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Monophyly of *nodA* and *nifH* Genes across Texan and Costa Rican Populations of *Cupriavidus* Nodule Symbionts[▽]

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***nodA* and *nifH* phylogenies for *Cupriavidus* nodule bacteria from native legumes in Texas and Costa Rica grouped all strains into a single clade nested among neotropical *Burkholderia* strains. Thus, *Cupriavidus* symbiotic genes were not acquired independently in different regions and are derived from other *Betaproteobacteria* rather than from α-rhizobial donors.**

Some lineages of *Betaproteobacteria* appear to have acquired the capacity for symbiotic interaction with legume plants by lateral transfer of symbiosis-related genes from rhizobia in the *Alphaproteobacteria* (4, 9). However, it is uncertain how many lateral gene transfer events have occurred to establish symbiotic lineages of β-rhizobia. *nodA* phylogenies for *Burkholderia* indicate that strains from South Africa and South America cluster with two distinct lineages of *Alphaproteobacteria* (4), implying an independent acquisition of symbiotic genes in the two locations. Less information is available for legume symbionts belonging to the genus *Cupriavidus*, which have so far been found within the native range of their *Mimosa* host species only in Costa Rica (3), although they also occur on *Mimosa* species introduced into Taiwan (4, 6) and India (12). *nod* genes of Taiwanese *Cupriavidus* strains proved to be highly similar to those of a Taiwanese *Burkholderia* strain (4, 6). Because these sequences nested within a clade of several other nodule-forming *Burkholderia* strains, it appeared that these *Cupriavidus nodA* genes were acquired from a *Burkholderia* strain rather than directly from an alphaproteobacterial ancestor (4, 6). However, because only three strains have been studied, from a symbiont population that is not known to be native to the region, it is important to analyze whether the same conclusions hold for indigenous *Cupriavidus* populations associated with *Mimosa* legumes in their native geographic ranges.

In this study, we analyzed nodule bacteria associated with the legume *Mimosa asperata* in south Texas. *M. asperata* was formerly considered to be a variety of the widely distributed invasive weed *Mimosa pigra* (*M. pigra* var. *berlandieri*) (11) but is now recognized as a distinct species. The range of *M. asperata* is centered in Mexico and extends slightly into south Texas, Cuba, and northern Central America (1). Central American populations of *M. pigra* associate almost exclusively with strains of *Burkholderia*, although *Cupriavidus* is also present at a low frequency in Costa Rica (2, 3). Thus, it is of interest to determine whether related legumes in nearby regions harbor *Cupriavidus* strains. We also obtained a small number of nodule bacterial strains from a second *Mimosa* species native to Texas (*Mimosa strigillosa*) that is classified as a close relative of *M. asperata* and *M. pigra* (1).

Eighty-one root nodules were collected from multiple plants of *M. asperata* at two sites 1 km apart within Santa Ana National Wildlife Refuge (Hidalgo County) in the lower Rio Grande River Valley, Texas, and one bacterial isolate per nodule was obtained as described previously (10). Eleven nodule isolates were also obtained from plants of *M. strigillosa* at two sites in the lower Rio Grande Valley. DNA purification and PCR experiments used standard protocols (3). BLAST searches on a 390-bp 5′ portion of the 23S rRNA gene sequenced in 14 Texan *M. asperata* isolates (GenBank accession no. DQ533663 to DQ533681) indicated that all had high affinities to various strains of *Cupriavidus* legume nodule symbionts. Eight distinct genotypes were observed among the 14 isolates, and all proved to be similar or identical (0 to 4 substitutions) to Costa Rican *Cupriavidus* legume nodule symbionts analyzed in a prior study (3).

None of the 11 nodule isolates obtained from *M. strigillosa* yielded a 5′ 23S rRNA PCR product of the same size as the *Cupriavidus* strains. Three of these PCR products were sequenced, and they proved to be similar either to *Sinorhizobium arboris* USDA 4878 (AY244381; 95 to 96% similarity) or to *Sinorhizobium saheli* USDA 4893 (AY244368; 95% similarity).

