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Bradyrhizobia Nodulating the *Acacia mangium* × *A. auriculiformis* Interspecific Hybrid Are Specific and Differ from Those Associated with Both Parental Species▽†

Christine Le Roux,[1]‡ Diana Tentchev,[1]‡§ Yves Prin,[1] Doreen Goh,[4] Yani Japarudin,[5] Marie-Mathilde Perrineau,[1] Robin Duponnois,[2] Odile Domergue,[3] Philippe de Lajudie,[2] and Antoine Galiana[1]*

CIRAD, UMR LSTM, F-34398 Montpellier Cedex 5, France[1]; IRD, UMR LSTM, F-34398 Montpellier Cedex 5, France[2]; INRA, UMR LSTM, F-34398 Montpellier Cedex 5, France[3]; YSG Biotech Sdn. Bhd., Plant Biotechnology Laboratory, P.O. Box 11623, 88817 Kota Kinabalu, Sabah, Malaysia[4]; and Sabah Softwoods Sdn. Bhd., P.O. Box 60966, 91019 Tawau, Sabah, Malaysia[5]

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In the context of an increasing utilization of the interspecific hybrid *Acacia mangium* × *A. auriculiformis* as a plantation tree in the tropical humid zone, its symbiotic characterization was carried out in comparison with that of its two parental species. Rhizobium strains of diverse geographical origins were isolated from root nodules of the hybrid and its parents. Almost all *Acacia* hybrid isolates were fast growing on yeast extract-mannitol medium, in contrast to those isolated from both parental species, which were mostly slow growing. The rhizobium strains were characterized through partial sequencing of the rRNA operon. In the phylogenetic tree, almost all strains isolated from the hybrid were grouped together in a clade close to *Bradyrhizobium japonicum*, while all strains isolated from both parental species were close to *Bradyrhizobium elkanii*. Inoculation experiments performed under in vitro or greenhouse conditions showed that all strains were infective with their original hosts but exhibited very variable degrees of effectivity according to the host plant tested. Thus, homologous strain-host associations were more effective than heterologous ones. This shows that there is still a high potential for isolating and testing new strains from hybrids to be used as inoculants in the context of large-scale afforestation programs.

Due to the abundance and diversity of tropical legume trees, many studies have investigated the nitrogen-fixing status of these trees from natural forests (7, 30, 37) and agroforestry systems (2). Concerning acacias, most of the studies were focused on the characterization of rhizobia associated with African dry-zone species (10, 11, 12, 23, 29, 41, 60), while only a few reports exist on rhizobia associated with Australasian *Acacia* spp. belonging to the *Phyllodineae* tribe (24, 28, 40, 57). In the last two decades, the acacias native to Australia and Papua New Guinea, *A. mangium* and *A. auriculiformis* in particular, have been extensively planted in the humid tropics, mostly in Southeast Asia for pulp production, reaching a total estimated area of about 2 million hectares (54). The first occurrence of spontaneous hybrids between *A. mangium* and *A. auriculiformis* was observed in 1972 in Malaysia (38), and hybrids were obtained later from controlled pollination (43, 47). More recently, the *A. mangium* × *A. auriculiformis* hybrid was identified as a very promising tree to be used in clonal plantations, since it was shown to have higher wood productivity than both parental species, as well as higher wood density and cellulose content and better tolerance for diseases, heart rot in particular, than *A. mangium* (38, 42).

The symbiotic properties of both parental species, *A. mangium* and *A. auriculiformis*, and the characteristics of the associated rhizobium strains have been reported in a few studies (5, 15, 33, 35, 53). Both *Acacia* species are generally considered to be poorly specific, as they form efficient nodules with any slow-growing *Bradyrhizobium* strain tested. However, it was shown that *A. mangium* was a specific species in terms of effectivity, since the effect of inoculation varied greatly according to the *Bradyrhizobium* strain inoculated, in contrast with *A. auriculiformis*, in which all the *Bradyrhizobium* strains tested had the same effectivity (15). Before the present study, no work has been done on the symbiotic characterization of the *A. mangium* × *A. auriculiformis* hybrid and its associated strains. Earlier inoculation trials performed on the hybrid in a nursery in Sabah (Malaysia) showed that a strain isolated from an hybrid, namely, AH12c, had a positive effect on plant growth, whereas inoculation with Aust13c, isolated from *A. mangium* and previously known to be one of the most high-performing and competitive strains with *A. mangium* (17, 19), had no effect on the growth of the hybrid (20). These prior observations thus suggested that the hybrid was symbiotically more specific than both parental species. Even though a very few studies of symbiotic specificity between rhizobia from interspecific hybrids versus parental species have been reported in the literature (27, 44, 45, 46), no phylogenetic study has been performed on such symbiotic models so far.

The objective of the present study was to evaluate the sym-

* Corresponding author. Mailing address: LSTM, Campus International de Baillarguet, TA A-82/J, 34398 Montpellier Cedex 5, France. Phone: (33) 4 67 59 38 51. Fax: (33) 4 67 59 38 02. E-mail: galiana@cirad.fr.

