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New Betaproteobacterial *Rhizobium* Strains Able To Efficiently Nodulate *Parapiptadenia rigida* (Benth.) Brenan

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Among the leguminous trees native to Uruguay, *Parapiptadenia rigida* (Angico), a Mimosoideae legume, is one of the most promising species for agroforestry. Like many other legumes, it is able to establish symbiotic associations with rhizobia and belongs to the group known as nitrogen-fixing trees, which are major components of agroforestry systems. Information about rhizobial symbionts for this genus is scarce, and thus, the aim of this work was to identify and characterize rhizobia associated with *P. rigida*. A collection of Angico-nodulating isolates was obtained, and 47 isolates were selected for genetic studies. According to enterobacterial repetitive intergenic consensus PCR patterns and restriction fragment length polymorphism analysis of their *nifH* and 16S rRNA genes, the isolates could be grouped into seven genotypes, including the genera *Burkholderia*, *Cupriavidus*, and *Rhizobium*, among which the *Burkholderia* genotypes were the predominant group. Phylogenetic studies of *nifH*, *nodA*, and *nodC* sequences from the *Burkholderia* and the *Cupriavidus* isolates indicated a close relationship of these genes with those from betaproteobacterial rhizobia (beta-rhizobia) rather than from alphaproteobacterial rhizobia (alpha-rhizobia). In addition, nodulation assays with representative isolates showed that while the *Cupriavidus* isolates were able to effectively nodulate *Mimosa pudica*, the *Burkholderia* isolates produced white and ineffective nodules on this host.

Parapiptadenia rigida (Benth.) Brenan, which is also known by its vernacular names Angico, Angico vermelho, and Gurucaia, belongs to the tribe Mimoseae within the Mimosoideae subfamily of the Fabaceae (Leguminosae) (5, 26, 27, 38). It is native to southern South America (south Brazil, Argentina, Paraguay, and Uruguay), where it can be found as one of the tallest species in the canopy of riverside forests, where it can reach heights of approximately 30 m and breast height diameters of from 30 to 120 cm. The wild tree is currently exploited by the locals owing to its economic value, although commercial cultivation of *P. rigida* has never been developed in Uruguay. Its main economic value is based on the excellence of its timber, which is appreciated for its high density (0.74 to 0.98 g/cm^3) and natural durability (26). It is mostly used for high-quality furniture, house construction, carpentry, and fire wood, and the reddish brown parquet floors built with its timber are deeply valued. Other reported uses are as a source of gums, tannins, and essential oils as well as for medicinal purposes (14, 37). Indeed, it is much appreciated by people in Brazil for its medicinal qualities and is duly included in the Brazilian Pharmacopeia.

This heliophyte species is part of the forest succession during the first steps of recovery of degraded areas, as it can grow under adverse and low-soil-fertility conditions. Its ability to establish a nitrogen-fixing association with rhizobia is well documented (18–20, 32), but information about the rhizobia associated with this leguminous tree is scarce. In an exhaustive list of inoculants for leguminous plants, Moreira (32) indicates the use of two strains of rhizobia for *P. rigida: Sinorhizobium fredii* Br827 (a bacterium from the *Alphaproteobacteria* group) and *Burkholderia* sp. strain Br9002 (a bacterium from the *Betaproteobacteria* group). Although most well-known rhizobia belong to the *Rhizobiaceae* or *Bradyrhizobiaceae* family of the *Alphaproteobacteria*, in 2001 the first publications of *Betaproteobacteria* which were able to nodulate legumes changed a century-old dogma that states that legumes can form nitrogen-fixing symbioses only with bacteria belonging to the *Alphaproteobacteria* (12, 33), and the terms alpha-rhizobia and beta-rhizobia were then coined to designate these two subgroups of symbionts (24). After Chen et al. (12) reported the isolation of *Ralstonia* (*Cupriavidus*) *taiwanensis* from *Mimosa pudica* (a mimosoid legume) and Moulin et al. (33) reported the isolation of *Burkholderia* strains (*Burkholderia phymatum* STM815 and *B. tuberum* STM678) from two papilionoid legumes, symbiotic nodulation by *Betaproteobacteria* was confirmed by studies showing effective nodulation on *Mimosa* (8, 10, 11). Further studies showed that *B. phymatum* STM815 was also a *Mimosa*-nodulating strain (17) but that *B. tuberum* STM678 could not nodulate *Mimosa* and could nodulate *Cyclopia*, a South African papilionoid legume (16). Therefore, with the exception of *B. tuberum* STM678 and other *Burkholderia* strains from South Africa (22), almost all nodulating *Betaproteobacteria* have been isolated from *Mimosa* (1–3, 8, 10–12), with Brazil being the main center of *Mimosa* diversification (38) and thus also being a major center for legume-nodulating *Burkholderia* (4, 15).

The main objective of this work was to find Uruguayan native rhizobia associated with *P. rigida* that are able to promote plant growth and thus be of potential use in forestry plantations. In order to achieve this goal, we identified locations in Uruguay in

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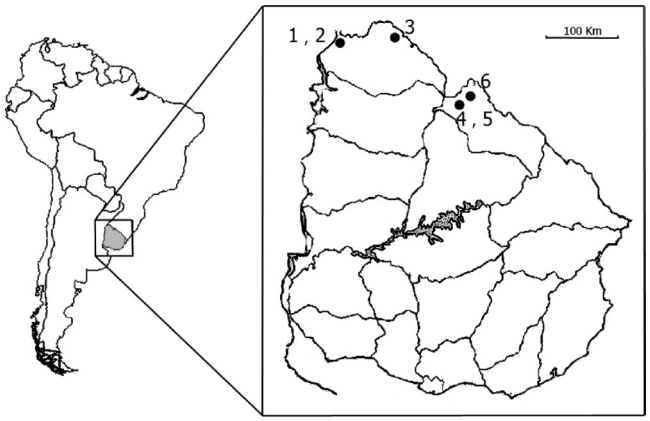

FIG 1 Geographical locations of nodule collection sites.

which Angico grows and generated a collection of seeds, as well as microsymbionts isolated from their nodules. The data obtained indicate a high genetic diversity among the Angico-nodulating bacteria and also highlight their potential as agroforestry inoculants.

MATERIALS AND METHODS

Rhizobial isolation and culture conditions. Bacterial strains were isolated from nodules collected from *P. rigida* plants found at different field sites in Uruguay or from plant trap assays with seedlings. Site locations or soil samples were selected from native forest where Angico trees are naturally present, from Angico plantations, or from pasture soils where Angico does not naturally grow. Geographical coordinates of each location were recorded using a Global Positioning System receiver. Site locations and the origins of the soil samples are shown in Fig. 1 and Table 1 (see also Table S1 in the supplemental material). Soil samples were also obtained from soil under pastures in the Tacuarembó and Treinta y Tres Departments in Uruguay. *S. fredii* strain Br827 was kindly provided by Sergio de Faria from EMBRAPA-Agrobiología, Seropédica, RJ, Brazil.

