for all strains is given in Table 1 and illustrated in supplementary Fig. S3. The top rankings of competitiveness were occupied by the 10 strains of *B. phymatum* (from 49.7% to 92.1%, Table 1). The rankings of *B. tuberum* were less clear, with five strains ranking from position 11 to 16 (27 to 39.6%), the others being poorly or not at all competitive (ranked 22, 26, 42 and 45 with 14%, 11.7%, 4.4% and 3.6%, respectively). Finally, *B. mimosarum* displayed a wide variability of competitiveness ranging from 19.6% (ranking 16) to 1.2% (ranking 49).

All the other strains (*Cupriavidus*, *Rhizobium*) were poorly competitive (0–19.4% of nodule occupancy) independently of their taxonomical classification, their geographical origin or their host of origin. Based on a Mann–Whitney test, *B. phymatum* is significantly the most competitive species ($P < 0.0001$), *B. tuberum* is significantly better than *C. taiwanensis* ($P = 0.023$) and α-rhizobia ($P = 0.010$) but not *B. mimosarum* ($P = 0.093$). All the other species (*B. mimosarum, C. taiwanensis*, *R.* sp1) are equally competitive. To determine whether the observed strain ranking was dependent on the choice of STM815-GFP as the shared competitor, we conducted

**Table 1.** List of rhizobial strains according to their species affiliation, geographical origin, host plant of origin and success in nodule formation on *M. pudica* when in competition against *B. phymatum* strain STM815-GFP.