§ Present address: INRA, Unité de Pathologie Végétale, Domaine Saint Maurice, BP 94, 84143 Montfavet Cedex, France.

‡ Christine Le Roux and Diana Tentchev contributed equally to this paper.

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TABLE 1. Bacterial strains isolated from *Acacia auriculiformis*, *Acacia mangium*, and *A. mangium* × *A. auriculiformis* hybrid

Strain name[a]	Original host plant	Accession no.	Geographical origin	Latitude, longitude	Growth[b]
Aa1a	*Acacia auriculiformis*	FJ025844	Brumas (Sabah), Malaysia	4°4′N, 117°8′E	S
Aa1d		FJ025845			S
Aa2c		FJ025846			S
Aa20103		FJ025850	Combi, French Guiana	5°3′N, 52°9′W	S
CGA1		None			S
Scia2		FJ025847	Sangoue, Côte d'Ivoire	7°5′N, 7°5′W	S
TAL1449		FJ025848	Hawaii	20°9′N, 156°4′E	S
Am1d	*Acacia mangium*	FJ025833	Brumas (Sabah), Malaysia	4°4′N, 117°8′E	S
Am2c		FJ025830			F
Am3b		FJ025832			S
Am3d		FJ025831			S
AG31d		FJ025849	Anguededou, Côte d'Ivoire	5°4′N, 4°0′W	S
Aust13c		AY603956[c]	Daintree, Queensland, Australia	16°1′S, 145°2′E	S
Am20101		FJ025853	Combi, French Guiana	5°3′N, 52°9′W	S
Am20102b		FJ025852			S
Am20102d		FJ025851			S
Ah1a	*A. mangium* × *A. auriculiformis* hybrid	FJ025835	Brumas (Sabah), Malaysia	4°4′N, 117°8′E	S
Ah4a		FJ025838			S
Ah5d		FJ025834			F
Ah6b		FJ025837			F
AH8c		FJ025836	Luasong (Sabah), Malaysia	4°6′N, 117°4′E	S
AH8i		FJ025840			F
AH8j		FJ025843			F
AH10		FJ025839			F
AH11a		GQ443307			F
AH11e		FJ025841			F
AH12c		FJ025842			F

[a] Strains are grouped by host plant and then geographical origin and latitude/longitude. For strains from a given host species, those having the same number in their name were isolated from the same tree but from different nodules, as differentiated by the last letter in the name.

[b] F, fast-growing isolates developing colonies >1 mm in diameter on YEM agar plates in 3 days at 27°C; S, slow-growing isolates developing visible colonies after 5 days in the same conditions.

[c] Accession number was previously published by Andre et al. (1).

biotic properties and specificity of the *A. mangium* × *A. auriculiformis* hybrid in comparison with those of both parental species at two different levels: (i) analysis of the molecular diversity of isolates from *A. mangium*, *A. auriculiformis*, and the hybrid in relation to their plant species and geographical origins and (ii) evaluation of symbiotic specificity between the rhizobial strains isolated and the three *Acacia* hosts through cross-inoculation experiments under controlled conditions.

MATERIALS AND METHODS

Origin and isolation of bacterial strains. The rhizobium strains used in the present study were isolated from root nodules of *A. mangium*, *A. auriculiformis*, and *A. mangium* × *A. auriculiformis* hybrids collected in different introduction zones, mostly in Malaysia, except for Aust13c that originates from Australia, the natural distribution area of *A. mangium* (Table 1). The root nodules from *A. mangium* and *A. auriculiformis* were collected from pure origins, while hybrids were genetically and/or phenotypically identified before nodule collection. Nodule isolates from genetically identified *Acacia* hybrids were labeled using the "AH" prefix, while those from phenotypically identified trees were labeled using "Ah" (Table 1). All "Ah" isolates originated from adult trees from genetic trials in Brumas (Sabah Softwoods Sdn. Bhd. Forest Company) which were identified according to easily distinguishable phenotypic traits, including leaf and pod shapes and flower color, all intermediary with those of parents. On the other hand, "AH" isolates were collected from 1.5-year-old trees planted in Luasong (Yayasan Sabah Group Sdn. Bhd.) that originated from phenotypically and genotypically identified hybrid clones produced as described below. Fresh root nodules were collected in different planting areas before direct isolation of bacteria the following day. The nodules were first rinsed in Eppendorf tubes containing tap water supplemented with a drop of Tween 20 and then immersed in 70% ethanol for 30 s before being surface sterilized in a solution of 0.1% $HgCl_2$ for 1 to 2 min according to the nodule size. After being washed in sterile distilled water, each nodule collected was crushed in a drop of sterile distilled water and then plated by bacterial streaking onto yeast extract-mannitol (YEM) agar plates (55). The agar plates were incubated in a dark room at 28°C for 5 days before a second transfer of the bacterial isolates onto fresh YEM agar medium. Before use, the bacterial isolates were stored in 25% glycerol at −80°C.