To screen the remaining 67 *M. asperata* isolates, PCR assays were performed with two sets of lineage-specific primers. Primers ralf2 and ralr2 have been shown to differentially amplify a 187-bp portion of the 23S rRNA gene in nodule-forming *Cupriavidus* (3). All 67 isolates exhibited an amplification product with these primers. A second pair of primers (ral16f and ral16r; Table 1) targeted a portion of the 16S rRNA gene characteristic of *Cupriavidus* and related genera such as *Ralstonia*. All 67 isolates yielded an amplification product with these primers as well. Together, these PCR assays imply that these *M. asperata* nodule isolates are likely to be strains of *Cupriavidus*.

A 1,453-bp portion of the 16S rRNA gene was sequenced in seven *M. asperata* strains. (Two cloned PCR products were sequenced separately for one of the strains [AMP18] due to apparent sequence heterogeneity among rRNA operons.) The eight sequences were all >99% similar to one another, differing by 1 to 13 nucleotides (GenBank accession no. DQ530643 to DQ530650). The two cloned PCR products from strain

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TABLE 1. Oligonucleotide primers used in this study

Primer	5′→3′ nucleotide sequence	Position	Product size (bp)	Gene
ral2f	GCGAACTGAAACATCTAAGTAGC	123918[a]	187	5′ 23S rRNA
ral2r	TGTCGYACACCTAGTTCCACA	124084[a]		
Ral16f	CAACTAGTTGTTGGGGATTCATTT	122518[a]	238	16S rRNA
Ral16r	CCATGCAGCACCTGTGTCCACTT	122733[a]		
nodAcf	GATCTTGAACTCTCCGACCATTT	434[b]	532	*nodA*
nodAcr	GTTCGATTGTTTCGCCGCTTG	965[b]		
nhcf3	AAGTCGACTACCTCGCAGAACAC	8[c]	519	*nifH*
nhcr4	CCTTCGCTCGTTACAGATCAACC	526[c]		

[a] Position of the 5′ primer nucleotide relative to *Ralstonia solanacearum* GMI1000 (AL646064 and AL646059).
[b] Position of the 5′ primer nucleotide relative to *Cupriavidus taiwanensis* (AJ505311).
[c] Position of the 5′ primer nucleotide relative to *Cupriavidus taiwanensis* (AJ505320).

AMP18 differed by one nucleotide substitution and one 2-bp insertion/deletion polymorphism. Maximum likelihood analysis of aligned sequences showed that all of the Texan *Cupriavidus* strains formed a well-supported group (61 to 96% bootstrap values) together with other *Cupriavidus* nodule symbionts from Costa Rica and Taiwan (data not shown). One Texan strain proved to have a 16S rRNA sequence identical to that of a *Cupriavidus* strain sampled from *Mimosa pudica* in Costa