| Strain[a] and species | Geographic origin | Host plant of origin | 16S RNA[b] | Rep-PCR[c] | % nodule occupancy[f] % stat. tests. | | Rank[g] | Reference |
|---|---|---|---|---|---|---|---|---|
| *Burkholderia phymatum* | | | | | | | | |
| Bp-815-GFP | | | | | | | | Elliott *et al.* (2007b) |
| Bp-815T | Fr. Guiana | *Mimosa* spp.[d] | AAA | BP1 | 91.5 (8.1) | a | 2 | Moulin *et al.* (2001) |
| Bp-3619 | Fr. Guiana | *M. pudica* | AAA | BP2 | 62.3 (5.8) | cd | 7 | Mishra *et al.* (2012) |
| Bp-3665 | Fr. Guiana | *M. pudica* | AAA | BP4 | 87.8 (4.4) | ab | 3 | Mishra *et al.* (2012) |
| Bp-3675 | Fr. Guiana | *M. pudica* | DEG | BP6 | 65.1 (8.3) | bcd | 5 | Mishra *et al.* (2012) |
| Bp-4211 | Fr. Guiana | *M. pudica* | AAA | BP7 | 52.9 (33.8) | de | 9 | Mishra *et al.* (2012) |
| Bp-4225 | Fr. Guiana | *M. pudica* | AAA | BP9 | 61.0 (8.1) | cd | 8 | Mishra *et al.* (2012) |
| Bp-4328 | Fr. Guiana | *M. pudica* | AAA | BP11 | 92.1 (2.8) | a | 1 | Mishra *et al.* (2012) |
| Bp-4343 | Fr. Guiana | *M. pudica* | AAA | BP12 | 82.2 (7.0) | abc | 4 | Mishra *et al.* (2012) |
| Bp-6022 | Fr. Guiana | *M. pudica* | AAA | BP14 | 49.7 (7.8) | de | 10 | Mishra *et al.* (2012) |
| Bp-3714 | Guinea | *M. pudica* | AAA | BP15 | 63.3 (12.3) | cd | 6 | This study |
| *Burkholderia tuberum* | | | | | | | | |
| Bt-3638 | Fr. Guiana | *M. pudica* | ABD | BT4 | 29.1 (23.1) | efghi | 14 | Mishra *et al.* (2012) |
| Bt-3649 | Fr. Guiana | *M. pudica* | ABF | BT2 | 27.0 (24.0) | fghij | 15 | Mishra *et al.* (2012) |
| Bt-3671 | Fr. Guiana | *M. pudica* | ABF | BT6 | 4.4 (3.9) | lmnop | 42 | Mishra *et al.* (2012) |
| Bt-4228 | Fr. Guiana | *M. pudica* | ABF | BT8 | 39.6 (9.2) | def | 11 | Mishra *et al.* (2012) |
| Bt-4252 | Fr. Guiana | *M. pudica* | ABF | BT7 | 14.0 (25.0) | klmnop | 22 | Mishra *et al.* (2012) |
| Bt-4287 | Fr. Guiana | *M. pudica* | ABF | BT1 | 30.1 (19.8) | efghi | 13 | Mishra *et al.* (2012) |
| Bt-3686 | Guinea | *M. pudica* | ABF | BT13 | 3.6 (3.5) | mnop | 45 | This study |
| Bt-3929 | Guinea | *M. pudica* | ABF | BT14 | 11.7 (16.6) | hijklmn | 26 | This study |
| Bt-4856 | Australia | *M. pudica* | XBF | BT15 | 36.5 (7.7) | defg | 12 | This study |
| *Burkholderia mimosarum* | | | | | | | | |
| Bm-3621 | Fr. Guiana | *M. pudica* | ABB | BM1 | 10.3 (5.0) | hijklmno | 31 | Mishra *et al.* (2012) |
| Bm-4215 | Fr. Guiana | *M. pudica* | ABB | BM2 | 19.6 (14.1) | fghijkl | 16 | Mishra *et al.* (2012) |
| Bm-4218 | Fr. Guiana | *M. pudica* | ABB | BM3 | 8.6 (8.1) | jklmnop | 36 | Mishra *et al.* (2012) |
| Bm-3699 | Guinea | *M. pudica* | GBB | BM5 | 4.4 (2.0) | klmnop | 43 | This study |
| Bm-3726 | Guinea | *M. pudica* | ABB | BM6 | 1.2 (2.0) | op | 49 | This study |
| Bm-3764 | Guinea | *M. pudica* | XBB | BM7 | 19.4 (11.1) | fghijk | 17 | This study |
| Bm-5615 | Australia | *M. pudica* | XBB | BM8 | 11.0 (3.9) | hijklmn | 29 | This study |
| Bm-5630 | Australia | *M. pudica* | XBB | BM9 | 11.0 (9.7) | hijklmno | 30 | This study |
| Bm-6824 (PAS 44T) | Taiwan | *M. pigra* | GBB | BM10 | 17.7 (12.0) | ghijklm | 19 | Chen *et al.* (2005a) |
| *Cupriavidus taiwanensis* | | | | | | | | |
| Ct-894 (LMG19424) | Taiwan | *M. pudica* | III/H[e] | CT4 | 14.8 (10.5) | ghijklm | 21 | Chen *et al.* (2001) |
| Ct-895 (LMG19425) | Taiwan | *M. diplotricha* | I/E[e] | CT5 | 9.1 (5.3) | ijklmno | 34 | Chen *et al.* (2001) |
| Ct-896 (LMG19426) | Taiwan | *M. pudica* | II/K[e] | CT6 | 9.0 (8.2) | jklmnop | 35 | Chen *et al.* (2001) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ct-1023 (TJ20) | Taiwan | *M. pudica* | I/A[e] | CT7 | 13.3 (11.4) | ghijklmn | 24 | Chen *et al.* (2003) |
| Ct-1025 (TJ17) | Taiwan | *M. pudica* | I/A[e] | CT8 | 2.3 (4.0) | nop | 48 | Chen *et al.* (2003) |
| Ct-1027 (TJ145) | Taiwan | *M. diplotricha* | I/A[e] | CT9 | 12.5 (11.4) | ijklmno | 25 | Chen *et al.* (2003) |
| Ct-1028 (TJ133) | Taiwan | *M. diplotricha* | II/F[e] | CT10 | 1.1 (1.9) | op | 50 | Chen *et al.* (2003) |
| Ct-1030 (TJ37) | Taiwan | *M. pudica* | I[e] | CT11 | 16.8 (5.0) | fghijklm | 20 | Chen *et al.* (2003) |
| Ct-1032 (TJ138) | Taiwan | *M. pudica* | I/A[e] | CT12 | 3.9 (4.2) | lmnop | 44 | Chen *et al.* (2003) |
| Ct-1038 (TJ151) | Taiwan | *M. pudica* | I/A[e] | CT13 | 7.0 (3.5) | jklmnop | 41 | Chen *et al.* (2003) |
| Ct-3711 | Guinea | *M. pudica* | HEI | CT14 | 19.4 (3.9) | fghijkl | 18 | This study |
| Ct-3718 | Guinea | *M. pudica* | HEI | CT15 | 10 (14.4) | klmnop | 32 | This study |
| Ct-6018 | Fr. Guiana | *M. pudica* | EGI | CT1 | 3.3 (5.8) | nop | 46 | Mishra *et al.* (2012) |
| Ct-6041 | Fr. Guiana | *M. pudica* | EGI | CT2 | 0.0 (0.0) | p | 52 | Mishra *et al.* (2012) |
| Ct-6123 | N. Caledonia | *M. pudica* | HEG | CT16 | 7.2 (6.6) | jklmnop | 39 | Klonowska *et al.* (2012) |
| *Rhizobium etli* (bv. *mimosae*) | | | | | | | | |
| Re-1020 (TJ169) | Taiwan | *M. diplotricha* | V/T[e] | RH1 | 7.1 (10.1) | klmnop | 40 | Chen *et al.* (2003) |
| Re-6822 (Mim7-4) | Mexico | *M. affinis* | | RH2 | 0.0 (0.0) | p | 53 | Wang *et al.* (1999) |
| *Rhizobium mesoamericanum* | | | | | | | | |
| Rm-3625 | Fr. Guiana | *M. pudica* | BCC | RH3 | 1.1 (1.9) | op | 51 | Mishra *et al.* (2012) |
| Rm-3629 | Fr. Guiana | *M. pudica* | BCC | RH4 | 11.3 (11.4) | ghijklmn | 28 | Mishra *et al.* (2012) |
| *Rhizobium tropici* | | | | | | | | |
| Rt-1022 (TJ172) | Taiwan | *M. diplotricha* | VII/W[e] | RH5 | 0.0 (0.0) p | 54 | 54 | Chen *et al.* (2003) |
| *Rhizobium sp. 1* | | | | | | | | |
| Rsp1-3696 | Guinea | *M. pudica* | FCD | RH6 | 8.3 (2.1) | ijklmno | 37 | This study |
| Rsp1-3716 | Guinea | *M. pudica* | FCD | RH6 | 13.6 (13.6) | ghijklmn | 23 | This study |
| Rsp1-3719 | Guinea | *M. pudica* | FCD | RH6 | 10.0 (3.3) | hijklmno | 33 | This study |
| Rsp-1–3730 | Guinea | *M. pudica* | FCD | RH6 | 7.9 (5.0) | jklmno | 38 | This study |
| Rsp1-3735 | Guinea | *M. pudica* | FCD | RH6 | 11.6 (6.7) | hijklmn | 27 | This study |
| Rsp1-3945 | Guinea | *M. pudica* | FCD | RH7 | 3.2 (0.1) | lmnop | 47 | This study |