Phenotypic characterization of bacterial strains. The bacterial strains were considered to be either fast growing or slow growing based on the criteria of Jordan (22). Fast-growing isolates developed colonies >1 mm in diameter on YEM agar plates in 3 days at 27°C, while slow-growing ones developed visible colonies after 5 days. Colony morphology and mucus production were evaluated at the same time.

16S-23S rRNA gene ITS sequencing and phylogenetic analysis. After 7 days of incubation at 28°C on YEM agar plates, rhizobial single colonies were suspended in 200 μl of sterile distilled water in Eppendorf tubes. The bacterial cells were lysed by several cycles of heat shocks programmed in a Perkin-Elmer model 2400 thermocycler. The tubes were held at 4°C overnight before the collection of 2 μl of supernatant for PCR. Twenty-five μl of reaction mixture containing 200 μM of each deoxynucleoside triphosphate, 0.8 μM of each primer (Eurogentec, Angers, France), 1.5 mM of $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Promega, France), and the buffer supplied with the enzyme was used for PCR amplification by a Perkin-Elmer model 2400 thermocycler. The internal transcribed spacer (ITS) of the 16S and 23S rRNA genes was amplified using primers 16S-870f CCTGGGGAGTACGGTCGCAAG (48) and FGPL132′ CCGGGTTTCCCCA TTCGG (39). The PCR cycles were performed as described by Leblanc et al. (25). Then, the PCR products were run on a 1% agarose gel (Sigma, France) in Tris-acetate-EDTA buffer with a DNA size standard (Eurogentec Smartladder). The amplified fragment of about 1,600 bp obtained (9) was purified with a QIAquick gel extraction kit (Qiagen, France). The purified PCR products were sequenced using primers BR5 CTTGTAGCTCAGTTGGTTAG (58) and FGPL132′. Sequencing reactions were analyzed on an Applied Biosystems model 310 DNA automated sequencer using a BigDye Terminator cycle se-

quencing kit (Perkin-Elmer Applied Biosystems). Multiple alignments were performed with Clustal_X (52), and phylogenetic analyses were performed with maximum parsimony using MEGA (Molecular Evolutionary Genetic Analysis version 4.0.1) (51). Confidence in the phylogenetic groupings was assessed by the bootstrap method, with 1,000 replications. Sequences of related organisms from the alphaproteobacteria were included in the analysis, in particular, one strain of each genospecies characterized by Willems et al. (58, 59).

Symbiotic specificity between rhizobial strains and host plants. We used the same origins of plant materials for both in vitro and greenhouse experiments: the *A. mangium* seeds, furnished by the forestry company Innoprise Corporation Sdn. Bhd., originated from a seed orchard located in Luasong (State of Sabah, Malaysia) which was initially set up using seeds of Papua New Guinean provenance. The *Acacia auriculiformis* seeds originated from Australia (CSIRO Tree Seed Center, seed lot no. 16142, Coen River provenance, Queensland). Both seed lots were pretreated with 95% H_2SO_4 during 1 h for *A. mangium* and 30 min for *A. auriculiformis* and then surface-sterilized for 5 min in a 5% (wt/vol) calcium hypochlorite solution before being rinsed in sterile distilled water. The sterilized seeds were transferred for germination into dishes containing sterile distilled water supplemented with 0.3% Phytagel (Sigma-Aldrich, France) before incubation in lighted culture rooms at 28°C under a 16-h photoperiod.

The *A. mangium* × *A. auriculiformis* hybrid plants tested were the following clones: no. 1-24, 3-26, 4-21, 6-27, 7-23, 10-23, 10-24, and 11-28. These eight clones were produced from seeds of a 5-year-old *A. mangium* mother tree planted in Oumé (Côte d'Ivoire) and selected based on phenotypic and genotypic identification criteria as described by Galiana et al. (20). The *Acacia* hybrid clones were propagated through microcutting and maintained in in vitro conditions before rhizobial inoculation experiments. Every 2 to 3 months, the microcuttings were subcultured into 13- by 10-cm glass flasks filled up with a basal Murashige and Skoog culture medium (32) with macroelements at half strength and supplemented with 0.1 mM NaFe-EDTA, vitamins (34), 1.07 μM of NAA (α-naphthalene acetic acid), and 58.4 mM of sucrose. The pH was adjusted to 5.7, and the medium solidified with 0.3% Phytagel. The microcuttings consisted of shoot segments 2 to 3 cm in height composed of two nodes. The culture flasks were placed in culture rooms at 28°C under a 16-h photoperiod and light intensity of 60 microeinsteins ($\mu E) \cdot m^{-2} \cdot s^{-1}$. Root initiation occurred at the shoot basis after 1 week of culture, and the mean rooting rate reached 100% after 2 weeks of culture regardless of the *Acacia* clone used.