Isolation of bacterial strains from nodules was performed using standard techniques (44). For each nodule, only one bacterial colony was selected. Strains were grown on yeast extract-mannitol (YEM) agar (44) at 30°C. Ninety-two isolates were obtained by this procedure. Cultures that originated from colonies yielding a *nifH* PCR amplification product of ca. 327 bp using *nifH*-specific primers (25, 34) were stored at −80°C in the presence of 25% (vol/vol) glycerol. The sequences of the primers used were 5′-ATYGTCGGYTGYGAYCCSAARGC-3′ for primer eufornif and 5′-ATGGTGTTGGCGGCRTAVAKSGCC-3′ for primer eurevnif.

ERIC. Amplification reactions using enterobacterial repetitive intergenic consensus (ERIC) primers were performed as described by Hulton et al. (25). Bacterial lysates obtained from the microsymbionts that yielded a *nifH* PCR product were used as a template, and independent PCRs were performed at least twice. The electrophoretic patterns were analyzed by the GelCompar program (version 4.2; Applied Math, Kortrijk, Belgium) using cluster correlation analysis. Similarities were clustered using the unweighted-pair group method using average linkages (UPGMA) algorithm. Final groups were manually revised.

RFLP analysis. Selected isolates were characterized by restriction fragment length polymorphism (RFLP) analysis of 16S rRNA and *nifH* genes. For 16S rRNA RFLP analysis, nearly full-length 16S rRNA genes were amplified using the universal primers 27f and 1522r (29). Amplicons of the 16S rRNA and *nifH* genes were digested with the endonucleases HinfI and RsaI, and polymorphism patterns were visualized after electrophoresis on a 1.5% (wt/vol) agarose gel with Tris-acetate-EDTA (pH 8.3) electrophoresis buffer (36).

Bacterial identification and phylogenetic analysis based on 16S rRNA, *nifH*, *nodA*, and *nodC* genes. Isolates that belonged to different ERIC and RFLP groups were selected for bacterial identification and phylogenetic analyses.

Sequences of the ca. 327-bp *nifH* intragenic fragment were obtained by using the aforementioned primers, while the almost complete 16S rRNA gene was sequenced using the 27f and 1492r universal primers. The thermocycler program for *nifH* and 16S rRNA amplification was as follows: 1 step at 95°C for 5 min, followed by 25 sequential cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final step at 72°C for 15 min.

Intragenic regions of *nodA* genes were amplified and sequenced using primers nodAforB (5′-CRGTGGARGGTBYGYTGGGA-3′) and nodArevB (5′-TCAYARCTCDGGBCCGTTBCG-3′) for *Burkholderia* isolates (L. Moulin, personal communication) or nodAforC (5′-GATCT TGAACTCTCCGACCATTT-3′) and nodArevC (5′-GTTCGATTGTTT CGCCGCTTG-3′) for *Cupriavidus* isolates (1). Amplification of *nodA* sequences was carried out as follows: an initial denaturation step (95°C, 5 min), followed by 35 cycles of denaturation (95°C, 30 s), annealing (60°C for *Burkholderia* strains and 58°C for *Cupriavidus* strains, 5 min), and extension (72°C, 1 min) and a final extension (72°C, 5 min).

Intragenic regions of *nodC* genes were amplified and sequenced using the primers nodCforB (5′-CTCAATGTACACARNGCRTA-3′) and nodCrevB (5′-GAYATGGARTAYTGGYT-3′) for *Burkholderia* isolates (17) or nodCforC (5′-GTCACGCACGTAGAGGGCAAACA-3′) and nodCrevC (5′-GGCCGCAATCAACACGACTTCT-3′) for *Cupriavidus* isolates (this work). Amplification of *nodC* sequences was carried out with an initial denaturation step (95°C, 5 min), followed by 35 cycles of denaturation (95°C, 30 s), annealing (49°C for *Burkholderia* strains and 61°C for *Cupriavidus* strains, 5 min), and extension (72°C, 1 min) and a final extension step (72°C, 5 min).

The amplicons obtained were sequenced by Macrogen Inc., South Korea. The forward and reverse sequences obtained were assembled using the DNA Baser Sequence Assembler (version 3.x, 2010). Nucleotide sequence identities (SIs) were determined using the BLAST tool of the National Center for Biotechnology Information (NCBI).

When necessary, gene sequences were obtained from the Complete

TABLE 1 Location of *P. rigida* nodules and soil samples and classification of isolates

Site	GPS[a] location	Relevant characteristic, location name	No. of isolates	Presumptive genus and genotype (no. of isolates)
1	30°30′47.0″S, 57°40′9.6″W	Angico plantation, Mandiyú	20	*Burkholderia* genotype B (20)
2	30°31′13.4″S, 57°41′53.8″W	Native forest, Mandiyú	10	*Burkholderia* genotype B (6), *Cupriavidus* genotype D (2), *Cupriavidus* genotype E (2)
3	30°24′58.14″S, 56°29′16.70″W	Small plantation, Artigas	3	*Burkholderia* genotype A (3)
4	31°07′52.4″S, 56°00′15.5″W	Isolated tree on a farm, Lunarejo	1	*Burkholderia* genotype A (1)
5	31°08′46.7″S, 55°53′55.9″W	Native forest, Subida de Pena	2	*Burkholderia* genotype C (2)
6	31°06′43.8″S, 55°39′48.0″W	Small plantation, Estación Ataques	5	*Burkholderia* genotype C (5)

[a] GPS, Global Positioning System.

Microbial Genomes database at the NCBI website (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html). Nucleotide alignments of 16S rRNA gene sequences were obtained with the Greengenes program using the NAST alignment tool (13) and were manually edited. Nucleotide alignments of the *nifH*, *nodA*, and *nod* sequences were carried out with the CLUSTALW tool of the NCBI and were manually edited. Phylogenetic trees were constructed with the MEGA4 program (41), and the neighbor-joining algorithm (35) with the Kimura two-parameter substitution model was used (28). The robustness of the tree branches was estimated with 1,000 bootstrap replications (39).

Collection and storage of *P. rigida* seeds. *P. rigida* seeds were collected from trees widely grown in site 1 (Table 1; Fig. 1). Seeds were stored at 4°C and used during the first year of storage. The seeds were surface sterilized with 0.1% (wt/vol) $HgCl_2$ in 0.1 N HCl for 2 min, followed by seven washes with sterile water. Surface-sterilized seeds were used for plant trap assays and for the determination of the nodulation ability of purified potential microsymbionts.