**a**. Strain coding: the first letters are a combination of the first letter of the bacterial genus and species, followed by the STM collection number of the strain; other names are indicated between parenthesis.
**b**. 16S rRNA ribotype according to Mishra and colleagues (2012) or produced in this study.
**c**. rep-PCR coding used according to Mishra and colleagues (2012).
**d**. The original host plant for STM815 is published as *Machaerium lunatum* (Moulin *et al.*, 2001; Vandamme *et al.*, 2002), but the strain has never been proved to nodulate this host. On the other hand, it nodulates many *Mimosa* spp. efficiently (Elliott *et al.*, 2007b; dos Reis *et al.*, 2010), and the *B. phymatum* species is frequently found as a *M. pudica* symbiont in French Guiana (Mishra *et al.*, 2012).
**e**. The alphabetical code corresponds to PFGE fingerprints from the Chen and colleagues (2003) study.
**f**. Arcsinus √p-transformed values of the proportions (p) were used for mean comparisons; LSD (5%) = 0.27; mean values followed by identical letters are not significantly different ($P > 0.05$). Standard deviations for mean values of % of nodule occupancy are given within parenthesis.
**g**. Rank indicates the competitiveness rank of strains among the whole collection.

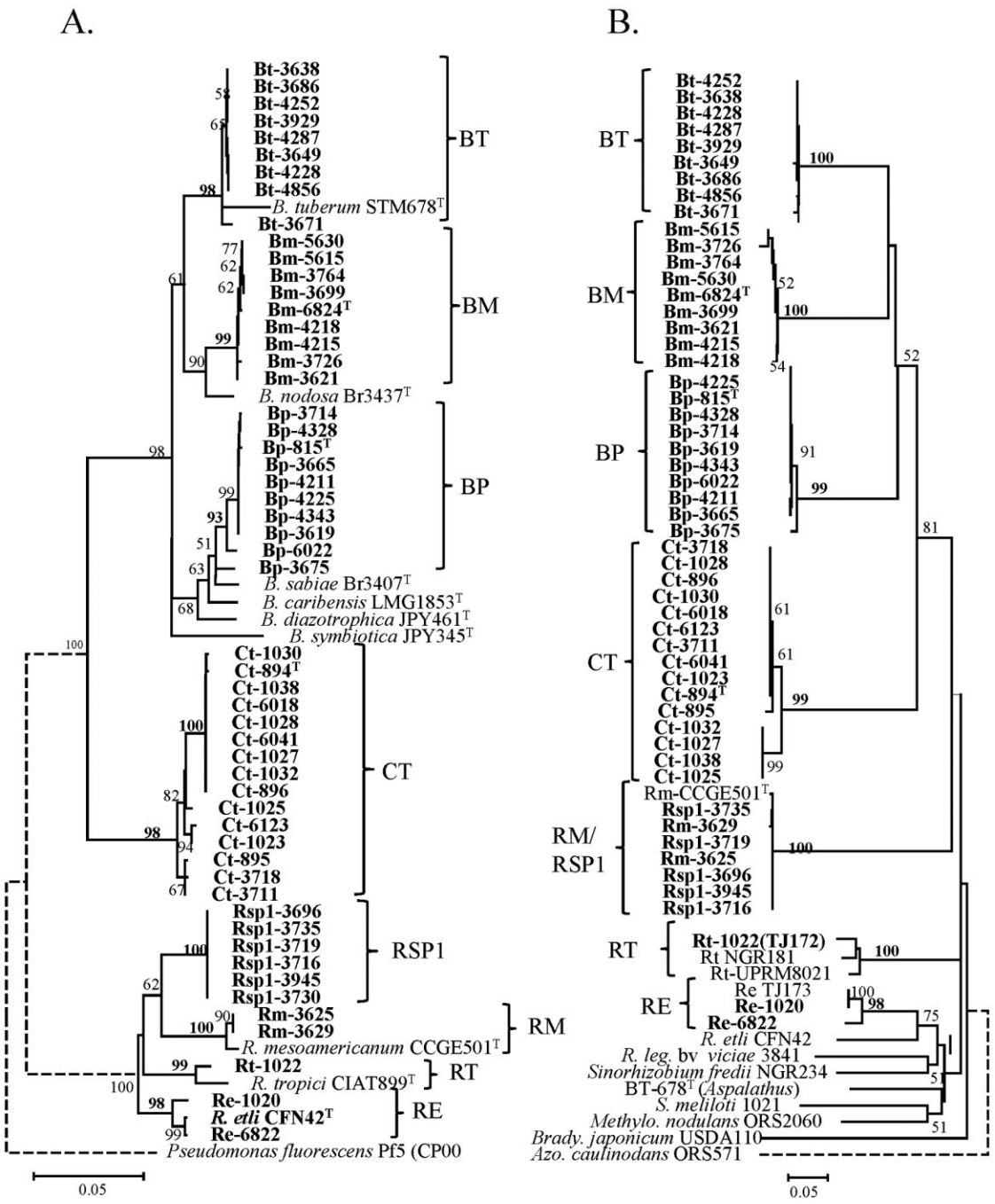


**Fig. 1.** Phylogenies of a 16S rDNA+*recA* combined data set (A) and the *nodC* symbiosis-related gene (B). Phylogenies were built by ML following a GTR + I + G model. The number at nodes indicates % of bootstrap (1000 replicates). Strains used in this study are in bold. GenBank accession numbers are given in Supplementary Table S1. Scale bars indicate number of substitutions per site. Broken lines indicate that the branch length is not drawn to scale. T, type strain of the species; Azo., *Azorhizobium*; BM, *B. mimosarum*; BP, *B. phymatum;* Brady, *Bradyrhizobium*; BT, *B. tuberum*; CT, *C. taiwanensis*; Methylo, *Methylobacterium*; R. leg.; *R. leguminosarum*; RE, *R. etli*; RM, *R. mesoamericanum*; RSP1, *Rhizobium* sp. 1; RT, *R. tropici*.

direct pairwise competition experiments by co-inoculation of wild-type strains. Rankings of strains were conserved between the two methods (Table 3), which validated our high throughput indirect method to assess strain competitiveness.