In the in vitro experiment, the culture device consisted of a 220- by 25-mm test tube containing a polypropylene support, as described by Galiana et al. (15). Each test tube contained 25 ml of a sterile N-free Broughton and Dilworth nutrient solution (4). Seven days after H_2SO_4 pretreatment, the germinated seeds of *A. mangium* and *A. auriculiformis* were transferred into the tubes, and 10 days later, they were inoculated with 1 ml of a washed rhizobium culture at a concentration of 10^9 bacteria per ml. The *A. mangium* × *A. auriculiformis* hybrid plants tested consisted of terminal microcuttings from the eight *Acacia* hybrid clones described above. Fourteen days after their transfer onto rooting medium, i.e., 7 days after root initiation, the rooted microcuttings were transferred from the flasks to the tubes and, 5 days after the transfer, inoculated with 1 ml of a washed rhizobium culture containing 10^9 bacteria. All plant cultures were placed in culture rooms at 28°C under a 16-h photoperiod and light intensity of 60 $\mu E \cdot m^{-2} \cdot s^{-1}$. The infectivity and effectivity of each of the nine following rhizobium strains were tested on *A. mangium*, *A. auriculiformis*, and their hybrid: Aust13c, AG31d, and Am1d originating from *A. mangium*; TAL1449, Scia2, and CGA1 from *A. auriculiformis*; and AH12c, AH10, and Ah1a from the hybrid (see "Geographical origin" in Table 1). Two additional hybrid-associated strains, namely, Ah4a and AH11a, were also tested on their homologous host. Eight replicates per treatment, i.e., per *Acacia* host and per strain tested, were used. Concerning the hybrid, each of the 11 strains tested was inoculated to eight plants comprising the eight different clones described above (i.e., one plant per clone), so that each strain tested was inoculated to the same clonal composition. For each of the three *Acacia* hosts tested, the strain treatments were compared to noninoculated control plants that consisted of eight replicates per *Acacia* host treated with 1 ml of an autoclaved rhizobium culture of the Aust13c strain.

In the greenhouse experiment, the plants were transferred to 12- by 8-cm polyethylene pots (modified Leonard jars) containing a 1/1 (vol/vol) perlite/vermiculite mixture and fed weekly with a N-free nutrient solution (4). The germinated seeds of *A. mangium* and *A. auriculiformis* were individually transferred into the pots 7 and 15 days, respectively, after seed scarification. Rooted microcuttings of the *Acacia* hybrid clone no. 3-21 were individually transferred into the culture jars 2 weeks after their transfer onto in vitro rooting medium, i.e., 7 days after root initiation. The day following their transfer into the pots, plants were inoculated with 1 ml of rhizobium culture at a concentration of 10^9 bacteria per ml. Both strain Aust13c from *A. mangium* and AH12c from the hybrid were tested on *A. mangium*, *A. auriculiformis*, and the hybrid with, respectively, 11, 10, and 8 replicates per inoculation treatment. Uninoculated control plants were treated with a 1-ml autoclaved rhizobium culture of the Aust13c strain.

After 4 and 4.5 months of growth for in vitro and greenhouse experiments, respectively, all plants were collected to determine the shoot height and number of nodules. The aerial parts and nodules were dried in an oven for 72 h at 60°C before weight measurements. For each of the three *Acacia* species studied and each parameter analyzed, all the data collected were subjected to a one-way analysis of variance. When the rhizobium strain factor had a significant effect on a given parameter, the means of the different treatments were ranked into homogeneous groups according to Duncan's multiple range test (6). All the statistical analyses were performed using SuperAnova software (Abacus Concepts, Inc., CA).

Nucleotide sequence accession numbers. The 16S-23S rRNA gene ITS partial sequences of 25 newly isolated *Acacia*-associated rhizobial strains were deposited in the GenBank database under accession numbers FJ025830 to FJ025853 and GQ443307. Strain Aust13c was previously sequenced under accession number AY603956 (1).

RESULTS

Phenotypic characterization of bacterial strains. As indicated in Table 1, all of the strains isolated from *Acacia auriculiformis* and *Acacia mangium*, except Am2c, were slow-growing and formed flat and soft colonies and strikes (see Fig. S1 in the supplemental material), like strain Aust13c, considered the *A. mangium* slow-growing reference strain here. Conversely, a majority of rhizobium strains isolated from the hybrid were considered comparatively fast growing in pure culture and produced convex and firm colonies and strikes (see Fig. S1 in the supplemental material). Among the *Acacia* hybrid-associated strains, only Ah1a, Ah4a, and AH8c were slow growing and had the same morphological type as Aust13c.

Phylogenetic analysis of bacterial strains. Based on 16S-23S rRNA gene ITS sequence comparisons that included reference bacterial species sequences, particularly, sequences of the *Bradyrhizobium* genospecies determined by Willems et al. (58, 59), all of the isolates studied, from both of the parental *Acacia* species and their hybrid, were shown to belong to the *Bradyrhizobium* genus, and no isolates of other genera were found. The phylogenetic tree showed two major clades (Fig. 1). The first clade included *B. elkanii* and *Bradyrhizobium* reference genospecies VII, IX, X, and XI. A majority of isolates (13 out of 15) from the parental species *A. mangium* and *A. auriculiformis* grouped diversely on this branch, which also contained only three isolates from the hybrid, i.e., Ah1a, Ah4a, and AH8c. A majority (8 out of 11) of isolates from the hybrid were closely related to *B. yuanmingense* in the second well-differentiated clade, which included *B. japonicum*, while only strain Am2c originating from *A. mangium* also grouped here.