Assessment of nodulation capacity. Surface-sterilized seeds were germinated on 0.8% (wt/vol) agar-water, and after germination, seedlings were transferred into glass tubes containing 15 ml of Jensen's N-free medium (44) plus 0.8% (wt/vol) agar. Alternatively, some seedlings were transferred into pots containing 1.5 kg of a sterile mixture of vermiculite and sand in a 1:1 (vol/vol) ratio. Seedlings were inoculated with 1 ml of a rhizobial suspension of ca. 1×10^7 CFU. One milliliter of sterile water was added to negative controls, while in other tubes, 0.05% KNO_3 was added to act as a positive control and to evaluate the response of *P. rigida* to N fertilization. Plants were grown under a photoperiod of 16 h light/8 h darkness at 26°C. Three months after inoculation, plants were harvested and presence of root nodules was evaluated.

Plant growth promotion under greenhouse conditions. Seeds were sown in pots containing 1.5 kg of a mixture of compost, soil, and sand in a 1:1:1 (vol/vol/vol) ratio. Approximately 1-week-old seedlings were inoculated with a rhizobial suspension of ca. 1×10^7 CFU per plant and were reinoculated 30 days later with a similar rhizobial suspension. A mixture of all strains (MIX) containing ca. 1×10^7 CFU of each strain was also included. One milliliter of sterile water was added to negative controls, while 0.05% KNO_3 was added to other pots as a positive control and to determine the response of *P. rigida* to N fertilization. Twelve repetitions per treatment were included, and the pots were randomly arranged in the greenhouse. Plants were watered with tap water as required. Thirty-four weeks after inoculation and before harvesting, plant height and stem diameter at ground level were determined with an electronic digital caliper. After harvesting, aerial dry weight was recorded, and differences between treatments were evaluated with Fisher's test (a P value of less than 0.1 indicated significance) and the multiple-comparison Tukey's test. The presence of nodules on inoculated plant roots was also evaluated, and some nodules were randomly selected to assess genetic group identification by RFLP analysis. The absence of nodules on uninoculated plants (N free and N fertilized) was confirmed.

Nucleotide sequence accession numbers. The 16S rRNA sequences were deposited in the GenBank database under the accession numbers JF683693, JF683694, JF683692, JF683699, JF683691, JF683695, JF683697, JF683698, and JF683696 for *Burkholderia* sp. strains UYPR1.413, UYPR2.522, UYPR1.3, UYPR1.45, UYPR1.313, UYPR3.611, UYPR5.94, UYPR6.101, and UYPR4.732, respectively; the accession numbers for *Cupriavidus* sp. strains UYPR2.54, UYPR2.55, UYPR2.56, and UYPR2.512 were JF683701, JF683702, JF683700, and JF683703, respectively, and the accession numbers for *Rhizobium sp.* strains UYPR8.331 and UYPR7.63 were JF683704 and JF683705, respectively. The *nifH* gene sequences were deposited in the GenBank database under accession numbers JF683716, JF683711, JF683710, JF683713, JF683715, JF683712, JF683714, JF683717, and JF683718 for *Burkholderia* sp. strains UYPR1.413, UYPR2.522, UYPR1.3, UYPR1.45, UYPR1.313, UYPR3.611, UYPR5.94, UYPR6.101, and UYPR4.732, respectively; the accession numbers for *Cupriavidus* sp. strains UYPR2.54, UYPR2.55, and UYPR2.512 were JF683708, JF683709, and JF683707, respectively, and the accession number for *Rhizobium* sp. strain UYPR7.63 was JF683706. Partial sequences for the acyltransferase gene sequences (*nodA*) were deposited in the GenBank database under accession numbers JF683729, JF683728, JF683722, JF683719, JF683721, and JF683720 for *Burkholderia* sp. strains UYPR1.413, UYPR2.522, UYPR1.3, UYPR1.45, UYPR1.313, and UYPR5.94, respectively; the accession numbers for *Cupriavidus* sp. strains UYPR2.54 and UYPR2.56 were JF683724 and JF683723, respectively. Partial sequences for the *N*-acetylglucosaminyltransferase nodulation gene sequences (*nodC*) were deposited in the GenBank database under accession numbers JF683725, JF683727, JF683726, JF683731, and JF683730 for *Burkholderia* sp. strains UYPR1.3, UYPR3.611, UYPR1.313, UYPR1.413, and UYPR2.522, respectively, and the accession number for *Rhizobium* sp. strain UYPR7.63 was JF683732.

RESULTS

The rhizobial symbionts of Angico are genetically diverse. The presence of root nodules was surveyed on young plants more than 10 cm tall or on superficial roots (less than ca. 20-cm depth) of older trees. The recovery of roots from an adult plant is extremely difficult, and very young plants were not nodulated. Nodules were mostly detected on trees in sites 1 and 2 (Table 1; Fig. 1). In order to explore the occurrence of Angico-nodulating rhizobia in soils with no history of Angico presence, we also analyzed trap plants grown in two different Uruguayan pasture soils. Ninety-three isolates were retrieved from nodules taken from different root samples (one isolate per nodule). Forty-seven isolates in which the *nifH* gene was detected by PCR, as well as the *S. fredii* Br827 Angico-nodulating strain (see Materials and Methods), were selected for further studies (see Table S1 in the supplemental material). To get a preliminary estimate of the bacterial diversity, we carried out an ERIC PCR analysis of isolates. The patterns obtained were highly heterogeneous, indicating a high genetic diversity among the isolates (data not shown). According to the results of these analyses, the isolates were classified into 6 different groups (see Table S1 in the supplemental material). ERIC group I contained 10 isolates: 2 isolates from site 3, the isolate from site 4, 1 isolate from site 5, and 6 isolates from site 6. ERIC group II and ERIC group IV consisted of 2 isolates each from site 2. ERIC group III included 20 isolates from site 1 and 6 isolates from site 2. The Angico-nodulating rhizobia found in the pasture soil from Tacuarembó belonged to group V (3 isolates out of 5) or to group VI (2 isolates). These results show that some groups of isolates were exclusive to certain locations. This is the case with the isolates which clustered in groups II and IV, which were found exclusively in the native forest present along the rivers Mandiyú and Uruguay (site 2). Moreover, three different ERIC groups were found in this site, meaning that it was the location where most genetic diversity was detected.