### *Effect of* M. pudica *genotype on nodulation competitiveness*

Based on morphology and random amplified polymorphic DNA (RAPD) analyses, commercial seeds of *M. pudica*

**Table 2.** Reproducibility of the method used to estimate the competitiveness of strains for nodulation of *M. pudica* (commercial seeds) based upon competition against a common competitor (strain STM815-GFP).

| | % of nodule occupancy in experiments[a]: | | |
|---|---|---|---|
| Strain | I (40 DAI) | II (55 DAI) | III (40 DAI) |
| Bp-815 | 93.6 (3.3) | 91.5 (8.1) | 84.4 (8.3) |
| Bp-3619 | 95.8 (1.9) | 62.3 (5.8) | nd |
| Bp-3714 | nd | 63.3 (12.3) | 75.3 (13.0) |
| Bp-6022 | nd | 49.7 (7.8) | 83.0 (8.1) |
| Bt-3649 | 55.2 (17.7) | 27.0 (24.0) | nd |
| Bm-3621 | 58.7 (13.5) | 10.3 (5.0) | nd |
| Bm-6824 | 50.0 (26.7) | 17.7 (12.0) | nd |
| Ct-3711 | nd | 19.4 (3.9) | 13.6 (3.4) |
| Ct-6018 | 16.6 (10.7) | 3.3 (5.8) | nd |
| Rm-3625 | 37.9 (9.9) | 1.1 (1.9) | nd |

**a**. Standard deviations for mean values of % of nodule occupancy are given within parentheses.
Data are from three independent experiments.
DAI, days after inoculation. nd, not determined.

were shown to consist of a mixture of two varieties, var. *hispida* and var. *unijuga* (approximately 40% and 60%, respectively). We sampled and identified other varieties from the field: var. *tetrandra* and an unidentified variety in French Guiana, and var. *unijuga* in Taiwan (supplementary Fig. S4). These seeds were used in another competition experiment (using the STM815-GFP method) in order to explore the effect of host genotype on competition for nodulation. Five strains of each *B. phymatum* and *C. taiwanensis* species were tested. All the plants were inoculated with the same batches of mixed inoculants (i.e. STM815-GFP + the strain tested). The range of nodule occupancy (i.e. 43–94%) remained similar for the *B. phymatum* strains whatever host plant variety was used (Fig. 3). In contrast, an interaction between the *C. taiwanensis* species and the host variety was found to be significant. As in the experiment with commercial seeds, all the *C. taiwanensis* strains were poorly competitive against strain STM815-GFP (3–36% of nodule occupancy) using the *M. pudica* varieties collected in French Guiana. In contrast, however, these same strains were on average more competitive than STM815-GFP on *M. pudica* var. *unijuga* collected in Taiwan, with a range of nodule occupancy (62–95%) similar to that of *B. phymatum* strains, whatever the geographical origin of the *C. taiwanensis* strains used (Taiwan, New Caledonia, French Guiana or Guinea).

**Table 3.** Comparison of two methods to estimate the competitiveness of strains for nodulation of *M. pudica* (commercial seeds): pairwise co-inoculation and ranking based upon competition against a common competitor, strain STM815-GFP.

| | | Pairwise comparison | |
|---|---|---|---|
| Strain A *versus* strain B | | (% of nodule occupancy by strain A)[a] | Indirect comparison[b] |
| Bp-3619 | Bt-3649 | 92*** | >*** |
| Bp-3619 | Bm-3621 | 100*** | >*** |
| Bt-3649 | Bt-3686 | 90*** | >** |
| Bt-3649 | Bm-3621 | 48 | NS |
| Bt-3686 | Bm-3699 | 53 | NS |
| Bt-3686 | Rsp1-3696 | 38 | NS |
| Bm-3621 | Bm-3699 | 41 | NS |
| Bm-3621 | Rm-3625 | 78*** | >* |
| Bm-3699 | Rsp1-3696 | 50 | NS |
| Ct-6018 | Rm-3625 | 75*** | NS |
| Ct-3711 | Bp-3714 | 3*** | <*** |

**a**. % significantly different from 50% are followed by asterisks.
**b**. Strain A showed a higher (>) or a lower (<) rank than strain B based upon competition experiments against strain STM815-GFP (% of nodules are given in Table 1).
*, **, *** significant at $P \leq 0.06$, $P \leq 0.05$ and $P \leq 0.01$, respectively.
NS, non-significant difference ($P > 0.06$).

## Discussion

### *Beta-rhizobial species are either more or equally competitive compared with alpha-rhizobia on* M. pudica *but vary widely*

In this study, we measured the nodulation competitiveness of symbiotic strains belonging to different species of α- and β-rhizobia. All studies published so far that have trapped symbiotic bacteria from *Mimosa* species worldwide have always recovered a strong majority of β-rhizobial isolates. In our study, using *M. pudica* as the host plant, the β-rhizobia strains were systematically either better (*B. tuberum*, *B. phymatum*) or equally competitive (*C. taiwanensis*, *B. mimosarum*) than α-rhizobia.