Symbiotic specificity between rhizobial strains and host plants. The in vitro experiment did not show any host specificity of the *Bradyrhizobium* strains tested in terms of infectivity, since they all formed nodules indifferently in *A. mangium*, *A. auriculiformis*, and their hybrid, regardless of the geographical or host plant origin of the strains (Table 2). However, as evidenced by the analysis of variance, significant differences (at $P = 0.05$) were observed in the number of nodules formed by the bacterial strains in *A. mangium* and the hybrid. On the other hand, no significant difference was observed in the number of nodules formed by the same strains in *A. auriculiformis*. The nodule dry weight varied according to the *Bradyrhizobium* strain in *A. mangium* and *A. auriculiformis* but not in the

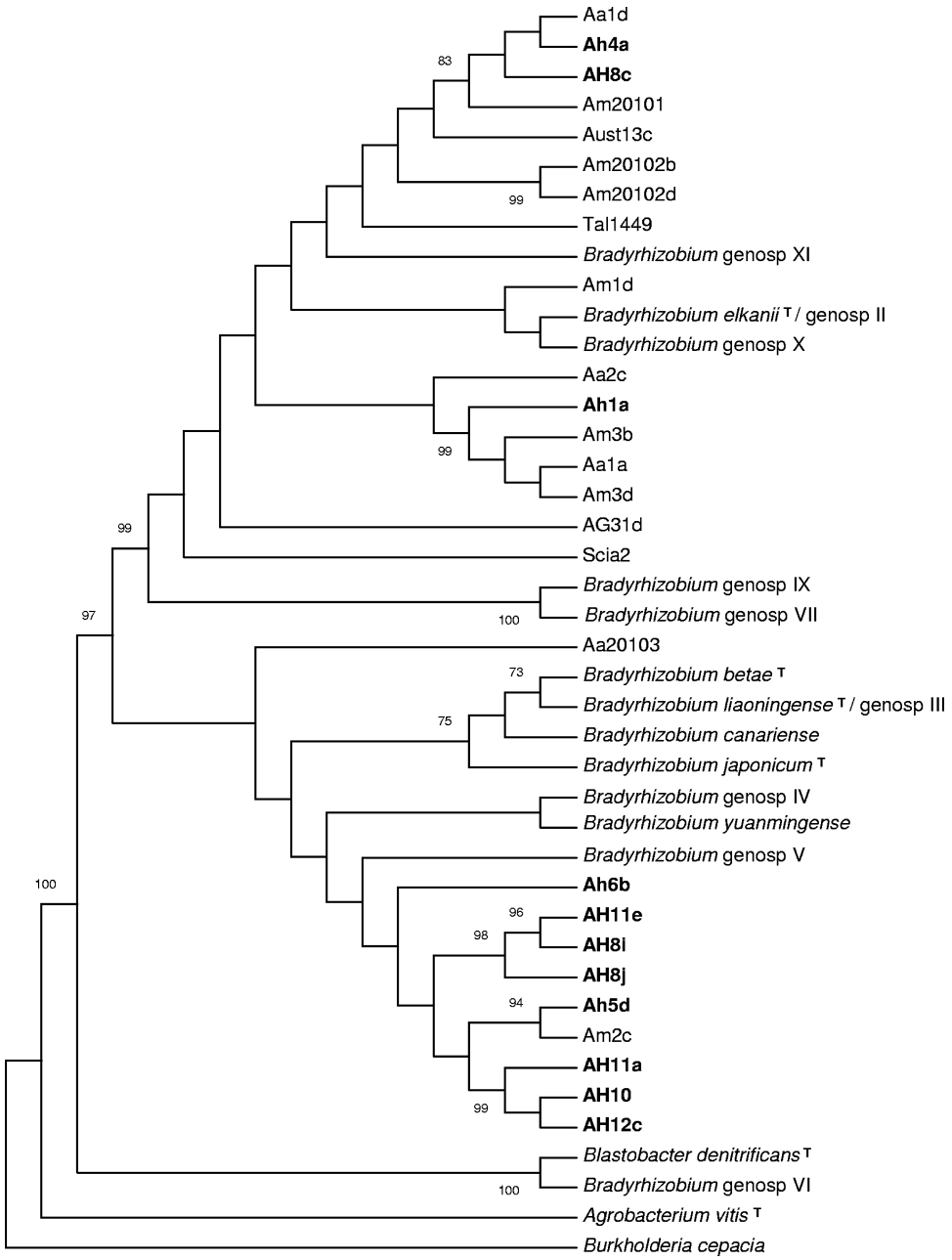

FIG. 1. Phylogenetic maximum parsimony tree based on 16S-23S rRNA gene ITS sequence of *Bradyrhizobium* spp. strains isolated from *Acacia mangium*, *A. auriculiformis*, and the *A. mangium* × *A. auriculiformis* hybrid (in bold). Only bootstrap probability values higher than 70% (1,000 replications) are given at the branching points. The type strains are indicated with the letter "T." The reference sequences from GenBank are *Agrobacterium vitis*^{T} (U45329), *Burkholderia cepacia* (DQ273266), *Blastobacter denitrificans*^{T} (AF338176), *Bradyrhizobium japonicum*^{T} (AJ279264), *B. canariense* (AY386706), *B. elkanii*^{T} (AJ279308), *B. liaoningense*^{T} (AJ279301), *B. betae*^{T} (AJ631967), *B. yuanmingense* (AJ534605), *Bradyrhizobium* genospecies IV (AJ279281), *Bradyrhizobium* genospecies V (AJ279287), *Bradyrhizobium* genospecies VI (AJ279312), *Bradyrhizobium* genospecies VII (AJ279272), *Bradyrhizobium* genospecies IX (AJ534597), *Bradyrhizobium* genospecies X (AJ534592), *Bradyrhizobium* genospecies XI (AJ534594), and *Bradyrhizobium* genospecies IV (AJ279317). genosp., genospecies.

hybrid. The dry weight of shoots varied significantly according to the strain in the two *Acacia* species and their hybrid (Table 2). In both *A. mangium* and *A. auriculiformis*, the host plant origin of a given strain had no effect on its degree of effectivity. For instance, strain Am1d, although isolated from *A. mangium*, formed inefficient nodules on this species. Concerning the hybrid, four out of five tested strains from the *Acacia* hybrid were the most efficient ones, even though the effectivity of some strains originating from *A. mangium* and *A. auriculiformis* was not statistically different according to the Duncan multiple range test (at $P = 0.5$). Three *Acacia* hybrid-associated strains were also ranked among the four most effective strains in *A. auriculiformis*.

The results of the greenhouse experiments reported in Table