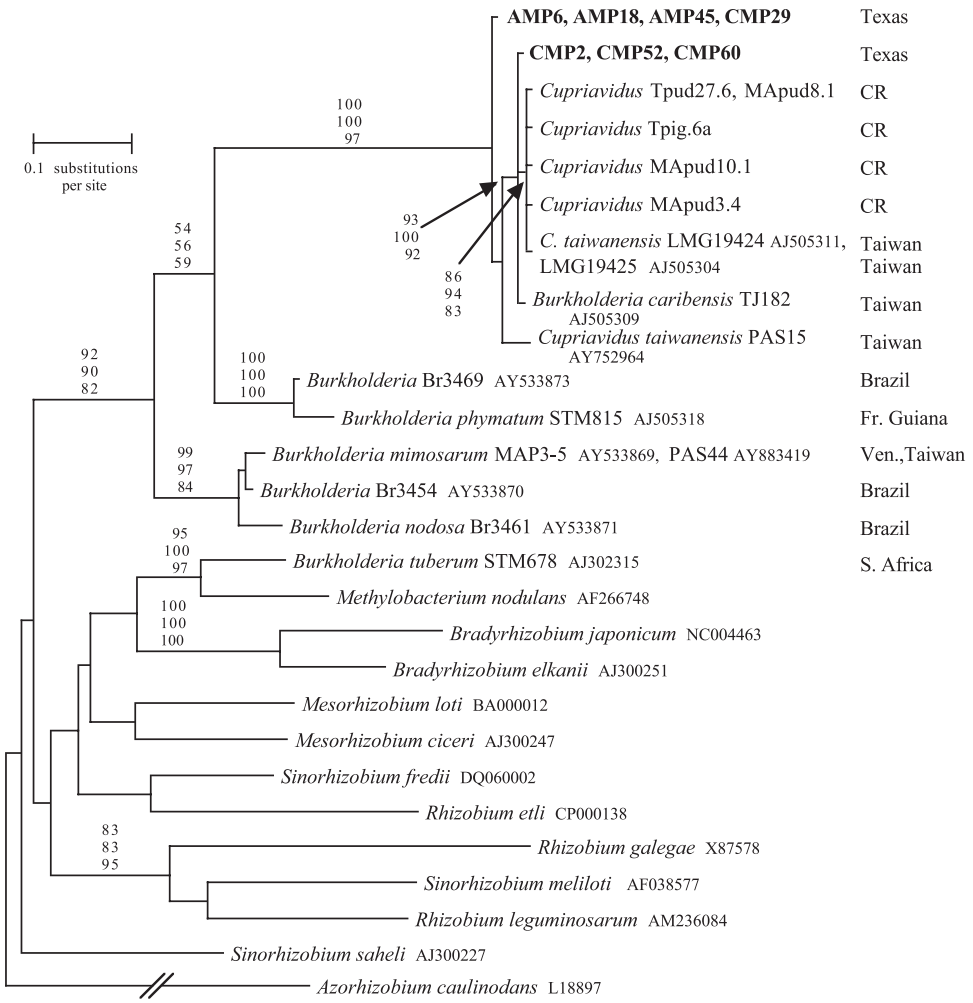

FIG. 1. Maximum likelihood phylogeny for *nodA* genes from Texan *Cupriavidus* strains (shown in bold) and other proteobacteria. The maximum likelihood model used a transition/transversion ratio estimated from the data, empirical base frequencies, and substitution rates estimated separately for each codon position. Numbers near branches are bootstrap percentages for parsimony (top), neighbor-joining (middle), and likelihood (bottom) analyses. For *Cupriavidus* and *Burkholderia* nodule bacteria, the place of origin is indicated (CR, Costa Rica; Fr. Guiana, French Guiana; Ven., Venezuela).