In a previous work, Elliott and colleagues (2009) showed that *B. mimosarum* PAS44 out-competed *C. taiwanensis* LMG19424 and three *Rhizobium* strains (*R. etli* TJ167, *R. tropici* NGR181 and UPRM8021) on

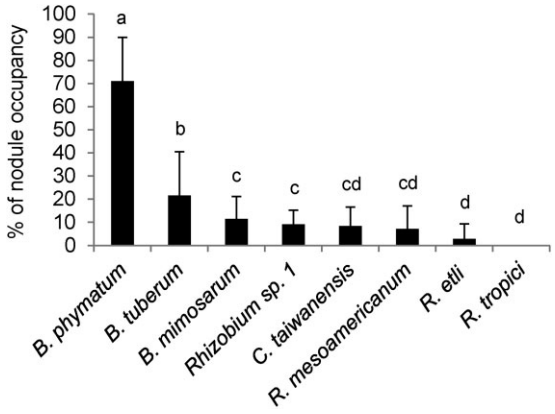


**Fig. 2.** Success in forming nodules on *M. pudica* (commercial seeds) according to the rhizobial species based upon competition experiments against strain STM815-GFP. Arcsinus √p-transformed values of the proportions were used for mean comparisons; identical letters indicate that the mean values are not significantly different ($P > 0.05$). Bars show standard deviations.

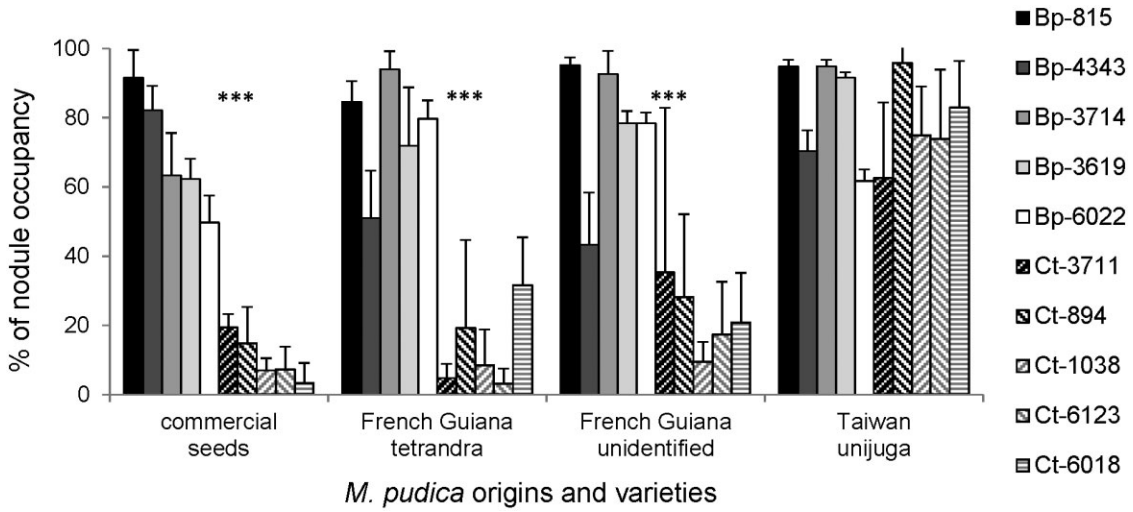


**Fig. 3.** Effect of the host plant variety and/or its origin on the success in forming nodules by β-rhizobial strains in competition against strain STM815-GFP. The three asterisks mean that the species effect for *B. phymatum* (Bp) and *C. taiwanensis* (Ct) is highly significant ($P \leq 0.0001$). Bars show standard deviations.

*M. pudica*, *M. pigra* and *M. diplotricha* hosts. We considerably enlarged this first result by including more than 50 strains using a robust and validated approach to estimate relative competitiveness among isolates. We observed a high affinity of *M. pudica* for β-rhizobial strains, whatever the genus, geographical origin and species of the strain. This result, together with previous studies, suggests that the genus *Mimosa* evolved towards a preferential association with *Burkholderia* and *Cupriavidus* symbionts before its species diversification in South America, although it should be noted that some species still retained an ability to nodulate with α-rhizobia. Although the genetical basis of this preferential association remains to be studied, their nodulation competitiveness mostly explains the high frequency of β-rhizobia in all previous diversity studies. On the other hand, the affinity of *Mimosa* towards β-rhizobia appears to be restricted to South American native species, as in other centres of endemism (such as Mexico and India), β-rhizobia are not the symbionts of endemic *Mimosa* species (Wang *et al*., 1999; Gehlot *et al*., 2013). It would thus be very interesting to test the competitiveness of a subset of strains on *Mimosa* species from the different centres of endemism to determine if there has been co-adaptation between each partner.

At a genus level, the supremacy of some *Burkholderia* species over *Cupriavidus* that we observed in our first set of experiments does not strictly reflect the situation in the field. Indeed, in Taiwan, *Cupriavidus* appears to be the main partner of *M. pudica*, even though *B. mimosarum* is present in the soil (this latter being more frequently isolated from another invasive species, *M. pigra*; Chen *et al*., 2005a,b). We thus explored in a second set of experiments the possibility that *M. pudica* genotypes had divergent symbiotic specificities and that these might explain variations in the ratio of bacterial genera recovered from this species.