TABLE 2. Infectivity and effectivity of rhizobium strains on *A. mangium*, *A. auriculiformis*, and *Acacia mangium* × *A. auriculiformis* hybrid grown under in vitro conditions

Strain name	Host plant of origin	Mean result for[a]:								
		No. of nodules per plant			Nodule dry wt (mg · plant^{-1})			Shoot dry wt (mg · plant^{-1})		
		A m	*A a*	*A* hyb	*A m*	*A a*	*A* hyb	*A m*	*A a*	*A* hyb
Aust13c	*A. mangium*	20.8 bc	19.1 a	20.8 bc	5.20 d	4.72 d	4.86 a	69.3 ab	79.8 c	68.4 abc
AG31d		23.4 ab	26.2 a	21.1 bc	7.05 abcd	8.95 a	4.81 a	69.4 ab	89.1 abc	57.4 abc
Am1d		1.9 e	31.5 a	30.8 b	0.18 e	6.55 bcd	5.06 a	3.8 d	91.1 abc	50.0 c
TAL 1449	*A. auriculiformis*	12.9 cd	28.2 a	19.6 bc	5.80 bcd	8.18 ab	5.35 a	56.8 bc	97.0 ab	69.3 abc
Scia2		32.0 a	24.0 a	51.5 a	7.58 abc	6.27 bcd	6.81 a	74.9 a	78.4 c	68.2 abc
CGA1		8.7 de	23.1 a	15.5 c	6.57 abcd	7.44 abc	4.64 a	74.2 a	83.0 bc	70.7 abc
AH12c	*Acacia* hybrid	24.2 ab	24.4 a	26.5 bc	8.00 ab	8.74 a	5.77 a	51.6 c	93.3 abc	54.9 bc
AH10		29.6 ab	23.2 a	32.1 b	8.61 a	9.04 a	6.95 a	56.0 bc	102.5 a	75.4 ab
Ah1a		19.2 bc	20.4 a	25.1 bc	5.41 bcd	5.89 cd	4.55 a	65.5 abc	103.7 a	71.3 abc
Ah4a		NT	NT	25.8 bc	NT	NT	4.96 a	NT	NT	71.7 abc
AH11a		NT	NT	27.4 bc	NT	NT	5.26 a	NT	NT	79.2 a
Uninoculated control[b]		0	0	0	—	—	—	4.0 d	13.3 d	25.2 d

[a] Values are means of the results obtained from eight replicates per strain tested 4 months after plant inoculation and growth under in vitro conditions. Means followed by different letters in the same column are significantly different according to the Duncan multiple range test at a *P* value of 0.05. *A m*, *A. mangium*; *A a*, *A. auriculiformis*; *A* hyb, *Acacia mangium* × *A. auriculiformis* hybrid; NT, not tested, —, not applicable.

[b] Uninoculated control plants consisted of eight replicates per *Acacia* host treated with 1 ml of an autoclaved rhizobium culture of the Aust13c strain.

3 show that the homologous strain-*Acacia* associations, i.e., *A. mangium* inoculated with Aust13c and the hybrid inoculated with AH12c, produced at least twice as many nodules as the heterologous associations 4 months after inoculation. In contrast, no significant difference (at $P = 0.05$) between strains was found for nodule dry weight in *A. mangium*, whereas the nodule biomass was higher with AH12c (72%) than with Aust13c in the *Acacia* hybrid. In *A. auriculiformis*, AH12c produced a higher nodule biomass (57%) and about three times more nodules than Aust13c. As for infectivity, strain effectivity was also higher in homologous strain-*Acacia* host associations, with shoot biomass increments of 79% and 78% in *A. mangium* inoculated with Aust13c and the *Acacia* hybrid inoculated with AH12c, respectively. No significant difference between strains was found for shoot dry weight in *A. auriculiformis*.