In an attempt to discriminate the bacterial isolates before proceeding to gene sequencing, RFLP analysis of their 16S rRNA and *nifH* genes was performed using two different restriction endonucleases (Table 2). For all isolates tested, the bacteria in ERIC groups I and III displayed the same 16S rRNA RFLP pattern, but discrimination according to the HinfI *nifH* RFLP pattern was possible. By using HinfI and RsaI endonucleases, the isolate from site 5 and the six isolates from site 6 (all from Rivera Department) could be differentiated from the isolates from sites 1 and 2 (all from Artigas Department). The four isolates of ERIC groups II and IV shared the same 16S rRNA RFLP and *nifH* RFLP patterns, which differentiated them from the other ERIC groups. A similar

TABLE 2 Bacterial genotypes according to ERIC and RFLP analyses of 16S rRNA and *nifH* genes

Genotype	RFLP pattern[a]				ERIC group	Genus contained in group
	16S rRNA		*nifH*			
	RsaI	HinfI	RsaI	HinfI		
A	1	1	1	1	I	*Burkholderia*
B	1	1	1	1	III	*Burkholderia*
C	1	1	1	2	I	*Burkholderia*
D	2	2	NR[b]	3	IV	*Cupriavidus*
E	2	2	NR	3	II	*Cupriavidus*
F	3	3	2	3	V	*Rhizobium*
G	3	3	2	3	VI	*Rhizobium*
H	4	4	ND[c]	ND	ND	*Sinorhizobium*

[a] 16S rRNA RFLP patterns were determined with a ca. 1,500-bp amplicon obtained by using the 27f and 1522r universal primers. *nifH* RFLP patterns were determined with a ca. 327-bp amplicon obtained by using the eufornif and eurevnif primers.
[b] NR, no restriction site.
[c] ND, not done.

scenario was observed for bacteria from ERIC groups V and VI, while the Brazilian isolate *S. fredii* Br827 had a distinctive and separate 16S rRNA RFLP pattern. Taken together, these data allowed the grouping of the Angico-nodulating rhizobia into eight genotypes: A to H (Table 2).

Representative isolates from each genotype were then assessed for their nodulation capacity with Angico plants grown under gnotobiotic conditions, and they were also selected for further genetic characterization. All the bacterial isolates tested were able to produce root nodules on Angico plants (see Table S1 and supporting information in the supplemental material).

Angico-nodulating rhizobia belong to the genera *Burkholderia*, *Cupriavidus*, and *Rhizobium*. Almost full-length 16S rRNA genes from 15 representative isolates of different bacterial clusters were amplified and sequenced. 16S rRNA gene sequences of genotype B isolates (UYPR1.313, UYPR1.3, UYPR1.45, UYPR1.413, and UYPR2.522) exhibited 99% SI among themselves, and the best hit corresponded to the *Burkholderia* sp. strain Hpig15.6 sequence (99% SI). Sequences of isolates with genotype A (UYPR3.611 and UYPR4.732) or with genotype C (UYPR5.94 and UYPR6.101) displayed 99% SI among themselves and 99% SI with *B. sabiae* Br3407^T. Sequences of isolates with genotype D (UYPR2.54 and UYPR2.55) or with genotype E (UYPR2.56 and UYPR2.512) exhibited 99% SI among them, and their highest SI (99%) corresponded to the sequence of *Cupriavidus necator* LMG8453^T, whereas that with the type strain of the beta-rhizobial species *C. taiwanensis* LMG19424^T was 98%. The sequences of isolates UYPR7.63 (genotype G) and UYPR8.331 displayed 99% SI with *Rhizobium tibeticum* LMG 24453^T, 98% SI with *Rhizobium etli* CFN 42^T, and 97% SI with *R. leguminosarum* USDA 2370^T.

The phylogenetic relationships of the 16S rRNA genes reinforce these affiliations of the Angico-nodulating rhizobia (Fig. 2). Moreover, these data support the genotypes defined in Table 2 and suggest that genotypes A, B, and C contain rhizobia in the genus *Burkholderia*, genotypes D and E contain *Cupriavidus*, and genotypes F and G contain *Rhizobium*.

Taken together, the results obtained in this work suggest that beta-rhizobia (*Burkholderia* and *Cupriavidus*) are the main Angico-nodulating rhizobia at sites where this leguminous tree is naturally present, with *Burkholderia* being the most commonly represented genus. Nevertheless, in spite of this apparent preponderance of beta-rhizobia, Angico can also be nodulated by alpha-rhizobia that belong to the genera *Rhizobium* and *Sinorhizobium*.

***nifH*, *nodA*, and *nodC* genes of the beta-rhizobia associated with Angico are clustered separately from the alpha-rhizobia.** With the aim of obtaining insight into the genetic diversity of symbiosis-related and nitrogen-fixing genes and to explore whether the ability to fix nitrogen and to nodulate legumes is an ancient or an early characteristic in these beta-rhizobial strains, phylogenetic analyses of their *nifH*, *nodA*, and *nodC* sequences were performed. A phylogenetic analysis of partial *nifH* gene sequences showed that the alpha- and beta-rhizobial sequences clustered separately with a bootstrap value of 75% (Fig. 3). The three *nifH* sequences from the Angico-nodulating *Cupriavidus* strains were clustered close to the *nifH* sequence of *C. taiwanensis* LMG19424^T, but in a separate group that was supported with a bootstrap value of 99%, suggesting a different evolutionary history. The *nifH* genes from the *Burkholderia* genotype C strains (UYPR5.94 and UYPR6.101) grouped apart from *Burkholderia* genotype A or B. The *nifH* sequences from the *Burkholderia* genotype A strains (UYPR4.732 and UYPR3.611) clustered in a branch closer to those of *Burkholderia* genotype B strains. The different clusters shown by the *nifH* phylogenies, such as those separating genotypes A/B, C/D/E, and G, agree with the *nifH* RFLP patterns obtained for these isolates (Fig. 3 and Table 2) and with the theoretical band sizes expected from them according to *in silico* analysis (data not shown).