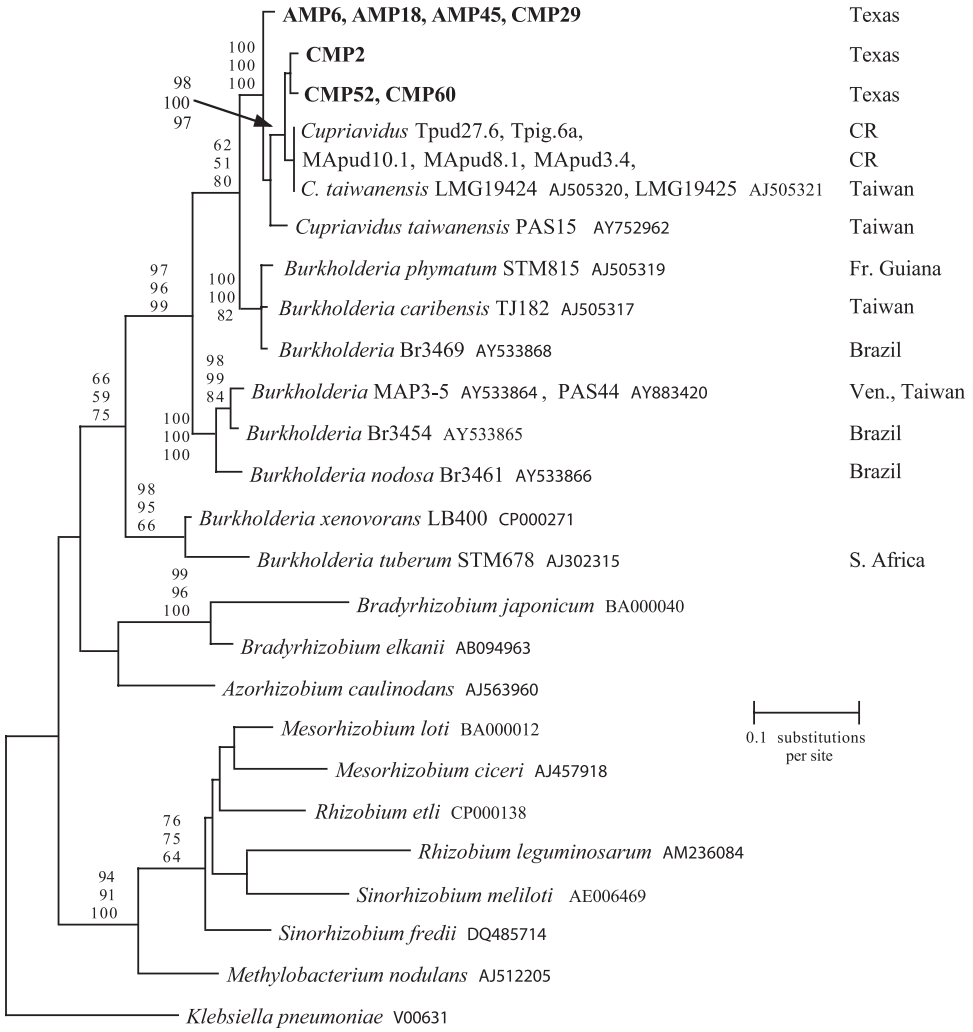

FIG. 2. Maximum likelihood phylogeny for *nifH* genes from Texan *Cupriavidus* strains (shown in bold) and other proteobacteria. Numbers near branches are bootstrap percentages for parsimony (top), neighbor-joining (middle), and likelihood (bottom) analyses. For *Cupriavidus* and *Burkholderia* nodule bacteria, the place of origin is indicated (CR, Costa Rica; Fr. Guiana, French Guiana; Ven., Venezuela).

Rica (3). The close relationship between *Cupriavidus* strains in sites 2,300 km apart in Texas and Costa Rica suggests that certain bacterial lineages may have spread across this region recently relative to the time scale of nucleotide substitution in this gene. Standard estimates of substitution rates in 16S rRNA genes (7) imply that the upper 95% confidence limit on divergence time for two strains that share an identical 1,453-bp portion of 16S rRNA ranges from 5.7 to 8.6 million years ago. This places an approximate upper bound on the time to common ancestry for Texan and Costa Rican strains.

A 488-bp portion of the *nodA* gene was amplified and sequenced in seven Texan and five Costa Rican *Cupriavidus* strains (chosen to represent all major lineages detected in a prior study) (3), using primers nodAcf and nodAcr (Table 1) designed from *Cupriavidus taiwanensis* sequences (4, 6). Four Texan strains (AMP6, AMP18, AMP45, and CMP29) shared one sequence, which differed by 15 nucleotides from a sequence shared by the other Texan strains (CMP2, CMP52, and CMP60). The five Costa Rican strains were highly similar, with *nodA* sequences that varied at 0 to 2 nucleotide sites. *C. taiwanensis* strains LMG19424 and LMG19425 differed by 1 or 2 nucleotides from the Costa Rican strains. Phylogenetic analysis of the *nodA* sequences indicated that all *Cupriavidus* nodule symbionts formed a highly supported group (97 to 100% bootstrap value) together with *Burkholderia caribensis* strain TJ182 (Fig. 1). Within this group, there was another strongly supported lineage comprised of *C. taiwanensis* strains LMG19424 and LMG19425, three Texan strains, and the five Costa Rican strains. The *Cupriavidus nodA* sequences nested within a larger clade encompassing all South American *Burkholderia* sequences (five sequences plus *Burkholderia* strain PAS44 from a *Mimosa* host introduced to Taiwan). These results are consistent with the interpretation that *Cupriavidus nodA* genes had a single origin and may be descended from a South American *Burkholderia* ancestor.

A 473-bp segment of the *nifH* gene was amplified using primers nhcf3 and nhcr4 (Table 1) designed from *C. taiwanensis* sequences (4, 6). Patterns of sequence variation in the *nifH* gene were highly similar to the *nodA* results (GenBank accession no. EF374065 to EF374088). Four Texan strains

TABLE 2. Nodule numbers of five *Mimosa* species inoculated with *Cupriavidus* strains

Cupriavidus strain	No. of nodules[a]				
	M. pigra	*M. pudica*	*M. invisa*	*M. strigillosa*	*M. quadrivalvis*
AMP6	25 ± 13	79 ± 5	51 ± 4	1 ± 1	0
AMP18	3 ± 1	86 ± 8	78 ± 5	0	0
AMP34	3 ± 0.2	60 ± 16	73 ± 23	3 ± 1	0.2 ± 0.2
AMP51	4 ± 2	26 ± 5	110 ± 29	4 ± 2	3 ± 2
CMP2	2 ± 0.8	56 ± 13	42 ± 7	2 ± 2	0
CMP29	1 ± 0.4	33 ± 4	18 ± 2	1.5 ± 0.1	0.8 ± 0.6
CMP60	49 ± 11	74 ± 10	144 ± 18	1 ± 0.8	0.2 ± 0.2

[a] Values are means ± 1 standard error.