DISCUSSION

Molecular characterization of bacterial strains. Based on the 16S-23S rRNA gene ITS, which shows more variability in length and in sequence (21) than the widely used 16S rRNA gene, the 25 new bacterial nodule isolates from the three *Acacia* hosts were shown to belong to *Bradyrhizobium*. The *Bradyrhizobium* isolates studied exhibited intrageneric diversity and ranged in either of the two widely recognized major clades of *Bradyrhizobium*, the *B. japonicum* and *B. elkanii* clades. The majority of the isolates from the hybrid and only one *A. mangium* isolate, Am2c, grouped on the *B. japonicum* branch close to *B. yuanmingense*. On the other hand, nearly all (except strain Am2c) rhizobial isolates from the parental tree species *A. mangium* and *A. auriculiformis* grouped on the *B. elkanii* branch; only three isolates (Ah1a, Ah4a, and AH8c) from the hybrid grouped on this branch. However, the plant origins of the two strains Ah1a and Ah4a remain questionable, since the hybrid status of their respective host plants of isolation has not been confirmed (uncertain phenotypic identification at harvesting in the plantation) and might rather be *A. mangium*. In contrast, the host plants from which the strains of the *B. japonicum* clade were isolated were genetically or phenotypically confirmed as hybrids (20). The strains studied could not be assigned to any particular *Bradyrhizobium* species. Their precise taxonomic position should be further clarified by DNA-DNA hybridization analysis or multilocus sequence analysis (56).

The few other studies reporting the characterization of rhi-

TABLE 3. Infectivity and effectivity of Aust13c and AH12c strains on *A. mangium*, *A. auriculiformis*, and *Acacia mangium* × *A. auriculiformis* hybrid grown under greenhouse conditions

Strain no.	Host plant of origin	Mean result for[a]:								
		No. of nodules per plant			Nodule dry wt (mg · plant^{-1})			Shoot dry wt (g · plant^{-1})		
		A m	*A a*	*A* hyb	*A m*	*A a*	*A* hyb	*A m*	*A a*	*A* hyb
Aust13c	*A. mangium*	219 a	98 b	231 b	162 a	90 b	220 a	2.65 a	1.97 a	1.72 b
AH12c	*Acacia* hybrid	111 a	285 a	596 a	171 a	141 a	128 a	1.48 b	1.79 a	3.06 a
Uninoculated control[b]		0	0	0	—	—	—	0.11 c	0.09 b	0.09 c

[a] Values are means of the results 4.5 months after plant inoculation and growth under greenhouse conditions; in *A. mangium* and *A. auriculiformis*, the means were calculated from the results for 5 replicates per strain tested for both nodule number and nodule dry weight and from 10 replicates for shoot dry weight. In the *A. mangium* × *A. auriculiformis* hybrid, all parameters were measured from eight plants per inoculation treatment. Means followed by different letters in the same column are significantly different according to the Duncan multiple range test at a *P* value of 0.05. *A m*, *A. mangium*; *A a*, *A. auriculiformis*; *A* hyb, *Acacia mangium* × *A. auriculiformis* hybrid; —, not applicable.

[b] Uninoculated control plants consisted of eight replicates per *Acacia* host treated with 1 ml of an autoclaved rhizobium culture of the Aust13c strain.

zobium isolates from *A. mangium* were restricted to limited areas of collection in its introduction zones but, surprisingly, showed more rhizobial diversity than the present study, although ours covered the four tropical continents. Thus, Nuswantara et al. (35) identified a few *Rhizobium* and *Sinorhizobium* strains and a majority of *B. elkanii* strains among *A. mangium* nodule isolates collected in Indonesian forest plantations. However, these authors did not perform any renodulation test to confirm the infectivity of these strains with their host of isolation. Clapp et al. (5) found about half *B. elkanii* and half *B. liaoningense/japonicum* with less than 5% *Mesorhizobium loti* among *A. mangium* isolates originating from Indonesian plantations through a combined PCR–restriction fragment length polymorphism–single-strand conformation polymorphism analysis. However, renodulation tests were not performed either, and the genetic identity of the sampled trees remains imprecise, without any information on the germ plasm origin, in which interspecific hybrids could easily occur. Similarly, other studies described nonbradyrhizobia, such as *Mesorhizobium*, *Rhizobium*, and even *Ochrobactrum* (*Brucellaceae* family), among a majority of *Bradyrhizobium* strains in *A. mangium* nodules (33, 57).