Phylogenetic analyses of the *nodA* and *nodC* sequences showed that the alpha- and beta-rhizobia clustered separately, with the exception of *B. tuberum* STM678^T (Fig. 4 and 5) sequences. Previous phylogenetic analyses (22, 33) also found that the nodulation gene sequences of *B. tuberum* STM678^T, a South African strain that nodulates the papilionoid legume *Cyclopia* (16), were more similar to those of the alpha-rhizobia than those of the beta-rhizobia. Out of 14 beta-rhizobial isolates tested (UYPR1.313, UYPR1.3, UYPR1.45, UYPR1.413, UYPR2.522, UYPR2.54, UYPR2.55, UYPR2.51, UYPR2.512, UYPR2.56, UYPR3.611, UYPR4.732, UYPR5.94, and UYPR6.101), only 8 *nodA* and 6 *nodC* gene sequences could be amplified. Similar results were previously reported for *nodC* PCR amplification of *Mimosa*-nodulating rhizobia (4). This feature might indicate that the sequences of these nodulation genes are highly diverse or that these strains have a different nodulation strategy. We cannot discard the possibility that the sets of primers used were not able to amplify slightly different *nodA-nodC* sequences, although we tested at least three different sets of primers (data not shown). In support of the former hypothesis, we found that *nodA-nodC* gene sequences of Angico-nodulating *Burkholderia* symbionts clustered separately from the other *nodA-nodC* sequences that were evaluated. The *nodA* gene from *C. taiwanensis* LMG19424^T clustered close to the *nodA* genes of isolates UYPR2.55 and UYPR2.56 (genotypes D and E, respectively), but after many attempts, we did not succeed in detecting *nodC*-like genes in the *Cupriavidus* isolates. Nonetheless, when genomic DNA obtained from *C. taiwanensis* LMG19424^T was used as template, a PCR product with the expected sequence for *nodC* was obtained (data not shown). This may indicate that the *nodC* sequences of this group are distant from known *nodC* sequences.

Some rhizobial strains promote the growth of Angico. Twelve representative strains from the different genotypes

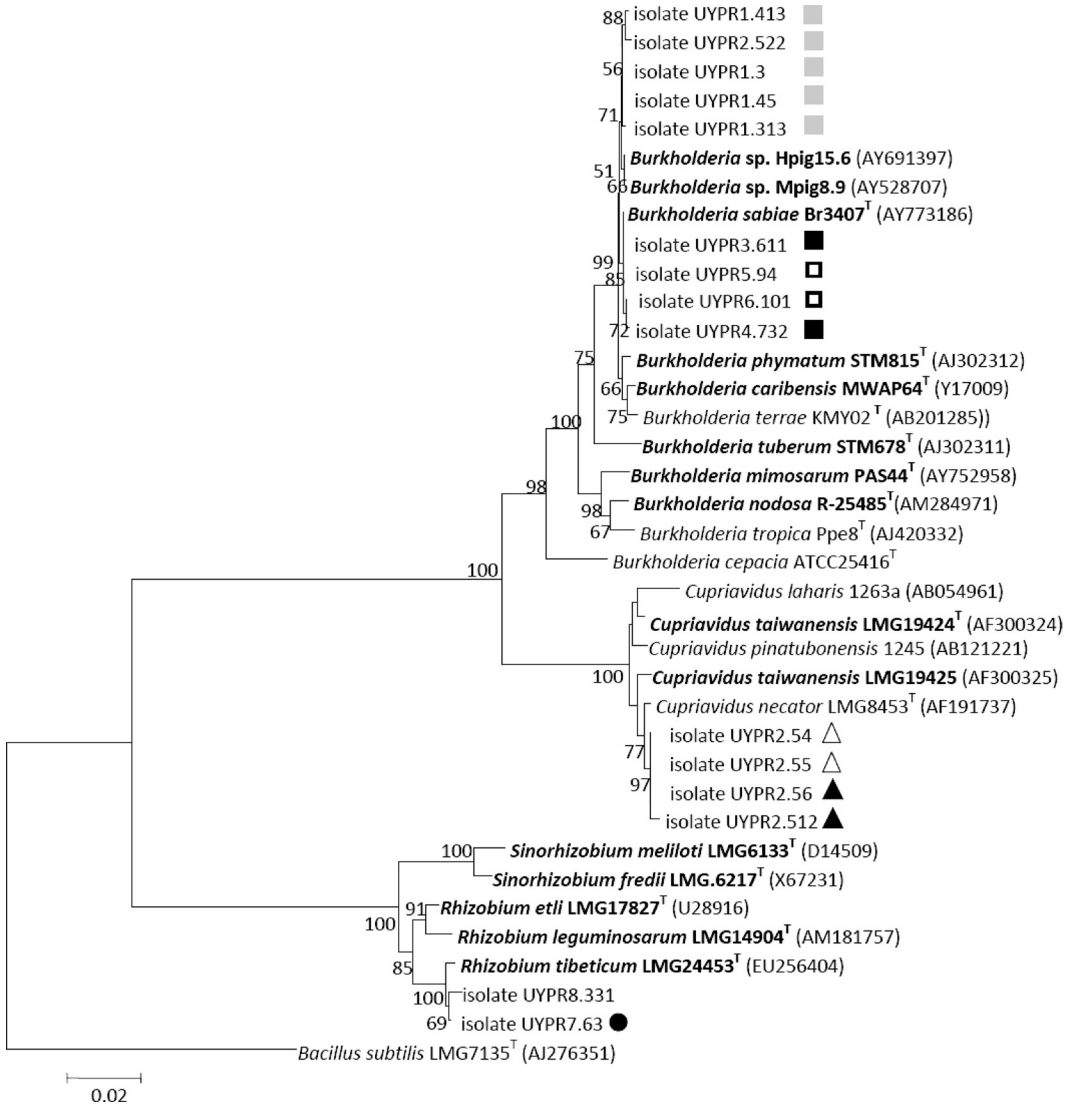

FIG 2 Phylogenetic affiliation of the almost entire (1,370-bp) 16S rRNA gene sequences of some representative Angico-nodulating rhizobia constructed using the MEGA4 program and the neighbor-joining algorithm with the Kimura two-parameter substitution model. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. *Bacillus subtilis* LMG7135 was used as an outgroup to root the tree. Black squares, *Burkholderia* genotype A; gray squares, *Burkholderia* genotype B; white squares, *Burkholderia* genotype C; white triangles, *Cupriavidus* genotype D; black triangles, *Cupriavidus* genotype E; black circle, *Rhizobium* genotype G. Legume symbionts are indicated in boldface type.

were chosen for greenhouse experiments with *P. rigida*. These were *Burkholderia* sp. UYPR3.611 (genotype A), *Burkholderia* sp. UYPR4.732 (genotype A), *Burkholderia* sp. UYPR2.522 (genotype B), *Burkholderia* sp. UYPR1.3 (genotype B), *Burkholderia* sp. UYPR1.413 (genotype B), *Burkholderia* sp. UYPR1.313 (genotype B), *Burkholderia* sp. UYPR6.101 (genotype C), *Burkholderia* sp. UYPR5.94 (genotype C), *Cupriavidus* sp. UYPR2.55 (genotype D), *Cupriavidus* sp. UYPR2.512 (genotype E), *Rhizobium* sp. UYPR7.63 (genotype G), and *S. fredii* Br827. Additionally, a mixed inoculum containing all the strains mentioned above was also included. In comparison with uninoculated plants, by the time of harvest at 34 weeks after inoculation, higher values for stem diameters at ground level and aerial dry weight were obtained for Angico plants inoculated with *Burkholderia* sp. UYPR3.611, *Burkholderia* sp. UYPR5.94, *Burkholderia* sp. UYPR2.522, *Cupriavidus* sp. UYPR2.512, *Burkholderia* sp. UYPR1.413, and the mixture of these strains (see Fig. S1A and B in the supplemental material) (Tukey's test, $P < 0.1$). The identity of the nodulating bacteria in randomly selected nodules was confirmed by isolation from the nodules and subjecting the isolates to 16S rRNA RFLP analysis.