### *Competitiveness of* Cupriavidus *on* M. pudica *is dependent on the host genotype and the origin of the seeds*

In *M. pudica*, several varieties have been described so far, but their worldwide distribution is unclear because of their natural invasive status and their use as ornamentals and in pastures (Barneby, 1991). Our tests showed that nodule occupancy of *C. taiwanensis* is significantly higher when estimated on *M. pudica* var. *unijuga* than on all the other *M. pudica* varieties tested. *Burkholderia phymatum* remained as competitive whatever the variety or the geographical origin of the seeds.

The *M. pudica* var. *unijuga* displayed a higher affinity for all the *C. taiwanensis* strains tested, whatever their geographical origin (Guinea, Taiwan, New Caledonia or French Guiana). Seeds used in this experiment were sampled from Taiwan, in the same sites where *C. taiwanensis* dominates in nodules of *M. pudica* (Chen *et al.,* 2003). The higher affinity between *M. pudica* var. *unijuga* and *Cupriavidus* is specific to this Taiwan accession, as the mix of varieties in the commercial seeds, which contains approximately 60% of var. *unijuga*, does not reflect any particular affinity in competition experiments. The high affinity for all strains, whatever their geographical origin, eliminates any hypothetical local co-adaptation between the plants and the symbiotic isolates in Taiwan. On the other hand, we may hypothesize

that Taiwanese accessions of *M. pudica* var. *unijuga* have evolved towards a higher affinity with all the *C. taiwanensis* isolates, involving a molecular basis shared by all isolates of this species. Tests using other seed accessions, from other geographical sites, should be done to confirm the specific mutation in Taiwanese accessions.

In 2009, Elliott *et al*. showed that different *Mimosa* species may result in varying competitiveness among rhizobial strains. Here, we show that it is not only the host species but also the variety of *M. pudica* and even the origin of the seeds that might impact drastically on the results of nodulation competitiveness tests.

A diversity of specificity and competitiveness among isolates between lines and varieties of the same plant species has been previously documented for other symbiotic partnerships (Martinez-Romero, 2009). A possible scenario of co-evolution might explain the β-rhizobial affinities towards *M. pudica*. As *B. phymatum* (as other *Mimoseae*-nodulating burkholderias) is most likely to be an ancient symbiont (Bontemps *et al*., 2010; Bournaud *et al*., 2013), its observed high competitiveness, whatever the genotypes used, could result from a long-term co-evolution with *M. pudica*, starting before its varietal diversification and worldwide dispersion. Conversely, *C. taiwanensis* is described as a recent symbiont (Amadou *et al*., 2008; Mishra *et al*., 2012), that might have co-evolved with specific varieties, thus explaining our results. As it is reported that *M. pudica* arrived in Taiwan from the America in the 17th century (Wu *et al*., 2003), the co-adaptation between *C. taiwanensis* and *M. pudica* var. *unijuga* might have occurred far earlier in another location. A survey of *M. pudica* accessions in America and Asia coupled with competition experiments is required to answer this question.

Finally, we cannot ruled out the hypothesis that the GFP tag in strain STM815-GFP is affecting a trait that is critically important for nodulation competitiveness in the *M. pudica* var. *unijuga*, making *C. taiwanensis* more competitive on this particular genotype. However, we consider this hypothesis as unlikely because the probability that the Tn5 insertion had fallen in a region involved in a variety-specificity region is very low, and that this would only concern the Taiwanese ecotype because the commercial seeds are composed of 60% of var. *unijuga* and the *C. taiwanensis* strains remain very low in competitiveness on these seeds.

### *Competition for nodulation on* M. pudica *is also variable among* Burkholderia *species*

Our first set of experiments showed that *B. phymatum* is by far and systematically the most competitive species on commercially produced *M. pudica* seeds. Elliott and colleagues (2009) suggested the same dominance of *B. phymatum* over *B. mimosarum* on *M. pudica* based on single isolates. Conversely, Liu and colleagues (2012), sampling *M. pudica* nodules from various sites in China, recovered more than 100 *Burkholderia* isolates, among which 95%, based on rDNA 16S sequence, were assigned to *B. mimosarum* and only 5% to *B. phymatum*. However, in their study, the *nodA* sequences of all these strains were similar and closely related to the one obtained from the *B. phymatum* type strain STM815. The high competitiveness of these Chinese *B. mimosarum* strains might thus be related to the lateral transfer of symbiotic genes from *B. phymatum*. Such horizontal gene transfer (HGT) seems uncommon because several authors previously described the vertical transfer of *nod* genes in *Mimosa*-associated β-rhizobial species (Bontemps *et al*., 2010; Mishra *et al*., 2012). Despite the inclusion of 28 *Burkholderia* strains in our data set, we similarly did not detect any evidence of HGT.

This trend of a higher competitiveness of *B. phymatum* on *M. pudica* should also be confirmed outside laboratory conditions. As suggested in Elliott and colleagues (2009), and Klonowska and colleagues (2012), growing conditions and abiotic parameters (such as pH or nitrogen content), in addition to host plant genetic diversity, might also explain why the prevalence of *B. phymatum* is not always high in the various studies that sampled from different geographical areas.