The *Bradyrhizobium* strains isolated from both parental *Acacia* species (except for Am2c), which belong to the *B. elkanii* clade, are phylogenetically close, although they originate from very distant regions distributed in the four tropical continents. In contrast, *Bradyrhizobium* strains originating from the same sites in Malaysia belong to distinct phylogenetic branches according to their host origin, i.e., *A. mangium* and *A. auriculiformis* versus hybrid. Such absence of correlation between the phylogeny of bacterial isolates and their geographical origin has already been reported for several tropical legume tree-rhizobium associations (3, 24). Although our isolates were obtained from very diverse origins worldwide, a small number of representative strains per origin were used for the phylogenetic analysis, except for the isolates from Malaysia, which were all analyzed. For the *A. mangium*-associated strains from Australia and Ivory Coast, i.e., Aust13c and AG31d, respectively, they were selected as representative based on previous diversity analyses performed from collections of about 80 (13, 14) and 60 (15, 18) isolates, respectively.

Although bradyrhizobia have largely been described as slow-growing bacteria in culture (26, 28, 31, 36, 49, 50), we distinguished two growing types in our study: almost all *Bradyrhizobium* isolates close to *B. japonicum* that were from the *Acacia* hybrid had fast growth, in comparison to those close to *B. elkanii* that were isolated from both parental *Acacia* species and were slow growing. Both growth types of bradyrhizobia also had distinct morphological aspects, with the former producing compact, convex, and firm colonies while the later formed very spread out, flat, and soft colonies. Such differences in growth rates and mucus production between *Bradyrhizobium* species have been described in a few cases (25, 61), but these phenotypic traits remain qualitative and relative.

Host specificity of *Bradyrhizobium* strains. The results obtained from the in vitro experiments confirm the lack of specificity for infectivity among the *Bradyrhizobium* sp. strains when inoculated to *Acacia* hybrids or both parental *Acacia* species, as reported earlier for the latter (15, 53). The hormonal treatment, i.e., the addition of NAA, used for root induction in the process of *Acacia* hybrid micropropagation probably did not affect the infectivity and effectivity of strains. Indeed, we already showed in earlier studies performed under the same in vitro conditions that the infectivity and effectivity of Aust13c versus those of TAL72 and ORS800, two less-effective *Bradyrhizobium* strains isolated from other host species, remained unchanged when the strains were inoculated to different *A. mangium* clones issued from selected micropropagated seedlings, although higher concentrations of auxins were used for rooting than here (16). Moreover, in another in vitro experiment, these three *Bradyrhizobium* strains exhibited the same behaviors and performances when inoculated to *A. mangium* seedlings (15).

The *Acacia* hybrid isolates were shown to be specific in terms of effectivity when the results of both in vitro and greenhouse experiments were considered, since the homologous strain-host associations tested were more effective than the heterologous ones. Strain Aust13c was more effective than AH12c on *A. mangium* in both growing conditions, whereas AH12c was more effective than Aust13c toward the *Acacia* hybrid in greenhouse conditions but not in vitro. In contrast, the four other *Acacia* hybrid-associated strains tested in vitro were more effective than the *A. mangium* ones when inoculated to *Acacia* hybrids. The reduced photosynthetic activity under in vitro conditions could thus affect *Acacia* hybrid clones more particularly, since the period of acclimatization time is longer than for *A. mangium* seedlings. However, the in vitro device used to assess both the infectivity and effectivity of rhizobium strains was proved to be a reliable method, at least for *A. mangium*, as confirmed by former studies showing a good correlation in strain performance between in vitro, greenhouse, nursery, and even field conditions (15, 17, 18). Compared to the results under in vitro conditions, the greenhouse experiment showed a far better performance of the homologous associations than the heterologous ones for the rhizobium-host plant species tested, at least in *A. mangium* and the hybrid, since *A. auriculiformis*, known as a promiscuous species, did not show any specificity. Nevertheless, greenhouse experiments should be extended to other hybrid clones and *Bradyrhizobium* strains to confirm the specificity observed here, especially as AH12c, the only hybrid strain tested in the greenhouse, exhibited the lowest efficiency among the five hybrid-associated strains tested under in vitro conditions. Actually, a similar cross-inoculation experiment was performed earlier in Malaysia under nursery conditions and showed significant superiority of strain AH12c over Aust13c on each of the three *Acacia mangium* × *A. auriculiformis* hybrid clones tested, i.e., clones 3-21, 5-13, and 7-23, as well as a significant clonal effect, while no interaction was found between clones and the two rhizobium strains (20).

So far, except through qualitative nodulation tests (8, 11, 36), very few studies have shown such specificity in the miscellaneous group of *Bradyrhizobium* spp. Moreover, to our knowledge, the phenotypic and genotypic differentiation observed between rhizobial isolates from two parental plant species and the corresponding interspecific hybrid has never been reported for legume species. Even though the symbiotic specificity of rhizobial isolates of such "hybrid versus parental" symbiotic models has already been investigated in a few studies (27, 44, 45, 46), they were performed with few isolates and without any phylogenetic analysis. This study shows that the isolation and

selection of new hybrid-specific and effective strains from other geographical origins is needed, so that they can be used as inoculants for the *Acacia* hybrid in the context of its increasing use in large-scale afforestation programs.

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