Angico-nodulating rhizobia exhibit some host specificity. With the aim of evaluating the specificity toward their symbiotic host, *P. rigida*, strains that exhibited the best performance in greenhouse experiments (*Burkholderia* sp. UYPR1.413, *Burkholderia* sp. UYPR3.611, *Burkholderia sp.* UYPR5.94, *Cupriavidus* sp. UYPR2.55, and *Cupriavidus* sp. UYPR 2.512) were used to inoculate different legumes grown on N-free plant nutrient medium. Root systems were examined at 2 months after inoculation, and it was found that none of the selected Angico-nodulating rhizobial isolates could form nodules on *Trifolium repens* (Papilionoideae),

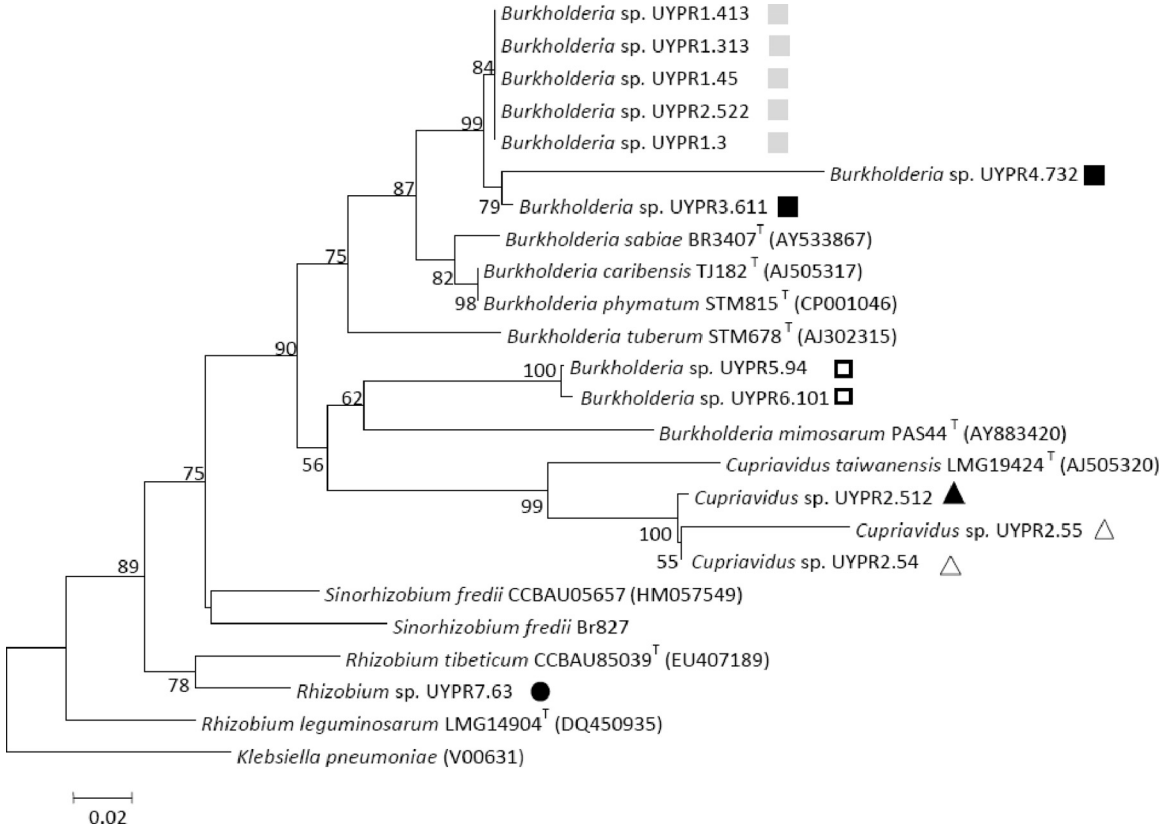

FIG 3 Phylogenetic affiliation of internal *nifH* gene sequences (ca. 307 bp) of some representative Angico-nodulating rhizobia constructed using the MEGA4 program and the neighbor-joining algorithm with the Kimura two-parameter substitution model. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. Nonrhizobial *Klebsiella pneumoniae* strain V00631 was used as an outgroup. Black squares, *Burkholderia* genotype A; gray squares, *Burkholderia* genotype B; white squares, *Burkholderia* genotype C; white triangles, *Cupriavidus* genotype D; black triangle, *Cupriavidus* genotype E; black circle, *Rhizobium* genotype G.

Medicago sativa (Papilionoideae), or *Peltophorum dubium* (Caesalpinioideae) seedlings grown under gnotobiotic conditions. Interestingly, however, all of the tested strains were able to nodulate *M. pudica* seedlings, but the degree of nodulation and nodule effectiveness, as well as plant growth promotion, varied according to the different bacterial genera. For example, while *Cupriavidus* isolates produced reddish nodules and were able to promote *M. pudica* plant growth, the *Burkholderia*-inoculated plants developed white and ineffective nodules (see Fig. S2 in the supplemental material). The kinetics of nodulation of *M. pudica* was much higher than that of *P. rigida*, with nodules forming on the former at only 4 days after inoculation but forming on the latter at 30 days after inoculation (data not shown). A rapid response by *M. pudica* roots to inoculation with *C. taiwanensis* LMG19424^T was also reported by Chen et al. (11).

DISCUSSION

In this study, we isolated, identified, and characterized bacterial strains from root nodules found on Angico (*P. rigida*) trees growing in native forests or in forestry plantations. Previous reports have demonstrated the presence of rhizobia able to nodulate leguminous trees in Uruguayan soils (20, 21). Of particular relevance to the present study, it was shown that Angico could be nodulated by rhizobial strains isolated from some other legumes (20), but until now there have been no reports on naturally occurring rhizobial strains associated with Angico growing in Uruguay. In the present study, we found that most of the Angico isolates belonged to the genus *Burkholderia*, although bacteria from the *Cupriavidus* and *Rhizobium* genera could also be recovered from Angico nodules. The presence of at least one *Burkholderia* strain (Br9002) able to nodulate Angico was previously reported from Brazil (32). Herein, we report for the first time that Angico can be efficiently nodulated by strains from the genus *Cupriavidus*. In addition, we identified Angico-nodulating rhizobia that belong to the genus *Rhizobium*, but as these strains were recovered only from trap plants grown in pasture soils with no history of Angico growing in them, these bacteria are unlikely to be the natural symbionts of Angico.