### *Comparison of symbiotic traits with biogeographical patterns of* M. pudica *symbionts*

Several studies have described contrasting diversity patterns of *M. pudica* symbionts, with for example *C. taiwanensis* dominating in Taiwan and the Philippines (Chen *et al*., 2003; Andrus *et al*., 2012), while *B. phymatum* and *B. tuberum* dominate in French Guiana (Mishra *et al*., 2012). A comparison of the competitive abilities of each species and diversity studies cannot show a strict agreement because every bacterial species is not present in every soil. For instance, *B. phymatum*, the best competitor in our analyses, has never been found in Taiwan in *M. pudica* nodules, neither in New Caledonia, Costa Rica nor Panama, nor even in Brazil on 47 species of *Mimosa* (Barrett and Parker, 2005; 2006; Bontemps *et al*., 2010; Klonowska *et al*., 2012; Mishra *et al*., 2012). When *B. phymatum* is present (as in soils of French Guiana), trapping experiments showed a dominance of this species over others. When it is not detected (and thus could be interpreted as absent or in very low proportion in soils), *B. tuberum* dominates (if it is present), in agreement with our competition results (Mishra *et al*., 2012). When both *B. tuberum* and *B. phymatum* are absent, as in Taiwan and the Philippines (Chen *et al*., 2003; 2005a;

Andrus *et al*., 2012), *C. taiwanensis*, *B. mimosarum* and α-rhizobial species are detected. This is what happens on a certain scale, as *C. taiwanensis* dominates in *M. pudica* nodules in Taiwan. *Burkholderia mimosarum* is present in Taiwan, but it is only found in *M. pigra* nodules (Chen *et al*., 2005a) and is absent in Taiwanese *M. pudica* (Chen *et al*., 2003) despite their host compatibility. These discrepancies might be linked to the symbionts affinity to particular plant genotypes or species but also to environmental factors, such as soil pH, nitrogen content or altitude, as observed in previous studies (Elliott *et al*., 2009; Bontemps *et al*., 2010; Mishra *et al*., 2012).

It is difficult to correlate strictly the areas of distribution of *M. pudica* varieties and the diversity patterns of their symbionts both because the exact distribution of *M. pudica* varieties is poorly described, apart from the broad distribution of all varieties in America and Asia observed today (Barneby, 1991), but also because the distribution of bacterial species is always extremely difficult to assess. A large sampling study of both *M. pudica* varieties and their associated symbionts is required to investigate this question. In particular, it would be interesting to assess the competitiveness of *Burkholderia* and *Cupriavidus* strains described in the Liu and colleagues (2012) study to further investigate variations in β-rhizobial symbiotic traits.

### *Conclusion*

Notwithstanding the possibility of a high competitiveness of α-rhizobia with various *M. pudica* accessions not tested here or with other *Mimosa* species, the better adaptation of *Burkholderia* species with *Mimosa* shown in this study reflects their greater overall frequency of isolation from natural *Mimosa* populations, certainly in relation to the long-term co-adaptation between the two partners (50 My, Bontemps *et al*., 2010; Bournaud *et al.,* 2013). *Cupriavidus*, for which environmental conditions might play a major role in their frequency in soils relative to other symbiotic bacteria, appears only to be well adapted to a few *Mimosa* species or varieties, reflecting a younger association that has probably arisen following HGT of nodulation genes from *Burkholderia* species (Bontemps *et al*., 2010; da Silva *et al*., 2012; Mishra *et al*., 2012).

## Experimental procedures

### *Bacterial strains and culture conditions*

The bacterial strains used in this study are listed in Table 1. Bacteria were grown at 28°C for 10 h in yeast mannitol (YM) broth or for 48 h on YM agar (Vincent, 1970). For long-term maintenance, strains were grown at 28°C in YM broth and preserved in 20% glycerol at −80°C.

### *Molecular methods*

Bacterial DNA extraction was carried out as previously described (Mishra *et al*., 2012). Various DNA fingerprinting methods for characterization of bacteria were used: restriction fragment length polymorphism (RFLP), analysis of the PCR-amplified 16S rRNA gene (ARDRA) and of the intergenic transcript spacer region between 16S and 23S rDNAs (rDNA ITS), and PCR fingerprinting of REPs. Procedures for ARDRA and REP-PCR fingerprinting were described in Mishra and colleagues (2012). PCR amplification of the variable rDNA ITS (300–1000 bp) was carried out using primers BR5 (CTTGTAGCTCAGTTGGTTAG; Willems *et al*., 2001) and FGPL132'-38 (CCGGGTTTCCCCATTCGG; Ponsonnet and Nesme, 1994). DNA templates were obtained by cellular burst (seven cycles of 94°C, 30 s, 4°C, 30 s). The PCR products were digested by *Cfo* I (Promega Corporation, Madison, WI, USA) in a 20 µl reaction volume according to the manufacturer's instructions and were analysed by agarose gel electrophoresis.

PCR amplification, sequencing and phylogenetic analyses of nearly full-length 16S rRNA genes, 800 bp *recA* fragments, 440 bp *nifH* fragments and 800 bp *nodC* fragments were carried out as described in Mishra and colleagues (2012).

Total genomic DNA was isolated from new leaves of *M. pudica* plants using the cetyltrimethylammonium bromide extraction method. RAPD fingerprints were obtained using primers OPAB7 (GTAAACCGCC) and GO3 (GAGCCCTCCA) designed by Operon Technologies (Alameda, CA, USA) using the following thermal cycling protocol: 4 min at 95°C; 40 cycles of 95°C for 1 min, 35°C for 1 min, and 72°C for 2 min. RAPD products were gel-electrophoresed in 1% agarose in TAE 1X buffer.

### *Plant material and growth conditions*

Various varieties of *M. pudica* were used in this study. Seeds of commercially-grown *M. pudica* were obtained from B&T World Seeds (Aigues-Vives, France). Seeds of *M. pudica* were collected in French Guiana, in Taiwan, and in Timor and Brazil. Plant varieties were identified following Barneby's classification (1991) and the results were correlated with RAPD fingerprinting (supplementary information Fig. S4). The commercial seeds were shown to consist of a mixture of *M. pudica* var. *hispida* (≈40%) and var. *unijuga* (≈60%) after the genotyping of 30 individual plants. One batch of seeds from French Guiana was identified as *M. pudica* var. *tetrandra*, and the other batch could not be identified but showed distinct features from the other seed batches. The seeds from Taiwan were identified as *M. pudica* var. *unijuga*.