TABLE 3. Acetylene reduction activity of five *Mimosa* species inoculated with *Cupriavidus* strains

Cupriavidus strain	Acetylation reduction activity[a]				
	M. pigra	*M. pudica*	*M. invisa*	*M. strigillosa*	*M. quadrivalvis*
AMP6	+	++	++	−	°
AMP18	−	++	++	°	°
AMP34	−	++	+	−	−
AMP51	−	++	++	−	−
CMP2	−	++	++	−	°
CMP29	−	−	−	−	−
CMP60	−	++	+++	−	−

[a] °, C_2H_2 reduction activity absent since no nodules formed; −, nodules formed but C_2H_2 reduction not detected; +, acetylene reduction between 0 and 0.1 μmol · min^{-1} · $plant^{-1}$; ++, acetylene reduction between 0.1 and 1.0 μmol · min^{-1} · $plant^{-1}$; +++, acetylene reduction greater than 1.0 μmol · min^{-1} · $plant^{-1}$.

(AMP6, AMP18, AMP45, and CMP29) shared an identical sequence, which differed by 12 or 13 nucleotides from the rest of the Texan and Costa Rican strains. All *Cupriavidus* strains from Costa Rica were found to be identical to one another and also to *C. taiwanensis* strains LMG19424 and LMG19425. Phylogenetic analysis of the *nifH* sequences (Fig. 2) indicated that all *Cupriavidus* nodule symbionts formed a highly supported lineage (100% bootstrap value). Within this lineage, there was the same subgroup evident in the *nodA* tree composed of *C. taiwanensis* strains LMG19424 and LMG19425, three Texan strains, and the five Costa Rican strains. In contrast to the *nodA* tree, the *nifH* sequence of *Burkholderia caribensis* strain TJ182 was grouped with South American *Burkholderia* strains (5) rather than within the *Cupriavidus* clade. Apart from the altered placement of *B. caribensis* TJ182, the *Cupriavidus* portions of the tree topology were highly similar for *nodA* and *nifH* phylogenies (Fig. 1 and 2). A partition homogeneity test (8) on all nodule-forming *Burkholderia* and *Cupriavidus* strains other than TJ182 indicated that there was no evidence for a difference in *nodA* and *nifH* tree topologies ($P = 0.99$). Thus, both genes appear to share a common genealogical history, and the placement of the *Cupriavidus nifH* clade suggests that their *nifH* gene may be derived from a *Burkholderia* ancestor that lived in tropical America (Fig. 2).

Nodulation and nitrogenase assays. Seeds from five *Mimosa* species (*M. pigra*, *M. pudica*, *M. invisa*, *M. strigillosa*, and *M. quadrivalvis*) were inoculated with seven Texan *Cupriavidus* isolates using standard protocols (13). The five *Mimosa* species developed nodules with most or all of the seven *Cupriavidus* strains tested (Tables 2 and 3). *M. invisa* formed the most nodules on average (18 to 144 nodules per plant across bacterial strains). *M. quadrivalvis* and *M. strigillosa* showed limited nodulation for all seven isolates.

Nodules of six out of the seven *Cupriavidus* strains showed substantial nitrogenase activity, as measured by acetylene reduction assays on plants of both *M. pudica* and *M. invisa* (Table 3). Plants of *M. pigra* exhibited little or no nodule nitrogenase activity with any bacterial strain. A consistent absence of nodule nitrogenase activity was also observed for plants of *M. quadrivalvis* and *M. strigillosa*.

The identity of nodule occupants was verified by reisolating one bacterial culture from a surface-sterilized nodule for each of the seven *Cupriavidus* strains and then sequencing a 390-bp portion of the 23S rRNA gene. Two of the reisolated cultures were obtained from nodules formed on *M. strigillosa*, and the others came from *M. pudica* or *M. invisa* hosts. For all seven strains, the 23S rRNA sequence from the nodule isolate was identical to that of the inoculant strain. This confirmed that these *Cupriavidus* isolates were responsible for inducing legume root nodule development in this experiment.