Seven genotypes of symbionts were distinguished according to the genetic characterization (Table 2). Genotypes A and C contained *Burkholderia* strains closely related to *B. sabiae* Br3407^T, a *Mimosa caesalpiniifolia*-nodulating strain isolated in Brazil (6). Genotype B contained *Burkholderia* strains whose 16S rRNA gene sequences are available at the NCBI database but which have not yet been identified to species level, i.e., *Burkholderia* sp. strain Hpig15 (isolated from nodules of *M. pigra* in Costa Rica) and *Burkholderia* sp. strain Mpig8.9 (isolated from nodules of *M. pigra* in Panama) (2). Six species of rhizobia that belong to the *Burkholderia* genus have been taxonomically identified to date. These are *B. tuberum* (42), *B. phymatum* (42), *B. caribensis* (11), *B. mi-*

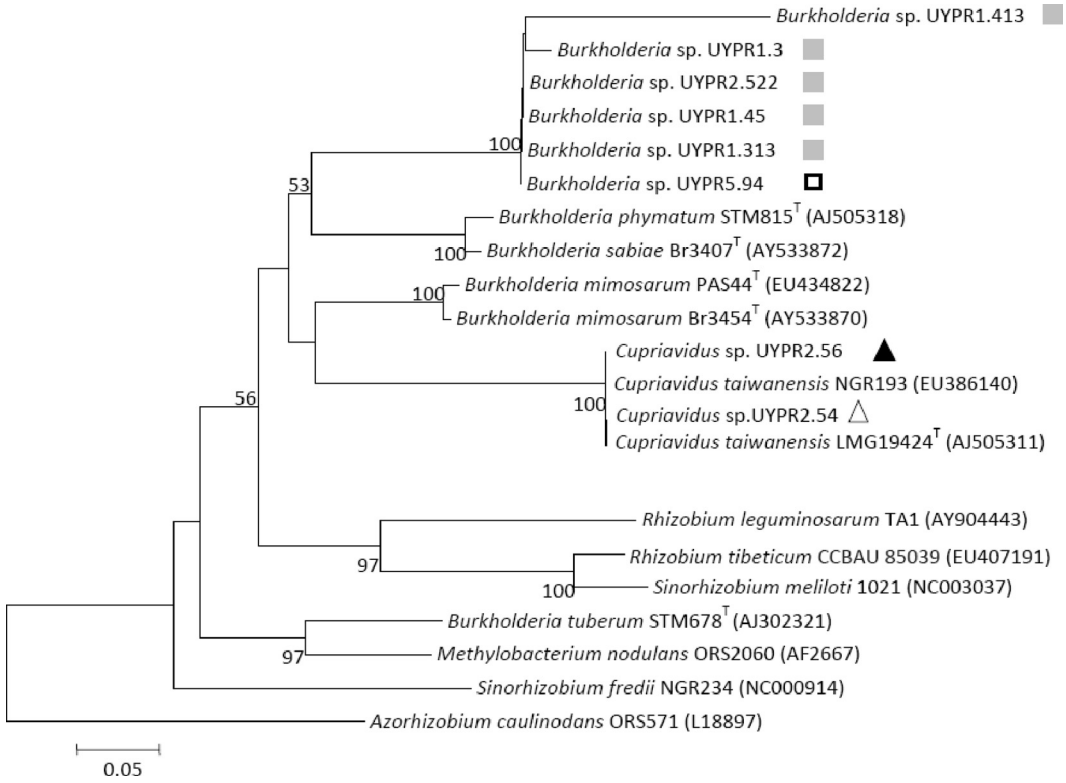

FIG 4 Phylogenetic affiliation of internal *nodA* gene sequences (ca. 460 bp) of some representative Angico-nodulating rhizobia constructed using the MEGA4 program and the neighbor-joining algorithm with the Kimura two-parameter substitution model. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. *Azorhizobium caulinodans* strain ORS571 was used as an outgroup. Gray squares, *Burkholderia* genotype B; white square, *Burkholderia* genotype C; white triangle, *Cupriavidus* genotype D; black triangle, *Cupriavidus* genotype E.

mosarum (9), *B. nodosa* (7), and *B. sabiae* (6). Beta-rhizobia from the *Burkholderia* genus appear to be widely distributed over the world; e.g., they have been found in the Americas (Brazil, Costa Rica, French Guiana, Mexico, Panama, the United States, and Venezuela), Africa (Morocco and South Africa), Asia (China, New Guinea, and Taiwan), and Western Australia. In general, they are the main beta-rhizobia identified (1–3, 8, 11, 22, 40), and although they have been dispersed along with their invasive *Mimosa* hosts to many other parts of the tropical world, they appear to have a major center of diversity in central Brazil (4) and have been reported as being ancient (>50 million years old) in symbiotic terms (4).

Genotypes D and E belonged to the genus *Cupriavidus*. Beta-rhizobia from the genus *Cupriavidus* are less frequently encountered, and so far, only a few *Cupriavidus* isolates have been found in Central America (Costa Rica) and North America (United States), with the vast majority being encountered in Asia (China, India, Taiwan, and Thailand), where they nodulate invasive *Mimosa* spp., such as *M. diplotricha*, *M. pigra*, and *M. pudica* (1, 2, 10–12, 30, 31, 43). In our survey, four *Cupriavidus* strains were recovered from Angico nodules, but even though they were related to the only *Cupriavidus* rhizobial type strain identified so far, *C. taiwanensis* LMG19424^T, the different phylogeny of the 16S rRNA sequences of our strains suggests that they might belong to a new species more closely related to *C. necator*, a species that has not hitherto been identified to be a legume symbiont. Clearly, further studies are required to identify these strains more definitively. Further to this, it is worth noting that the genome of *Cupriavidus* sp. strain UYPR2.512 (previously named 5v12) is being sequenced as part of the Genome Encyclopedia of Bacteria and Archaea on Root Nodulating Bacteria (GEBA-RNB) Project at the Joint Genome Institute, U.S. Department of Energy (www.jgi.doe.gov), with the Gi08830 Gold Card identifier, as well as *Burkholderia* sp. strain UYPR1.413 (previously named 4.13) with the Gi08829 Gold Card identifier.