Seedlings were prepared as previously described (Mishra *et al*., 2012). The seedlings were transferred to pots filled with sterilized attapulgite substrate (Oil dry US-special Type III R, Damolin Etrechy S.A., Etrechy, France), supplied with an N-free nutrient solution (Broughton and Dilworth, 1971), and were inoculated with bacterial cell suspensions from cultures in exponential growth phase ($10^7$ per plant) seven days after germination. Randomized complete block designs were used. The pots were placed in sterile transparent bags (pore size 0.02 µm) to maintain humidity and to avoid cross-contamination (Sun bag, Sigma-Aldrich Chemie, Saint-

Quentin Fallavier, France). Growth conditions were used according to each experiment, as described in the following sections.

### *Competition studies*

The plants were grown in 1 l plastic pots (three replicates, four plants per pot) filled with a sterilized mixture (1:1 v : v) of attapulgite and sand in a growth chamber (day/night temperature of 27°C, 60 μE $m^{-2}$ $s^{-1}$, day/night photoperiod of 16/24 h). Seedlings were inoculated with a mixture (50:50 cell ratio) of each strain and a GFP-tagged common competitor strain, *B. phymatum* STM815-GFP (Elliott *et al*., 2007b). Direct cell counting prior to inoculant preparation was carried out by light microscopy using cell counter slides (KOVA® Glasstic® Slide) according to the manufacturer's instructions (Hycor BIOMEDICAL, Inc., Garden Grove, California, USA). Nodules were collected 40–55 DAI depending on the experiment. Rhizobial strains were isolated from crushed nodules (32 per pot; 96 per treatment) as described previously (Mishra *et al*., 2012). Morphological traits and colony fluorescence were used for strain identification.

Plants grown in a closed system (Magenta Vessel GA-7, Sigma-Aldrich Chemie) filled with a sterilized mixture of sand and vermiculite (1:1, v : v) were also used to carry out pairwise competition experiments by co-inoculation of wild-type strains. Nodules were collected at 33 DAI, and rhizobial strains were isolated from crushed nodules (eight nodules per plant, four replicates). The strains were identified by RFLP of rDNA ITS.

### *Phylogenetic and sequence analyses*

Nucleotide sequences from 16S rDNA, *recA*, *nodC* and *nifH* genes were corrected with CHROMAS PRO v1.33 (Technelysium Pty Ltd, Brisbane, Australia), aligned using Muscle3.6 (Edgar, 2004), and alignments were manually curated with GENEDOC (Nicholas and Nicholas, 1997). Phylogenetic trees were constructed by distance and ML methods using MEGA5 (Tamura *et al*., 2011). Distance matrices were corrected used the Kimura-2 parameter method, and trees were built by neighbour joining. ML parameters followed a GTR + I + G model by estimating all parameters under MEGA5. Bootstrapping analyses were conducted on MEGA5.

### *Statistical analyses*

Data analyses by ANOVAs and correlation were performed using XLSTAT software v2011.1.03 (Addinsoft™, Paris, France). Means were classified using a *post hoc* least-significant difference (LSD) Fisher test. For comparison of proportions (p), arcsinus √p-transformed values were used. *Z* tests for one proportion were used to test whether the % of nodule occupancy differed from 50%.

### *Nucleotide sequence accession numbers*

The nucleotide sequences have been deposited in EMBL database under accession numbers HE864332 to HE864414 and are listed by gene for each strain in supplementary material Table S1. Sequence alignments are available upon request.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** *nifH* gene phylogeny of the collection. The tree was built by neighbour-joining from a distance matrix corrected by the Kimura-2 parameter method, and the robustness of nodes was assessed with 1000 bootstrap replicates.

**Fig. S2.** Micrographs under epifluorescence macroscopy of the root system of *Mimosa pudica* infected by a dual inoculation of fluorescent (STM815 gfp-reference strain) and non-fluorescent rhizobia, leading to fluorescent and non-fluorescent nodules. A, C and B, D are the same pictures under either gfp or cherry filter excitation/visualization. Scale bar is 1 mm (A, C) or 5 mm (B, D). White arrows indicates non-fluorescent nodules.

**Fig. S3.** Success in forming nodules on *M. pudica* (commercial seeds) according to the rhizobial strains based on competition experiments against strain STM815-GFP. Bars show standard deviations.

**Fig. S4.** RAPD fingerprints of *Mimosa pudica* varieties with the rep-PCR primers OPAB7 (A) or G03 (B). Lane 1, 2, 3, 4: *M. pudica* plants from the commercial seeds (source from B&T World Seeds). Lane 1: var. *hispida*, Lane 2–4: var. *unijuga*; Lane 5–6: French Guianan *M. pudica* var. *tetrandra*; Lane 7–8: Taiwanese *M. pudica* var. *unijuga*; Line 9: Smart ladder (200 bp per band); Lane 10–11: *M. pudica* var. *hispida* from Timor; Lane 12–13: *M. pudica* var *hispida* from Brazil; Lane 14: *M. pudica* unidentified variety from French Guiana. The order on the gel is the same for both the OPAB7 and G03 primers. Letters at the bottom of the gels indicate the plant varieties: H, *hispida*; U, *unijuga*; T, *tetrandra*.

**Table S1.** Nucleotide sequence accession numbers.