Three strains from *M. strigillosa* that had 5′ 23S rRNA sequences similar to those of *Sinorhizobium arboris* or *S. saheli* were also tested for nodule formation ability on *M. strigillosa*. The mean number of nodules per plant ranged from 6 to 16 for the three strains, and nodules of all three strains exhibited positive acetylene reduction activity (0.04 to 0.16 μmol per plant per minute).

Conclusions. This is the first report of *Cupriavidus* strains being the predominant root nodule symbionts for a legume population in its native geographic range and is also the first report of *Cupriavidus* nodule symbionts for a legume indigenous to the continental United States. The results help to clarify the origins of nodule symbiosis in *Cupriavidus*. All *Cupriavidus* nodule symbionts, including strains indigenous to two distant regions in the New World and strains isolated from *Mimosa* plants introduced to Taiwan (4, 6), formed a strongly supported monophyletic group in both the *nodA* and *nifH* phylogenies (Fig. 1 and 2). This suggests that different regional populations of *Cupriavidus* nodule bacteria did not independently acquire symbiotic genes from different sources but rather may be derived from a single ancestor. The *Cupriavidus* clade was found to nest within a set of *Burkholderia* strains indigenous to South America for both the *nodA* and *nifH* trees (Fig. 1 and 2). Thus, it appears that *Cupriavidus* acquired these genes by horizontal transfer from *Burkholderia* rather than from alphaproteobacterial nodule bacteria and that this event most likely occurred in tropical America.

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REFERENCES

1. **Barneby, R. C.** 1991. Sensitive Censitae: a description of the genus *Mimosa* Linnaeus (Mimosaceae) in the New World. The New York Botanical Garden, New York, NY.
2. **Barrett, C. F., and M. A. Parker.** 2005. Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. Syst. Appl. Microbiol. **28:**57–65.

3. **Barrett, C. F., and M. A. Parker.** 2006. Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in Costa Rica. Appl. Environ. Microbiol. **72:**1198–1206.
4. **Chen, W.-M., L. Moulin, C. Bontemps, P. Vandamme, G. Béna, and C. Boivin-Masson.** 2003. Legume symbiotic nitrogen fixation by β-proteobacteria is widespread in nature. J. Bacteriol. **185:**7266–7272.
5. **Chen, W.-M., S. M. de Faria, R. Straliotto, R. M. Pitard, J. L. Simões-Araüjo, J.-H. Chou, Y.-J. Chou, E. Barrios, A. R. Prescott, G. N. Elliot, J. I. Sprent, J. P. W. Young, and E. K. James.** 2005. Proof that *Burkholderia* forms effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from South America. Appl. Environ. Microbiol. **71:**7461–7471.
6. **Chen, W. M., E. K. James, J. H. Chou, S. Y. Sheu, S. Z. Yang, and J. I. Sprent.** 2005. β-Rhizobia from *Mimosa pigra*, a newly discovered invasive plant in Taiwan. New Phytol. **168:**661–675.
7. **Clark, M. A., N. A. Moran, and P. Baumann.** 1999. Sequence evolution in bacterial endosymbionts having extreme base pair composition. Mol. Biol. Evol. **16:**1586–1598.
8. **Farris, J. S., M. Kallersjo, A. G. Kluge, and C. Bult.** 1995. Testing significance of incongruence. Cladistics **10:**315–319.
9. **Moulin, L., A. Munive, B. Dreyfus, and C. Boivin-Masson.** 2001. Nodulation of legumes by members of the beta-subclass of Proteobacteria. Nature **411:** 948–950.
10. **Spoerke, J. M., H. H. Wilkinson, and M. A. Parker.** 1996. Nonrandom genotypic associations in a legume-*Bradyrhizobium* mutualism. Evolution **50:**146–154.
11. **Turner, B. L.** 1959. The legumes of Texas. University of Texas Press, Austin.
12. **Verma, S. C., S. P. Chowdhury, and A. K. Tripathi.** 2004. Phylogeny based on 16S rDNA and *nifH* sequences of *Ralstonia taiwanensis* strains isolated from nitrogen-fixing nodules of *Mimosa pudica*, in India. Can. J. Microbiol. **50:** 313–322.
13. **Wilkinson, H. H., J. M. Spoerke, and M. A. Parker.** 1996. Divergence in symbiotic compatibility in a legume-*Bradyrhizobium* mutualism. Evolution **50:**1470–1477.