Symbiotic nitrogen-fixing genes are mainly represented by *nif* and *nod* genes, albeit a few Nod factor-independent rhizobia have been found (23). To get insight into the genetic diversity of symbiotic nitrogen-fixing genes found in Angico symbionts, we wanted to analyze *nifH*, *nodA*, and *nodC* sequences. Unfortunately, after many attempts we did not succeed in amplify *nod* genes in most of the isolates tested. According to the sequences obtained, symbiotic nitrogen-fixing genes clustered separately the alpha- and beta-rhizobial sequences and, thus, might indicate an ancient acquisition of these genes, as has been suggested in an exhaustive study done by Bontemps et al. (4) with *Mimosa*-nodulating beta-rhizobia in Central Brazil.

RsaI and HinfI *nifH* RFLP patterns clustered the isolates into four groups (Table 2). Group 1 comprises *Burkholderia* genotypes A and B, group 2 contains *Burkholderia* genotype C, group 3 consists of *Cupriavidus* genotypes D and E, and group 4 contains *Rhizobium* spp. Moreover, phylogenetic analysis reinforced these *nifH* patterns (Fig. 3). Additionally, we found that *nifH* sequences from *Burkholderia* genotype C clustered apart from *Burkholderia* genotypes A and B and in a branch closer to the branch with those

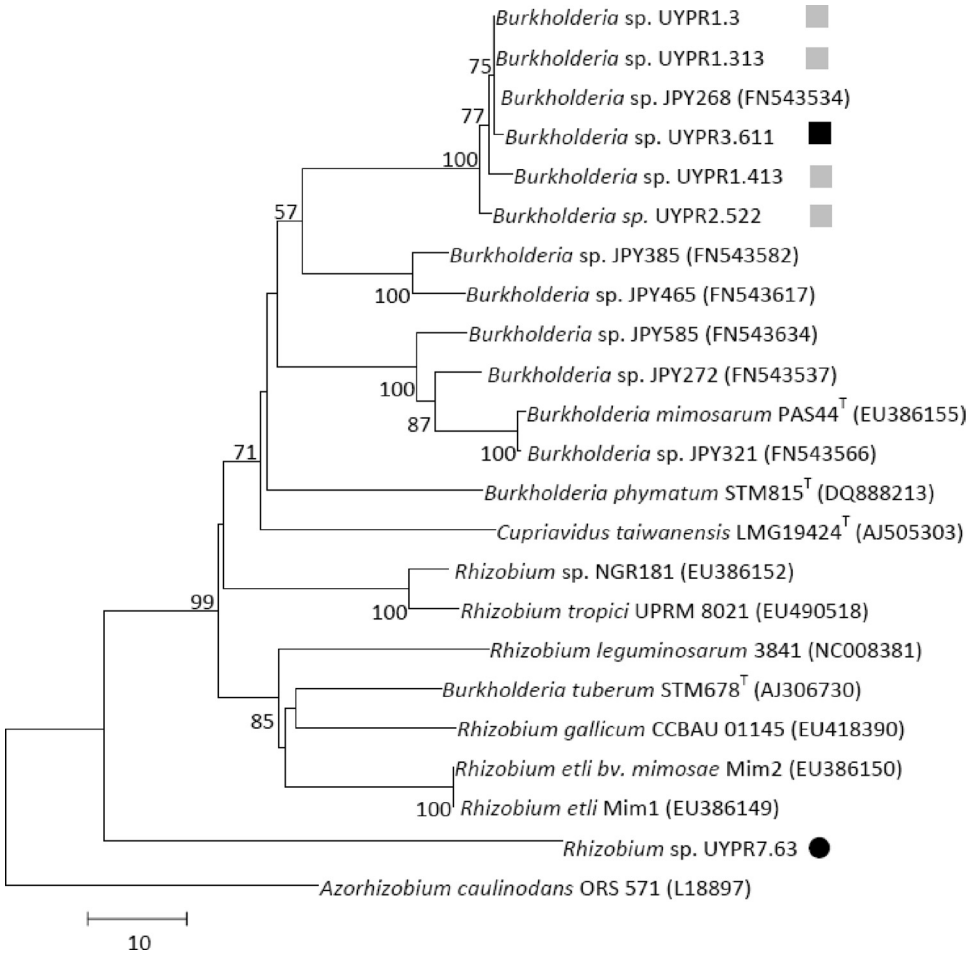

FIG 5 Phylogenetic affiliation of internal *nodC* gene sequences (ca. 600 bp) of some representative Angico-nodulating rhizobia constructed using the MEGA4 program and the neighbor-joining algorithm with the Kimura two-parameter substitution model. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. *Azorhizobium caulinodans* strain ORS571 was used as an outgroup. Gray squares, *Burkholderia* genotype B; black square, *Burkholderia* genotype C; black circle, *Rhizobium* genotype G.

of *B. mimosarum* PAS44 and *Cupriavidus* strains (Fig. 3), suggesting that *nifH* genes from genotypes C, D, and E might have a common evolutionary history.

Phylogenetic analysis of *nod* gene sequences clustered the isolates into three groups (Fig. 4 and 5). Group 1 contains the *Rhizobium* sp. UYPR7.63 isolate, group 2 the *Cupriavidus* sp. isolates, and group 3 the *Burkholderia* sp. isolates. Similarly to *nifH* sequences, the *nodA* sequences from *Cupriavidus* grouped close to *C. taiwanensis* LMG19424 and in a branch close to *B. mimosarum* stains, while *Burkholderia* sp. isolates grouped apart from the other *nod* genes analyzed.

C. taiwanensis LMG19424 and *B. mimosarum* stains are well-known symbionts of *M. pudica* (8, 12). Taking into account the close relationship of nitrogen-fixing symbiotic genes from Angico symbionts to those from *Mimosa* symbionts, we evaluated the nodulation and growth response of Angico and *M. pudica* to beta-rhizobial inoculation. We found that both legumes developed effective nodules and plant growth was promoted after inoculation with some selected Angico strains belonging to the *Cupriavidus* group (see Fig. S1 and S2 in the supplemental material). However, when plants grown on N-free medium were inoculated with *Burkholderia* sp. strain UYPR 1.413 or UYPR3.611, only nodules produced on Angico plants were effective. *M. pudica* plants inoculated with *Burkholderia* sp. strain UYPR 1.413 or UYPR3.611 produced small and white nodules, and no plant growth promotion could be detected. These results might suggest that these *Burkholderia* sp. strains are natural symbionts of Angico and are not natural symbionts of the closely related *M. pudica* plant. The observed response of Angico to inoculation supports the possibility that these *Burkholderia* and *Cupriavidus* strains may be exploited as inoculants for this multipurpose tree.

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